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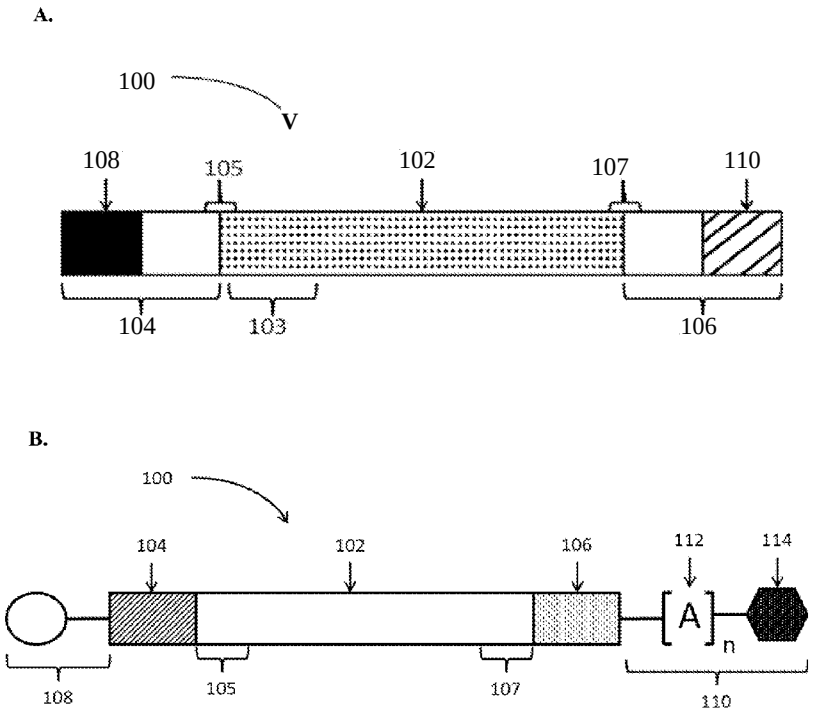
(54) Title: TERMINAL MODIFICATIONS OF POLYNUCLEOTIDES

(57) Abstract: The present invention relates to compositions and methods for the preparation, manufacture and therapeutic use of polynucleotides comprising at least one terminal modification.



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TERMINAL MODIFICATIONS OF POLYNUCLEOTIDES

REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Patent Application No. 62/025,985 filed July 17, 2014, entitled Terminal Modifications of Polynucleotides, the contents of each of which are herein incorporated by reference in its entirety.

REFERENCE TO THE SEQUENCE LISTING

[0002] The present application is being filed along with a Sequence Listing in electronic format. The Sequence Listing is provided as a file entitled M119PCTSL.txt created on July 17, 2015 which is 181,234 bytes in size. The information in the electronic format of the sequence listing is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

[0003] The invention relates to polynucleotides comprising at least one terminal modification, methods, processes, kits and devices using the polynucleotides comprising at least one terminal modification.

BACKGROUND OF THE INVENTION

[0004] In the early 1990's Bloom and colleagues successfully rescued vasopressin-deficient rats by injecting in vitro-transcribed vasopressin mRNA into the hypothalamus (Science 255: 996-998; 1992). However, the low levels of translation and the immunogenicity of the molecules hampered the development of mRNA as a therapeutic and efforts have since focused on alternative applications that could instead exploit these pitfalls, *i.e.* immunization with mRNAs coding for cancer antigens.

[0005] More recently, others have investigated the use of mRNA to deliver a construct encoding a polypeptide of interest and have shown that certain chemical modifications of mRNA molecules, particularly pseudouridine and 5-methyl-cytosine, have reduced immunostimulatory effect.

[0006] Notwithstanding these reports which are limited to a selection of chemical modifications including pseudouridine and 5-methyl-cytosine where the modifications are uniformly present in the RNA, there remains a need in the art for therapeutic modalities to address the myriad of barriers surrounding the efficacious modulation of intracellular translation and processing of polynucleotides encoding polypeptides including the barrier to selective incorporation of different chemical modifications or incorporation of chemical modifications not previously possible in order to fine tune or tailor physiologic responses and outcomes.

[0007] The present invention addresses provides nucleic acid based compounds or polynucleotides (both coding and non-coding and combinations thereof) and formulations thereof which have structural and/or chemical features that avoid one or more of the problems in the art, for example, features which are useful for optimizing nucleic acid-based therapeutics while retaining structural and functional integrity, overcoming the threshold of expression, improving expression rates, half life and/or protein concentrations, optimizing protein localization, and avoiding deleterious bio-responses such as the immune response and/or degradation pathways. These barriers may be reduced or eliminated using the present invention.

SUMMARY OF THE INVENTION

[0008] Described herein are polynucleotides comprising at least one terminal modification, methods, processes, kits and devices using the polynucleotides comprising at least one terminal modification in one or more untranslated regions. Such untranslated regions may be a 5' or 3' untranslated region.

[0009] In some aspects, the untranslated region is a synthetic 5' untranslated region having a length of 3 - 13 nucleotides.

[00010] In some aspects the length of the synthetic 5' untranslated region is 10-12 nucleotides in length.

[00011] In some aspects the 3' untranslated region has a length of 20-50 nucleotides in length.

[00012] In some aspects the length of the 3' untranslated region is 30 nucleotides.

[00013] In some aspects the untranslated region is a polyA tailing region of approximately 80 nucleotides in length.

[00014] In some aspects, the tailing region comprises at least one miR sequence.

[00015] In some aspects the miR sequence is located at a position selected from the group consisting of the beginning of the polyA tail, the middle of the polyA tail and the end of the polyA tail.

[00016] In some aspects a second terminal region comprises at least one miR sequence.

[00017] In some aspects, the second terminal region comprises a 3' untranslated region and said 3' untranslated region comprises the at least one miR sequence.

[00018] In some aspects the at least one miR sequence is selected from the group consisting of miR-142-3p, miR-122, miR-133, miR-1, miR-206, miR-126, miR-132, miR-125, miR-124, miR-21, miR-484, miR-17, miR-34a and fragments thereof.

[00019] In some aspects the at least one miR sequence is specific for a tissue selected from the group consisting of muscle, endothelium, lung, ovarian, colorectal, prostate, liver and spleen.

[00020] In some aspects, the tissue is muscle and the at least one miR sequence is selected from the group consisting of miR-133, miR-1 and miR-206.

[00021] In some aspects, the at least one miR sequence is miR-206.

[00022] In some aspects, the tissue is endothelium and the at least one miR sequence is miR-126.

[00023] In some aspects, the tissue is lung and the at least one miR sequence is miR-21.

[00024] In some aspects, the tissue is ovarian and the at least one miR sequence is miR-484.

[00025] In some aspects, the tissue is colorectal and the at least one miR sequence is miR-17.

[00026] In some aspects, the tissue is prostate and the at least one miR sequence is miR-34a.

[00027] In some aspects, the at least one miR sequence is specific for the central nervous system.

[00028] In some aspects, the at least one miR sequence is selected from the group consisting of miR-132, miR-125 and miR-124.

[00029] In some aspects, the 5' untranslated region comprises at least one miR sequence.

[00030] In some aspects, the at least one miR sequence is miR-10a.

[00031] In some aspects, the polynucleotides comprising the untranslated region comprises at least one chemical modification.

[00032] In some aspects, a method of producing a polypeptide of interest in a cell or tissue comprising contacting said cell or tissue with the polynucleotide having an untranslated region disclosed herein is provided.

[00033] In some aspects, the contacting is a route of administration selected from the group consisting of intramuscular, intravenous, intradermal, and subcutaneous.

[00034] The details of various embodiments of the invention are set forth in the description below. Other features, objects, and advantages of the invention will be apparent from the description and the drawings, and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[00035] The foregoing and other objects, features and advantages will be apparent from the following description of particular embodiments of the invention, as illustrated in the accompanying drawings in which like reference characters refer to the same parts throughout the different views. The drawings are not necessarily to scale, emphasis instead being placed upon illustrating the principles of various embodiments of the invention.

[00036] FIG. 1 comprises Figure 1A and Figure 1B showing schematics of an IVT polynucleotide construct. Figure 1A is a schematic of a polynucleotide construct taught in commonly owned co-pending US Patent Application 13/791,922 filed March 9, 2013, the

contents of which are incorporated herein by reference. Figure IB is a schematic of a polynucleotide construct.

[00037] FIG. 2 is a schematic of a series of chimeric polynucleotides of the present invention.

[00038] FIG. 3 is a schematic of a series of chimeric polynucleotides illustrating various patterns of positional modifications and showing regions analogous to those regions of an mRNA polynucleotide.

[00039] FIG. 4 is a schematic of a series of chimeric polynucleotides illustrating various patterns of positional modifications based on Formula I.

[00040] FIG. 5 is a is a schematic of a series of chimeric polynucleotides illustrating various patterns of positional modifications based on Formula I and further illustrating a blocked or structured 3' terminus.

[00041] FIG. 6 comprises Figure 6A-6G which are schematics of a circular polynucleotide construct of the present invention. Figure 6A and 6B are circular polynucleotides with and without a non-nucleic acid moiety. Figure 6C is a circular polynucleotide with at least one spacer region. Figure 6D is a circular polynucleotide with at least one sensor region. Figure 6E is a circular polynucleotide with at least one spacer and sensor region. Figures 6F and 6G are non-coding circular polynucleotides.

DETAILED DESCRIPTION

[00042] It is of great interest in the fields of therapeutics, diagnostics, reagents and for biological assays to be able design, synthesize and deliver a nucleic acid, e.g., a ribonucleic acid (RNA) inside a cell, whether *in vitro, in vivo, in situ* or *ex vivo,* such as to effect physiologic outcomes which are beneficial to the cell, tissue or organ and ultimately to an organism. One beneficial outcome is to cause intracellular translation of the nucleic acid and production of at least one encoded peptide or polypeptide of interest. In like manner, non-coding RNA has become a focus of much study; and utilization of non-coding polynucleotides, alone and in conjunction with coding polynucleotides, could provide beneficial outcomes in therapeutic scenarios.

[00043] Described herein are compositions (including pharmaceutical compositions) and methods for the design, preparation, manufacture and/or formulation of nucleic acids comprising at least one terminal modification. The nucleic acids comprising at least one terminal modification may be IVT polynucleotides, chimeric polynucleotides and/or circular polynucleotides. The terminal modification of a nucleic acid is located in one or more terminal regions of the nucleic acid. Such terminal region include regions to the 5' or 3' of the coding

region such as, but not limited to, the 5'untranslated region (UTR), and 3'UTR, the capping region e.g., the 5'cap and tailing region of the nucleic acid.

[00044] Also provided are systems, processes, devices and kits for the selection, design and/or utilization of the polynucleotides described herein.

[00045] According to the present invention, the polynucleotides may be modified in a manner as to avoid the deficiencies of other molecules of the art.

[00046] The use of polynucleotides encoding polypeptides (e.g., polynucleotides, modified polynucleotides or modified mRNA) in the fields of human disease, antibodies, viruses, and a variety of *in vivo* settings has been explored previously and polypeptides of interest are disclosed in for example, in Table 6 of International Publication Nos. WO2013151666, WO2013151668, WO2013151663, WO2013151669, WO2013151670, WO2013151664, WO2013151665, WO2013151736; Tables 6 and 7 International Publication No. WO2013151672; Tables 6, 178 and 179 of International Publication No. WO2013151671; Tables 6, 185 and 186 of International Publication No WO2013 15 1667; the contents of each of which are herein incorporated by reference in their entireties. Any of the foregoing may be synthesized as an IVT polynucleotide, chimeric polynucleotide or a circular polynucleotide, and each may comprise at least one terminal modification and such embodiments are contemplated by the present invention.

[00047] Provided herein in part are polynucleotides encoding polypeptides capable of modulating a cell's status, function and/or activity, and methods of making and using these nucleic acids and polypeptides. As described herein and in co-pending and co-owned International Publication No WO2012019168 filed August 5, 201 1, International Publication No WO2012045082 filed October 3, 201 1, International Publication No WO2012045075 filed October 3, 2011, International Publication No WO2013052523 filed October 3, 2012, and International Publication No. WO2013090648 filed December 14, 2012, the contents of each of which are incorporated by reference herein in their entirety, these polynucleotides are capable of reducing the innate immune activity of a population of cells into which they are introduced, thus increasing the efficiency of protein production in that cell population.

[00048] In one embodiment, the polynucleotides described herein may comprise at least one terminal modification and may also comprise at least one chemical modification such as, but not limited to, a non-natural nucleoside and nucleotide. Non-limiting examples of chemical modifications are described in International Patent Publication No. WO2012045075, filed October 3, 201 1, US Patent Publication No US201301 15272, filed October 3, 2012 and International Patent Publication No. WO2014093924 (Attorney Docket No. M036.20), filed December 13, 2013, the contents of each of which are herein incorporated by reference in its

entirety. In one embodiment, the utilization of at least one terminal modification and at least one chemical modification may increase protein production from a cell population.

[00049] Provided herein, therefore, are polynucleotides which have been designed to improve one or more of the stability and/or clearance in tissues, receptor uptake and/or kinetics, cellular access, engagement with translational machinery, mRNA half-life, translation efficiency, immune evasion, immune induction (for vaccines), protein production capacity, secretion efficiency (when applicable), accessibility to circulation, protein half-life and/or modulation of a cell's status, function and/or activity.

I. Compositions of the Invention

Polynucleotides

[00050] The present invention provides nucleic acid molecules, specifically polynucleotides which, in some embodiments, encode one or more peptides or polypeptides of interest. The term "nucleic acid," in its broadest sense, includes any compound and/or substance that comprise a polymer of nucleotides. These polymers are often referred to as polynucleotides.

[00051] Exemplary nucleic acids or polynucleotides of the invention include, but are not limited to, ribonucleic acids (RNAs), deoxyribonucleic acids (DNAs), threose nucleic acids (TNAs), glycol nucleic acids (GNAs), peptide nucleic acids (PNAs), locked nucleic acids (LNAs, including LNA having a β- D-ribo configuration, a-LNA having an a-L-ribo configuration (a diastereomer of LNA), 2'-amino-LNA having a 2'-amino functionalization, and 2'-amino- a-LNA having a 2'-amino functionalization), ethylene nucleic acids (ENA), cyclohexenyl nucleic acids (CeNA) or hybrids or combinations thereof.

[00052] In one embodiment, polynucleotides of the present invention which are made using only *in vitro* transcription (IVT) enzymatic synthesis methods are referred to as "IVT polynucleotides." Methods of making IVT polynucleotides are known in the art and are described in co-pending International Publication Nos. WO2013 151666, WO20 13 151668, WO2013151663, WO2013151669, WO2013151670, WO2013151664, WO2013151665, WO20 13151671, WO20 13151 672, WO20 13 15 1667 and WO20 13 15 1736; the contents of each of which are herein incorporated by reference in their entireties.

[00053] In another embodiment, the polynucleotides of the present invention which have portions or regions which differ in size and/or chemical modification pattern, chemical modification position, chemical modification percent or chemical modification population and combinations of the foregoing are known as "chimeric polynucleotides." A "chimera" according to the present invention is an entity having two or more incongruous or heterogeneous parts or

regions. As used herein a "part" or "region" of a polynucleotide is defined as any portion of the polynucleotide which is less than the entire length of the polynucleotide.

[00054] In yet another embodiment, the polynucleotides of the present invention that are circular are known as "circular polynucleotides" or "circP." As used herein, "circular polynucleotides" or "circP" means a single stranded circular polynucleotide which acts substantially like, and has the properties of, an RNA. The term "circular" is also meant to encompass any secondary or tertiary configuration of the circP.

[00055] In some embodiments, the polynucleotide includes from about 30 to about 100,000 nucleotides (e.g., from 30 to 50, from 30 to 100, from 30 to 250, from 30 to 500, from 30 to 1,000, from 30 to 1,500, from 30 to 3,000, from 30 to 5,000, from 30 to 7,000, from 30 to 10,000, from 30 to 25,000, from 30 to 50,000, from 30 to 70,000, from 100 to 250, from 100 to 500, from 100 to 1,000, from 100 to 1,500, from 100 to 3,000, from 100 to 5,000, from 100 to 7,000, from 100 to 10,000, from 100 to 25,000, from 100 to 50,000, from 100 to 70,000, from 100 to 100,000, from 500 to 1,000, from 500 to 1,500, from 500 to 2,000, from 500 to 3,000, from 500 to 5,000, from 500 to 7,000, from 500 to 10,000, from 500 to 25,000, from 500 to 50,000, from 500 to 70,000, from 500 to 100,000, from 1,000 to 1,500, from 1,000 to 2,000, from 1,000 to 3,000, from 1,000 to 5,000, from 1,000 to 7,000, from 1,000 to 10,000, from 1,000 to 25,000, from 1,000 to 50,000, from 1,000 to 70,000, from 1,000 to 100,000, from 1,500 to 3,000, from 1,500 to 5,000, from 1,500 to 7,000, from 1,500 to 10,000, from 1,500 to 25,000, from 1,500 to 50,000, from 1,500 to 70,000, from 1,500 to 100,000, from 2,000 to 3,000, from 2,000 to 5,000, from 2,000 to 7,000, from 2,000 to 10,000, from 2,000 to 25,000, from 2,000 to 50,000, from 2,000 to 70,000, and from 2,000 to 100,000).

[00056] In one embodiment, the polynucleotides of the present invention may encode at least one peptide or polypeptide of interest. In another embodiment, the polynucleotides of the present invention may be non-coding.

[00057] In one embodiment, the length of a region encoding at least one peptide polypeptide of interest of the polynucleotides present invention is greater than about 30 nucleotides in length (e.g., at least or greater than about 35, 40, 45, 50, 55, 60, 70, 80, 90, 100, 120, 140, 160, 180, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1,000, 1,100, 1,200, 1,300, 1,400, 1,500, 1,600, 1,700, 1,800, 1,900, 2,000, 2,500, and 3,000, 4,000, 5,000, 6,000, 7,000, 8,000, 9,000, 10,000, 20,000, 30,000, 40,000, 50,000, 60,000, 70,000, 80,000, 90,000 or up to and including 100,000 nucleotides). As used herein, such a region may be referred to as a "coding region" or "region encoding."

[00058] In one embodiment, the polynucleotides of the present invention is or functions as a messenger RNA (mRNA). As used herein, the term "messenger RNA" (mRNA) refers to any polynucleotide which encodes at least one peptide or polypeptide of interest and which is capable of being translated to produce the encoded peptide polypeptide of interest *in vitro, in vivo, in situ* or *ex vivo.*

[00059] In one embodiment, the polynucleotides of the present invention may be structurally modified or chemically modified. As used herein, a "structural" modification is one in which two or more linked nucleosides are inserted, deleted, duplicated, inverted or randomized in a polynucleotide without significant chemical modification to the nucleotides themselves. Because chemical bonds will necessarily be broken and reformed to effect a structural modification, structural modifications are of a chemical nature and hence are chemical modifications. However, structural modifications will result in a different sequence of nucleotides. For example, the polynucleotide "ATCG" may be chemically modified to "AT-5meC-G". The same polynucleotide may be structurally modified from "ATCG" to "ATCCCG". Here, the dinucleotide "CC" has been inserted, resulting in a structural modification to the polynucleotide.

[00060] In one embodiment, the polynucleotides of the present invention, such as IVT polynucleotides or circular polynucleotides, may have a uniform chemical modification of all or any of the same nucleoside type or a population of modifications produced by mere downward titration of the same starting modification in all or any of the same nucleoside type, or a measured percent of a chemical modification of all any of the same nucleoside type but with random incorporation, such as where all uridines are replaced by a uridine analog, e.g., pseudouridine. In another embodiment, the polynucleotides may have a uniform chemical modification of two, three, or four of the same nucleoside type throughout the entire polynucleotide (such as all uridines and all cytosines, etc. are modified in the same way).

[00061] When the polynucleotides of the present invention are chemically and/or structurally modified the polynucleotides may be referred to as "modified polynucleotides."

[00062] In one embodiment, the polynucleotides of the present invention may include a sequence encoding a self-cleaving peptide. The self-cleaving peptide may be, but is not limited to, a 2A peptide. As a non-limiting example, the 2A peptide may have the protein sequence: GSGATNFSLLKQAGDVEENPGP (SEQ ID NO: 1), fragments or variants thereof. In one embodiment, the 2A peptide cleaves between the last glycine and last proline. As another non-limiting example, the polynucleotides of the present invention may include a polynucleotide sequence encoding the 2A peptide having the protein sequence GSGATNFSLLKQAGDVEENPGP (SEQ ID NO: 1) fragments or variants thereof.

[00063] One such polynucleotide sequence encoding the 2A peptide is GGAAGCGGAGCTACTAACTTCAGCCTGCTGAAGCAGGCTGGAGACGTGGAGGAGAA CCCTGGACCT (SEQ ID NO: 2). Further, the polynucleotide sequence of the 2A peptide may be modified or codon optimized by the methods described herein and/or are known in the art.

[00064] In one embodiment, this sequence may be used to separate the coding region of two or more polypeptides of interest. As a non-limiting example, the sequence encoding the 2A peptide may be between a first coding region A and a second coding region B (A-2Apep-B). The presence of the 2A peptide would result in the cleavage of one long protein into protein A, protein B and the 2A peptide. Protein A and protein B may be the same or different peptides or polypeptides of interest. In another embodiment, the 2A peptide may be used in the polynucleotides of the present invention to produce two, three, four, five, six, seven, eight, nine, ten or more proteins.

IVT Polynucleotide Architecture

[00065] Traditionally, the basic components of an mRNA molecule include at least a coding region, a 5'UTR, a 3'UTR, a 5' cap and a poly-A tail. The IVT polynucleotides of the present invention may function as mRNA but are distinguished from wild-type mRNA in their functional and/or structural design features which serve to overcome existing problems of effective polypeptide production using nucleic-acid based therapeutics.

[00066] Figure 1 shows a primary construct **100** of an IVT polynucleotide of the present invention. As used herein, "primary construct" refers to a polynucleotide of the present invention which encodes one or more polypeptides of interest and which retains sufficient structural and/or chemical features to allow the polypeptide of interest encoded therein to be translated.

[00067] According to FIG. **1A** and **IB,** the primary construct **100** of an IVT polynucleotide here contains a first region of linked nucleotides **102** that is flanked by a first flanking region **104** and a second flaking region **106.** The first flanking region **104** may include a sequence of linked nucleosides which function as a 5' untranslated region (UTR) such as the 5' UTR of any of the nucleic acids encoding the native 5'UTR of the polypeptide or a non-native 5'UTR such as, but not limited to, a heterologous 5'UTR or a synthetic 5'UTR. The polypeptide of interest may comprise at its 5' terminus one or more signal sequences encoded by the signal sequence region **103** of the polynucleotide. The flanking region **104** may comprise a region of linked nucleotides comprising one or more complete or incomplete 5' UTRs sequences which may be completely codon optimized or partially codon optimized. The flanking region **104** may include at least one nucleic acid sequence including, but not limited to, miR sequences, TERZAK™ sequences and translation control sequences. The flanking region **104** may also comprise a 5' terminal cap **108.**

The 5' terminal capping region **108** may include a naturally occurring cap, a synthetic cap or an optimized cap. Non-limiting examples of optimized caps include the caps taught by Rhoads in US Patent No. US7074596 and International Patent Publication No. WO2008 157668, WO2009149253 and WO2013 103659, the contents of each of which are herein incorporated by reference in its entirety. The second flanking region **106** may comprise a region of linked nucleotides comprising one or more complete or incomplete 3' UTRs which may encode the native 3' UTR of the polypeptide or a non-native 3'UTR such as, but not limited to, a heterologous 3'UTR or a synthetic 3' UTR. The flanking region **106** may also comprise a 3' tailing sequence **110.** The second flanking region **106** may be completely codon optimized or partially codon optimized. The flanking region **106** may include at least one nucleic acid sequence including, but not limited to, miR sequences and translation control sequences. The 3' tailing sequence **110** may be, but is not limited to, a polyA tail, a polyC tail, a polyA-G quartet and/or a stem loop sequence.

[00068] Bridging the 5' terminus of the first region **102** and the first flanking region **104** is a first operational region **105.** Traditionally this operational region comprises a Start codon. The operational region may alternatively comprise any translation initiation sequence or signal including a Start codon.

[00069] Bridging the 3' terminus of the first region **102** and the second flanking region **106** is a second operational region **107.** Traditionally this operational region comprises a Stop codon. The operational region may alternatively comprise any translation initiation sequence or signal including a Stop codon. Multiple serial stop codons may also be used in the IVT polynucleotide. In one embodiment, the operation region of the present invention may comprise two stop codons. The first stop codon may be "TGA" or "UGA" and the second stop codon may be selected from the group consisting of "TAA," "TGA," "TAG," "UAA," "UGA" or "UAG."

[00070] Figure 1 shows a representative IVT polynucleotide primary construct **100** of the present invention. IVT polynucleotide primary construct refers to a polynucleotide transcript which encodes one or more polypeptides of interest and which retains sufficient structural and/or chemical features to allow the polypeptide of interest encoded therein to be translated. Non-limiting examples of polypeptides of interest and polynucleotides encoding polypeptide of interest are described in Table 6 of International Publication Nos. WO2013151666, WO2013151668, WO2013151663, WO2013151669, WO2013151670, WO2013151664, WO2013151665, WO2013151736; Tables 6 and 7 International Publication No. WO2013151672; Tables 6, 178 and 179 of International Publication No. WO2013151671;

Tables 6, 185 and 186 of International Publication No WO2013151667, the contents of each of which are incorporated herein by reference in their entirety.

[00071] Returning to FIG. **1,** the IVT polynucleotide primary construct **130** here contains a first region of linked nucleotides **132** that is flanked by a first flanking region **134** and a second flaking region **136.** As used herein, the "first region" may be referred to as a "coding region" or "region encoding" or simply the "first region." This first region may include, but is not limited to, the encoded polypeptide of interest. In one aspect, the first region **132** may include, but is not limited to, the open reading frame encoding at least one polypeptide of interest. The open reading frame may be codon optimized in whole or in part. The flanking region **134** may comprise a region of linked nucleotides comprising one or more complete or incomplete 5' UTRs sequences which may be completely codon optimized or partially codon optimized. The flanking region **134** may include at least one nucleic acid sequence including, but not limited to, miR sequences, TERZAK™ sequences and translation control sequences. The flanking region **134** may also comprise a 5' terminal cap **138.** The 5' terminal capping region **138** may include a naturally occurring cap, a synthetic cap or an optimized cap. Non-limiting examples of optimized caps include the caps taught by Rhoads in US Patent No. US7074596 and International Patent Publication No. WO2008157668, WO2009149253 and WO2013 103659. The second flanking region **106** may comprise a region of linked nucleotides comprising one or more complete or incomplete 3' UTRs. The second flanking region **136** may be completely codon optimized or partially codon optimized. The flanking region **134** may include at least one nucleic acid sequence including, but not limited to, miR sequences and translation control sequences. After the second flanking region **136** the IVT polynucleotide primary construct may comprise a 3' tailing sequence **140.** The 3' tailing sequence **140** may include a synthetic tailing region **142** and/or a chain terminating nucleoside **144.** Non-liming examples of a synthetic tailing region include a polyA sequence, a polyC sequence, a polyA-G quartet. Non-limiting examples of chain terminating nucleosides include 2'-0 methyl, F and locked nucleic acids (LNA).

[00072] Bridging the 5' terminus of the first region **132** and the first flanking region **134** is a first operational region **144.** Traditionally this operational region comprises a Start codon. The operational region may alternatively comprise any translation initiation sequence or signal including a Start codon.

[00073] Bridging the 3' terminus of the first region **132** and the second flanking region **136** is a second operational region **146.** Traditionally this operational region comprises a Stop codon. The operational region may alternatively comprise any translation initiation sequence or signal

including a Stop codon. According to the present invention, multiple serial stop codons may also be used.

[00074] The shortest length of the first region of the primary construct of the IVT polynucleotide of the present invention can be the length of a nucleic acid sequence that is sufficient to encode for a dipeptide, a tripeptide, a tetrapeptide, a pentapeptide, a hexapeptide, a heptapeptide, an octapeptide, a nonapeptide, or a decapeptide. In another embodiment, the length may be sufficient to encode a peptide of 2-30 amino acids, e.g. 5-30, 10-30, 2-25, 5-25, 10-25, or 10-20 amino acids. The length may be sufficient to encode for a peptide of at least 11, 12, 13, 14, 15, 17, 20, 25 or 30 amino acids, or a peptide that is no longer than 40 amino acids, e.g. no longer than 35, 30, 25, 20, 17, 15, 14, 13, 12, 11 or 10 amino acids. Examples of dipeptides that the polynucleotide sequences can encode or include, but are not limited to, carnosine and anserine.

[00075] The length of the first region of the primary construct of the IVT polynucleotide encoding the polypeptide of interest of the present invention is greater than about 30 nucleotides in length (e.g., at least or greater than about 35, 40, 45, 50, 55, 60, 70, 80, 90, 100, 120, 140, 160, 180, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1,000, 1,100, 1,200, 1,300, 1,400, 1,500, 1,600, 1,700, 1,800, 1,900, 2,000, 2,500, and 3,000, 4,000, 5,000, 6,000, 7,000, 8,000, 9,000, 10,000, 20,000, 30,000, 40,000, 50,000, 60,000, 70,000, 80,000, 90,000 or up to and including 100,000 nucleotides).

[00076] In some embodiments, the IVT polynucleotide includes from about 30 to about 100,000 nucleotides (e.g., from 30 to 50, from 30 to 100, from 30 to 250, from 30 to 500, from 30 to 1,000, from 30 to 1,500, from 30 to 3,000, from 30 to 5,000, from 30 to 7,000, from 30 to 10,000, from 30 to 25,000, from 30 to 50,000, from 30 to 70,000, from 100 to 250, from 100 to 500, from 100 to 1,000, from 100 to 1,500, from 100 to 3,000, from 100 to 5,000, from 100 to 7,000, from 100 to 10,000, from 100 to 25,000, from 100 to 50,000, from 100 to 70,000, from 100 to 100,000, from 500 to 1,000, from 500 to 1,500, from 500 to 2,000, from 500 to 3,000, from 500 to 5,000, from 500 to 7,000, from 500 to 10,000, from 500 to 25,000, from 500 to 50,000, from 500 to 70,000, from 500 to 100,000, from 1,000 to 1,500, from 1,000 to 2,000, from 1,000 to 3,000, from 1,000 to 5,000, from 1,000 to 7,000, from 1,000 to 10,000, from 1,000 to 25,000, from 1,000 to 50,000, from 1,000 to 70,000, from 1,000 to 100,000, from 1,500 to 3,000, from 1,500 to 5,000, from 1,500 to 7,000, from 1,500 to 10,000, from 1,500 to 25,000, from 1,500 to 50,000, from 1,500 to 70,000, from 1,500 to 100,000, from 2,000 to 3,000, from 2,000 to 5,000, from 2,000 to 7,000, from 2,000 to 10,000, from 2,000 to 25,000, from 2,000 to 50,000, from 2,000 to 70,000, and from 2,000 to 100,000).

[00077] According to the present invention, the first and second flanking regions of the IVT polynucleotide may range independently from 15-1,000 nucleotides in length (e.g., greater than 30, 40, 45, 50, 55, 60, 70, 80, 90, 100, 120, 140, 160, 180, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, and 900 nucleotides or at least 30, 40, 45, 50, 55, 60, 70, 80, 90, 100, 120, 140, 160, 180, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, and 1,000 nucleotides).

[00078] According to the present invention, the tailing sequence of the IVT polynucleotide may range from absent to 500 nucleotides in length (e.g., at least 60, 70, 80, 90, 120, 140, 160, 180, 200, 250, 300, 350, 400, 450, or 500 nucleotides). Where the tailing region is a polyA tail, the length may be determined in units of or as a function of polyA Binding Protein binding. In this embodiment, the polyA tail is long enough to bind at least 4 monomers of PolyA Binding Protein. PolyA Binding Protein monomers bind to stretches of approximately 38 nucleotides. As such, it has been observed that polyA tails of about 80 nucleotides and 160 nucleotides are functional.

[00079] According to the present invention, the capping region of the IVT polynucleotide may comprise a single cap or a series of nucleotides forming the cap. In this embodiment the capping region may be from 1 to 10, e.g. 2-9, 3-8, 4-7, 1-5, 5-10, or at least 2, or 10 or fewer nucleotides in length. In some embodiments, the cap is absent.

[00080] According to the present invention, the first and second operational regions of the IVT polynucleotide may range from 3 to 40, e.g., 5-30, 10-20, 15, or at least 4, or 30 or fewer nucleotides in length and may comprise, in addition to a Start and/or Stop codon, one or more signal and/or restriction sequences.

[00081] In one embodiment, the IVT polynucleotides of the present invention may be structurally modified or chemically modified. When the IVT polynucleotides of the present invention are chemically and/or structurally modified the polynucleotides may be referred to as "modified IVT polynucleotides."

[00082] In one embodiment, if the IVT polynucleotides of the present invention are chemically modified they may have a uniform chemical modification of all or any of the same nucleoside type or a population of modifications produced by mere downward titration of the same starting modification in all or any of the same nucleoside type, or a measured percent of a chemical modification of all any of the same nucleoside type but with random incorporation, such as where all uridines are replaced by a uridine analog, e.g., pseudouridine. In another embodiment, the IVT polynucleotides may have a uniform chemical modification of two, three, or four of the same nucleoside type throughout the entire polynucleotide (such as all uridines and all cytosines, etc. are modified in the same way).

[00083] In one embodiment, the IVT polynucleotides of the present invention may include a sequence encoding a self-cleaving peptide, described herein, such as but not limited to the 2A peptide. The polynucleotide sequence of the 2A peptide in the IVT polynucleotide may be modified or codon optimized by the methods described herein and/or are known in the art.

[00084] In one embodiment, this sequence may be used to separate the coding region of two or more polypeptides of interest in the IVT polynucleotide.

[00085] In one embodiment, the IVT polynucleotide of the present invention may be structurally and/or chemically modified. When chemically modified and/or structurally modified the IVT polynucleotide may be referred to as a "modified IVT polynucleotide."

[00086] In one embodiment, the IVT polynucleotide may encode at least one peptide or polypeptide of interest. In another embodiment, the IVT polynucleotide may encode two or more peptides or polypeptides of interest. Non-limiting examples of peptides or polypeptides of interest include heavy and light chains of antibodies, an enzyme and its substrate, a label and its binding molecule, a second messenger and its enzyme or the components of multimeric proteins or complexes.

[00087] In one embodiment, the IVT polynucleotide may include modified nucleosides such as, but not limited to, the modified nucleosides described in US Patent Publication No. US201301 15272 including pseudouridine, 1-methylpseudouridine, 5-methoxyuridine and 5-methylcytosine. As a non-limiting example, the IVT polynucleotide may include 1-methylpseudouridine and 5-methylcytosine. As another non-limiting example, the IVT polynucleotide may include 1-methylpseudouridine. As yet another non-limiting example, the IVT polynucleotide may include 5-methoxyuridine and 5-methylcytosine. As a non-limiting example, the IVT polynucleotide may include 5-methoxyuridine.

[00088] IVT polynucleotides (such as, but not limited to, primary constructs), formulations and compositions comprising IVT polynucleotides, and methods of making, using and administering IVT polynucleotides are described in co-pending International Publication Nos. WO2013151666, WO2013151668, WO2013151663, WO2013151669, WO2013151670, WO2013151664, WO2013151665, WO2013151671, WO2013151672, WO2013151667 and WO2013 15 1736; the contents of each of which are herein incorporated by reference in their entireties.

Chimeric Polynucleotide Architecture

[00089] The chimeric polynucleotides or RNA constructs of the present invention maintain a modular organization similar to IVT polynucleotides, but the chimeric polynucleotides comprise one or more structural and/or chemical modifications or alterations which impart useful

properties to the polynucleotide. As such, the chimeric polynucleotides which are modified mRNA molecules of the present invention are termed "chimeric modified mRNA" or "chimeric mRNA."

[00090] Chimeric polynucleotides have portions or regions which differ in size and/or chemical modification pattern, chemical modification position, chemical modification percent or chemical modification population and combinations of the foregoing.

[00091] Examples of parts or regions, where the chimeric polynucleotide functions as an mRNA and encodes a polypeptide of interest include, but are not limited to, untranslated regions (UTRs, such as the 5' UTR or 3' UTR), coding regions, cap regions, polyA tail regions, start regions, stop regions, signal sequence regions, and combinations thereof. Figure 2 illustrates certain embodiments of the chimeric polynucleotides of the invention which may be used as mRNA. Figure 3 illustrates a schematic of a series of chimeric polynucleotides identifying various patterns of positional modifications and showing regions analogous to those regions of an mRNA polynucleotide. Regions or parts that join or lie between other regions may also be designed to have subregions. These are shown in the figure.

[00092] In some embodiments, the chimeric polynucleotides of the invention have a structure comprising Formula I.

$$5' \ [A_n]_x\text{–}L1\text{-}[\mathbf{Bo}]_y\text{–}L2\text{-}[Cp]_z\text{-}L3 \ 3'$$

Formula I

[00093] wherein:

[00094] each of A and **B** independently comprise a region of linked nucleosides;

[00095] C is an optional region of linked nucleosides;

[00096] at least one of regions A, **B,** or C is positionally modified, wherein the positionally modified region comprises at least two chemically modified nucleosides of one or more of the same nucleoside type of adenosine, thymidine, guanosine, cytidine, or uridine, and wherein at least two of the chemical modifications of nucleosides of the same type are different chemical modifications;

[00097] n, o and p are independently an integer between 15-1000;

[00098] x and y are independently 1-20;

[00099] z is 0-5;

[000100] L I and L2 are independently optional linker moieties, the linker moieties being either nucleic acid based or non-nucleic acid based; and

[000101] L3 is an optional conjugate or an optional linker moiety, the linker moiety being either nucleic acid based or non-nucleic acid based.

[000102] In some embodiments the chimeric polynucleotide of Formula I encodes one or more peptides or polypeptides of interest. Such encoded molecules may be encoded across two or more regions.

[000103] Also provided are methods of making and using the chimeric polynucleotides in research, diagnostics and therapeutics.

[000104] In another aspect, the invention features a chimeric polynucleotide encoding a polypeptide, wherein the polynucleotide has a sequence including Formula II:

$$[A_n]\text{-}L^1\text{-}[B_o]$$

Formula II

[000105] wherein each A and B is independently any nucleoside;

[000106] n and o are, independently 10 to 10,000, e.g., 10 to 1000 or 10 to 2000; and

[000107] L^1 has the structure of Formula III:

$$\text{-}(R^1)_a\text{-}(R^2)_b\text{-}(R^3)_c\text{-}R^4\text{-}(R^5)_d\text{-}(R^6)_e\text{-}(R^7)_f\text{-}$$

Formula III

[000108] wherein a, b, c, d, e, and f are each, independently, 0 or 1;

[000109] each of R^1, R^3, R^5, and R^7, is, independently, selected from optionally substituted Ci-C_6 alkylene, optionally substituted C1-C6 heteroalkylene, O, S, and NR^8;

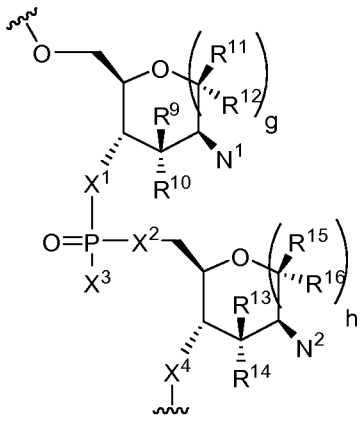
[000110] R^2 and R^6 are each, independently, selected from carbonyl, thiocarbonyl, sulfonyl, or phosphoryl;

[000111] R^4 is optionally substituted Ci-Cio alkylene, optionally substituted C2-$C1_0$ alkenylene, optionally substituted C2-C10 alkynylene, optionally substituted C2-C9 heterocyclylene, optionally substituted C_6-C12 arylene, optionally substituted C2-$C1_{00}$ polyethylene glycolene, or optionally substituted C_1-$C1_0$ heteroalkylene, or a bond linking $(R^1)_a$-$(R^2)_b$-$(R^3)_c$ to $(R^5)_d$-$(R^6)_e$-$(R^7)_f$, wherein if a, b, c, d, e, and f are 0, R^4 is not a bond; and

[000112] R^8 is hydrogen, optionally substituted C1-C4 alkyl, optionally substituted C2-C4 alkenyl, optionally substituted C2-C4 alkynyl, optionally substituted C2-C6 heterocyclyl, optionally substituted C_6-C12 aryl, or optionally substituted C1-C7 heteroalkyl;

[000113] wherein L^1 is attached to $[A_n]$ and $[B_0]$ at the sugar of one of the nucleosides (e.g., at the 3' position of a five-membered sugar ring or 4' position of a six membered sugar ring of a nucleoside of $[A_n]$ and the 5' position of a five-membered sugar ring or 6' position of of a six membered sugar ring of a nucleoside of $[B_0]$ or at the 5' position of a five-membered sugar ring or 6' position of of a six membered sugar ring of a nucleoside of $[A_n]$ and the 3' position of a five-membered sugar ring or 4' position of a six membered sugar ring of a nucleoside of $[B_0]$).

[000114] In some embodiments, at least one of [A_n] and [B_0] includes the structure of Formula IV:



Formula IV

[000115] wherein each of N^1 and N^2 is independently a nucleobase;

[000116] each of R^9, R^{10}, R^{11}, R^{12}, R^{13}, R^{14}, R^{15}, and R^{16} is, independently, H, halo, hydroxy, thiol, optionally substituted Ci-C_6 alkyl, optionally substituted Ci-C_6 heteroalkyl, optionally substituted C2-C6 heteroalkenyl, optionally substituted C2-C6 heteroalkynyl, optionally substituted amino, azido, or optionally substituted C_6-$C1_0$ aryl;

[000117] each of g and h is, independently, 0 or 1;

[000118] each X^1 and X^4 is, independently, O, NH, or S;

[000119] each X^2 is independently O or S; and

[000120] each X^3 is OH or SH, or a salt thereof.

[000121] In another aspect, the invention features a chimeric polynucleotide encoding a polypeptide, wherein the polynucleotide has a sequence including Formula II:

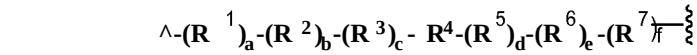
[A_n]-L^1-[B_0]

Formula II

[000122] wherein each A and B is independently any nucleoside;

[000123] n and 0 are, independently 10 to 10,000, e.g., 10 to 1000 or 10 to 2000; and

[000124] L^1 is a bond or has the structure of Formula III:



Formula III

[000125] wherein a, b, c, d, e, and f are each, independently, 0 or 1;

[000126] each of R^1, R^3, R^5, and R^7, is, independently, selected from optionally substituted C1-C6 alkylene, optionally substituted C1-C6 heteroalkylene, O, S, and NR^8;

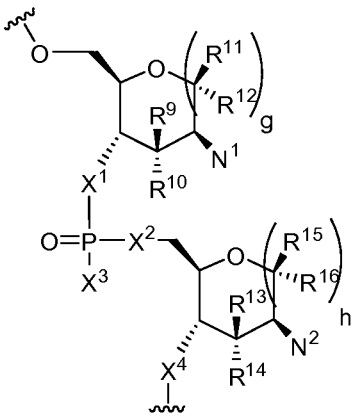
[000127] R^2 and R^6 are each, independently, selected from carbonyl, thiocarbonyl, sulfonyl, or phosphoryl;

[000128] R^4 is optionally substituted Ci-Cio alkylene, optionally substituted C2-$C1_0$ alkenylene, optionally substituted C2-$C1_0$ alkynylene, optionally substituted C2-C9 heterocyclylene, optionally substituted C_6-C12 arylene, optionally substituted C2-$C1_{00}$ polyethylene glycolene, or optionally substituted C_1-$C1_0$ heteroalkylene, or a bond linking $(R^1)_a$-$(R^2)_b$-$(R^3)_c$ to $(R^5)_d$-(R^6)e-$(R^7)_f$; and

[000129] R^8 is hydrogen, optionally substituted C_1-C_4 alkyl, optionally substituted C2-C4 alkenyl, optionally substituted C2-C4 alkynyl, optionally substituted C2-C6 heterocyclyl, optionally substituted C_6-C12 aryl, or optionally substituted C1-C7 heteroalkyl;

[000130] wherein L^1 is attached to $[A_n]$ and $[B_0]$ at the sugar of one of the nucleosides (e.g., at the 3' position of a five-membered sugar ring or 4' position of a six membered sugar ring of a nucleoside of $[A_n]$ and the 5' position of a five-membered sugar ring or 6' position of of a six membered sugar ring of a nucleoside of $[B_0]$ or at the 5' position of a five-membered sugar ring or 6' position of of a six membered sugar ring of a nucleoside of $[A_n]$ and the 3' position of a five-membered sugar ring or 4' position of a six membered sugar ring of a nucleoside of $[B_0]$).

[000131] wherein at least one of $[A_n]$ or $[B_0]$ includes the structure of Formula IV:



Formula IV

[000132] wherein each of N^1 and N^2 is independently a nucleobase;

[000133] each of R^9, R^{10}, R^{11}, R^{12}, R^{13}, R^{14}, R^{15}, and R^{16} is, independently, H, halo, hydroxy, thiol, optionally substituted C1-C6 alkyl, optionally substituted C1-C6 heteroalkyl, optionally substituted C2-C6 heteroalkenyl, optionally substituted C2-C6 heteroalkynyl, optionally substituted amino, azido, or optionally substituted C_6-$C1_0$ aryl;

[000134] each of g and h is, independently, 0 or 1;

[000135] each X^1 and X^4 is, independently, O, NH, or S; and

[000136] each X^2 is independently O or S; and

[000137] each X^3 is OH or SH, or a salt thereof;

[000138] wherein at least one of X^1, X^2, or X^4 is NH or S.

[000139] In some embodiments, X^1 is NH. In other embodiments, X^4 is NH. In certain embodiments, X^2 is S.

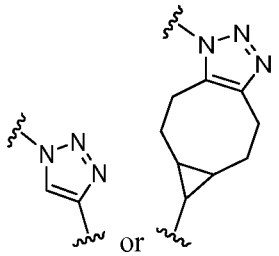
[000140] In some embodiments, the polynucleotide includes: (a) a coding region; (b) a 5' UTR including at least one Kozak sequence; (c) a 3' UTR; and (d) at least one 5' cap structure. In other embodiments, the polynucleotide further includes (e) a poly-A tail.

[000141] In some embodiments, one of the coding region, the 5' UTR including at least one Kozak sequence, the 3' UTR, the 5' cap structure, or the poly-A tail includes $[A_n]$-L^1-$[B_0]$.

[000142] In other embodiments, one of the coding region, the 5' UTR including at least one Kozak sequence, the 3' UTR, the 5' cap structure, or the poly-A tail includes $[A_n]$ and another of the coding region, the 5' UTR including at least one Kozak sequence, the 3' UTR, the 5' cap structure, or the poly-A tail includes $[B_0]$.

[000143] In certain embodiments, the polynucleotide includes at least one modified nucleoside (e.g., a nucleoside described herein).

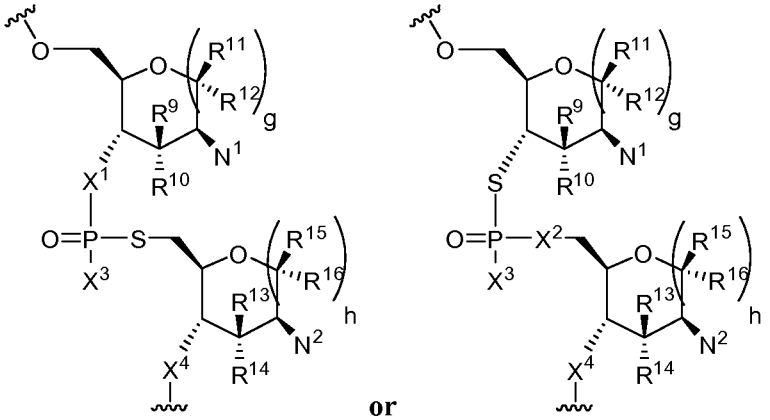
[000144] In some embodiments, R^4 is optionally substituted $_{C2-9}$ heterocyclylene, for example, the heterocycle may have the structure:



[000145] In certain embodiments, L^1 is attached to $[A_n]$ at the 3' position of a five-membered sugar ring or 4' position of a six membered sugar ring of one of the nucleosides and to $[B_0]$ at the 5' position of a five-membered sugar ring or 6' position of of a six membered sugar ring of one of the nucleosides.

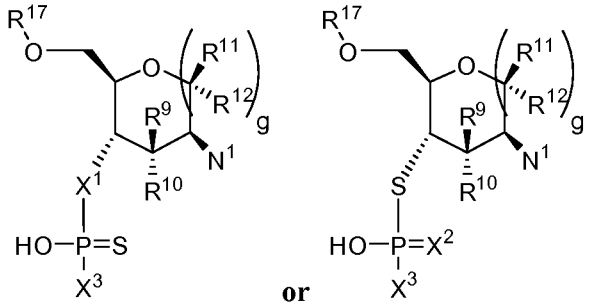
[000146] In some embodiments, the polynucleotide is circular.

[000147] In another aspect, the invention features a method of producing a composition including a chimeric polynucleotide encoding a polypeptide, wherein the polynucleotide includes the structure of Formula Va or Vb:



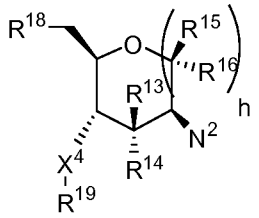
Formula Va **Formula Vb**

[000148] This method includes reacting a compound having the structure of Formula Via or VIb:



Formula Via **Formula VIb**

with a compound having the structure of Formula VII:



Formula VII

[000149] wherein each of N^1 and N^2 is, independently, a nucleobase;

[000150] each of R^9, R^{10}, R^{11}, R^{12}, R^{13}, R^{14}, R^{15}, and R^{16} is, independently, H, halo, hydroxy, thiol, optionally substituted Ci-C_6 alkyl, optionally substituted Ci-C_6 heteroalkyl, optionally substituted C2-C6 heteroalkenyl, optionally substituted C2-C6 heteroalkynyl, optionally substituted amino, azido, or optionally substituted C_6-$C1_0$ aryl;

[000151] each of g and h is, independently, 0 or 1;

[000152] each X^1 and X^4 is, independently, O, NH, or S;

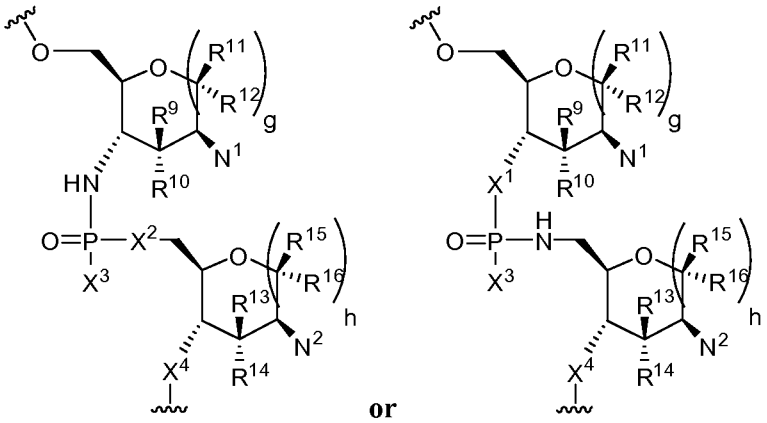
[000153] each X^2 is O or S; and

[000154] each X^3 is independently OH or SH, or a salt thereof;

[000155] each of $\mathbf{R^{17}}$ and $\mathbf{R^{19}}$ is, independently, a region of linked nucleosides; and

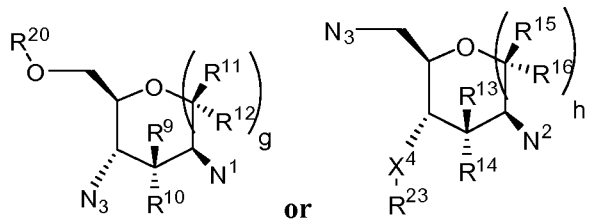
[000156] $\mathbf{R^{18}}$ is a halogen.

[000157] In another aspect, the invention features a method of producing a composition including a chimeric polynucleotide encoding a polypeptide, wherein the polynucleotide includes the structure of Formula Villa or Vlllb:



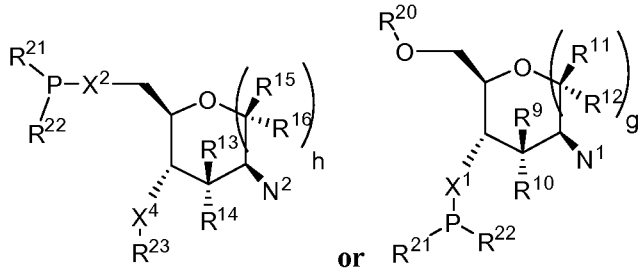
Formula Villa **or** **Formula Vlllb**

[000158] This method includes reacting a compound having the structure of Formula IXa or IXb:



Formula IXa **or** **Formula IXb**

with a compound having the structure of Formula Xa or Xb:



Formula Xa **or** **Formula Xb**

[000159] wherein each of N^1 and N^2 is, independently, a nucleobase;

[000160] each of $\mathbf{R^9}$, $\mathbf{R^{10}}$, $\mathbf{R^{11}}$, $\mathbf{R^{12}}$, $\mathbf{R^{13}}$, $\mathbf{R^{14}}$, $\mathbf{R^{15}}$, and $\mathbf{R^{16}}$ is, independently, H, halo, hydroxy, thiol, optionally substituted Ci-C_6 alkyl, optionally substituted Ci-C_6 heteroalkyl, optionally

substituted C2-C6 heteroalkenyl, optionally substituted C2-C6 heteroalkynyl, optionally substituted amino, azido, or optionally substituted C_6-C_{10} aryl;

[000161] each of g and h is, independently, 0 or 1;

[000162] each X^4 is, independently, O, NH, or S; and

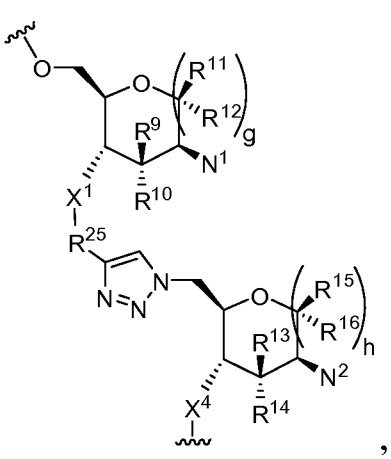
[000163] each X^1 and X^2 is independently O or S;

[000164] each X^3 is independently OH, SH, or a salt thereof;

[000165] each of R^{20} and R^{23} is, independently, a region of linked nucleosides; and

[000166] each of R^{21} and R^{22} is, independently, optionally substituted ***Ci-Ce*** alkoxy.

[000167] In another aspect, the invention features a method of producing a composition including a chimeric polynucleotide encoding a polypeptide, wherein the polynucleotide includes the structure of Formula XIa, XIb, XIIa, or XIIb:



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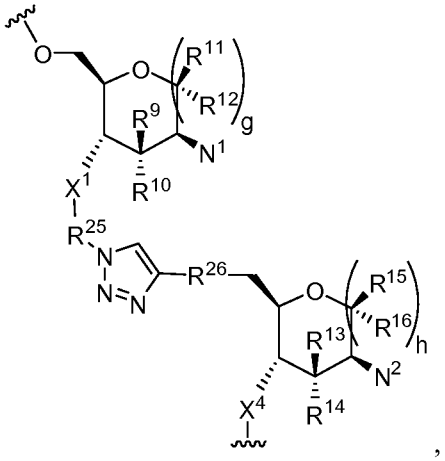
Formula XIa



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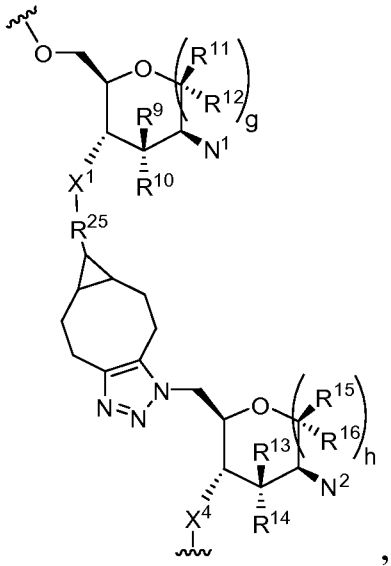
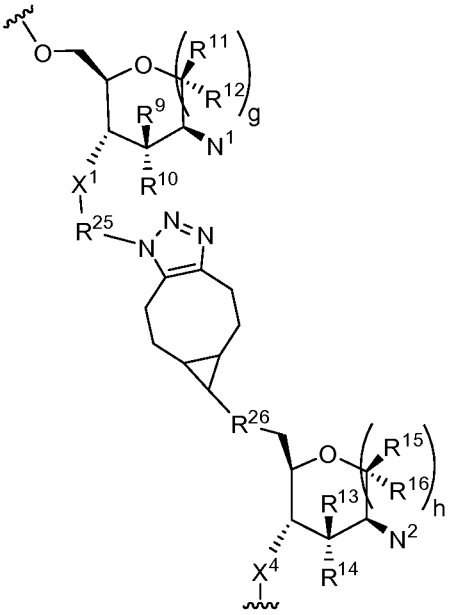
Formula XIb

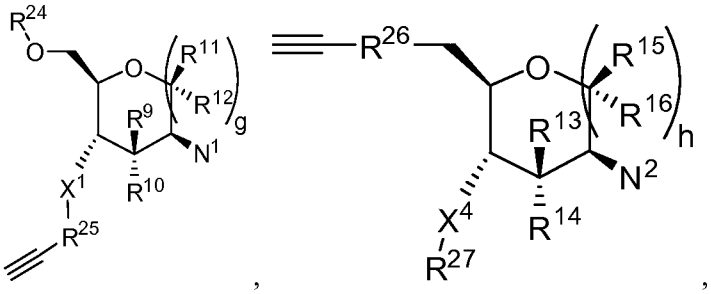


, or

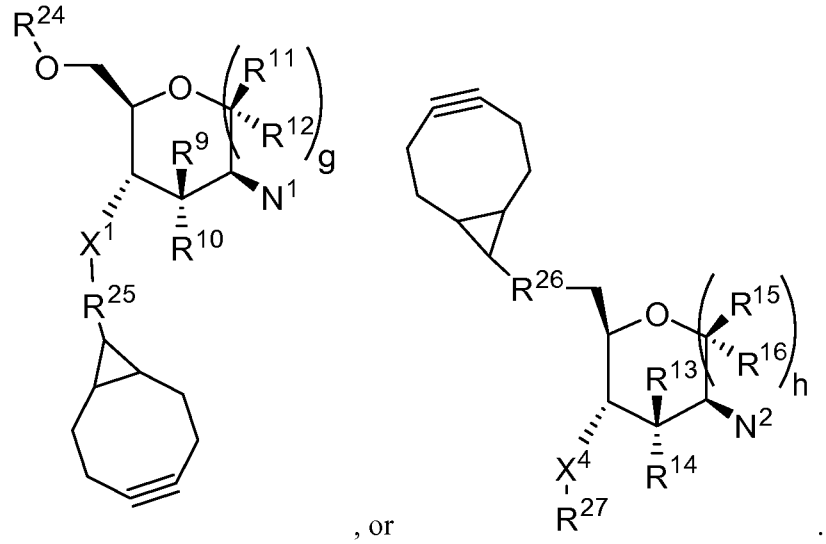


Formula XIIa **Formula XIIb.**

[000168] This method includes reacting a compound having the structure of Formula XIIIa, XHIb, XlVa, or XlVb:

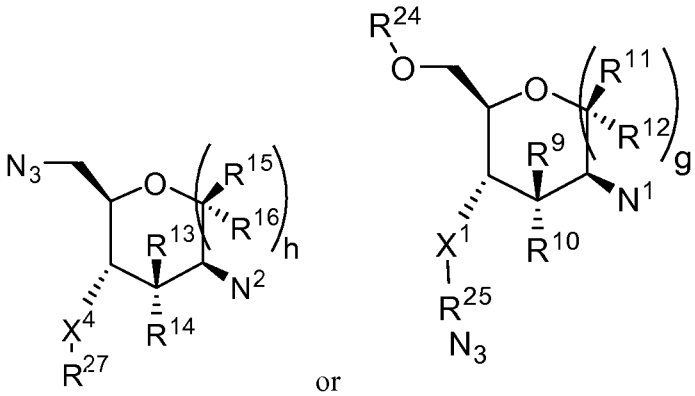
[000169] 

Formula XIIIa **Formula XHIb**

[000170] 

Formula XlVa **Formula XlVb**

[000171] with a compound having the structure of Formula XVa or XVb:

[000172] 

Formula XVa **Formula XVb**

[000173] wherein each of N^1 and N^2 is, independently, a nucleobase;

[000174] each of R^9, R^{10}, R^{11}, R^{12}, R^{13}, R^{14}, R^{15}, and R^{16} is, independently, H, halo, hydroxy, thiol, optionally substituted Ci-C_6 alkyl, optionally substituted Ci-C_6 heteroalkyl, optionally substituted C2-C6 heteroalkenyl, optionally substituted C2-C6 heteroalkynyl, optionally substituted amino, azido, or optionally substituted C_6-C_{10} aryl;

[000175] each of g and h is, independently, 0 or 1;

[000176] each X^1 and X^4 is, independently, absent, O, NH, or S or a salt thereof;

[000177] each of R^{24} and R^{27} is, independently, a region of linked nucleosides; and

[000178] each of R^{25} and R^{26} is absent or optionally substituted ***Ci-Ce*** alkylene or optionally substituted ***Ci-Ce*** heteroalkylene or R^{25} and the alkynyl group together form optionally substituted cycloalkynyl.

[000179] In another aspect, the invention features a method of producing a composition including a chimeric polynucleotide encoding a polypeptide, wherein the polynucleotide has a sequence including Formula II:

$$[A_\eta]\text{-}L^1\text{-}[B_0],$$

Formula II

[000180] This method includes reacting a compound having the structure of Formula XVI

$$[A_n]\text{-}(R^1)_a\text{-}(R^2)_b\text{-}(R^3)_c\text{-}N_3$$

Formula XVI

with a compound having the structure of Formula XVII:

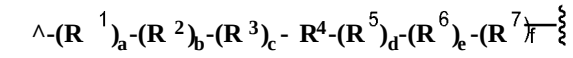
$$R^{27}\text{-}(R^5)_d\text{-}(R^6)_e\text{-}(R^7)_f\text{-}[B_0]$$

Formula XVII

[000181] wherein each A and B is independently any nucleoside;

[000182] n and 0 are, independently 10 to 10,000, e.g., 10 to 1000 or 10 to 2000; and

[000183] L^1 has the structure of Formula III:



Formula III

[000184] wherein a, b, c, d, e, and f are each, independently, 0 or 1;

[000185] R^1, R^3, R^5, and R^7 each, independently, is selected from optionally substituted ***Ci-Ce*** alkylene, optionally substituted ***Ci-Ce*** heteroalkylene, O, S, and NR^8;

[000186] R^2 and R^6 are each, independently, selected from carbonyl, thiocarbonyl, sulfonyl, or phosphoryl;

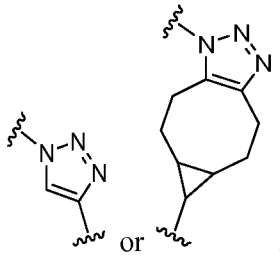
[000187] R^4 is an optionally substituted triazolene; and

[000188] R^8 is hydrogen, optionally substituted C1-C4 alkyl, optionally substituted $_{C3-C4}$ alkenyl, optionally substituted C2-C4 alkynyl, optionally substituted C2-C6 heterocyclyl, optionally substituted $_{C6}$-C12 aryl, or optionally substituted C1-C7 heteroalkyl; and

[000189] R^{27} is an optionally substituted C2-C3 alkynyl or an optionally substituted C_8-C_{12} cycloalkynyl,

[000190] wherein L^1 is attached to $[A_n]$ and $[B_0]$ at the sugar of one of the nucleosides.

[000191] In some embodiments, the optionally substituted triazolene has the structure:



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[000192] The details of various embodiments of the invention are set forth in the description below. Other features, objects, and advantages of the invention will be apparent from the description and the drawings, and from the claims.

[000193] In one embodiment, at least one of the regions of linked nucleosides of A may comprise a sequence of linked nucleosides which can function as a 5' untranslated region (UTR). The sequence of linked nucleosides may be a natural or synthetic 5' UTR. As a non-limiting example, the chimeric polynucleotide may encode a polypeptide of interest and the sequence of linked nucleosides of A may encode the native 5' UTR of a polypeptide encoded by the chimeric polynucleotide or the sequence of linked nucleosides may be a non-heterologous 5' UTR such as, but not limited to a synthetic UTR.

[000194] In another embodiment, at least one of the regions of linked nucleosides of A may be a cap region. The cap region may be located 5' to a region of linked nucleosides of A functioning as a 5'UTR. The cap region may comprise at least one cap such as, but not limited to, CapO, Capl, ARCA, inosine, Nl-methyl-guanosine, 2'fluoro-guanosine, 7-deaza-guanosine, 8-oxo-guanosine, 2-amino-guanosine, LNA-guanosine, 2-azido-guanosine, Cap2 and Cap4.

[000195] In one embodiment, at least one of the regions of linked nucleosdies of B may comprise at least one open reading frame of a nucleic acid sequence. The nucleic acid sequence may be codon optimized and/or comprise at least one modification.

[000196] In one embodiment, at least one of the regions of linked nucleosides of C may comprise a sequence of linked nucleosides which can function as a 3' UTR. The sequence of linked nucleosides may be a natural or synthetic 3' UTR. As a non-limiting example, the chimeric polynucleotide may encode a polypeptide of interest and the sequence of linked

nucleosides of C may encode the native 3' UTR of a polypeptide encoded by the chimeric polynucleotide or the sequence of linked nucleosides may be a non-heterologous 3' UTR such as, but not limited to a synthetic UTR.

[000197] In one embodiment, at least one of the regions of linked nucleosides of A comprises a sequence of linked nucleosides which functions as a 5' UTR and at least one of the regions of linked nucleosides of C comprises a sequence of linked nucleosides which functions as a 3' UTR. In one embodiment, the 5' UTR and the 3' UTR may be from the same or different species. In another embodiment, the 5' UTR and the 3' UTR may encode the native untranslated regions from different proteins from the same or different species.

[000198] In one aspect the chimeric polynucleotides has a sequence or structure comprising Formula I,

$$5' [A_n]_x\text{–}L1\text{-}[B_0]_y\text{–}L2\text{-}[C_p]_z\text{-}L3\ 3'$$

Formula I

[000199] wherein:

[000200] each of A and B independently comprise a region of linked nucleosides;

[000201] C is an optional region of linked nucleosides;

[000202] at least one of regions A, B, or C is positionally modified, wherein said positionally modified region comprises at least two chemically modified nucleosides of one or more of the same nucleoside type of adenosine, thymidine, guanosine, cytidine, or uridine, and wherein at least two of the chemical modifications of nucleosides of the same type are different chemical modifications;

[000203] n, o and p are independently an integer between 15-1000;

[000204] x and y are independently 1-20;

[000205] z is 0-5;

[000206] L1 and L2 are independently optional linker moieties, said linker moieties being either nucleic acid based or non-nucleic acid based; and

[000207] L3 is an optional conjugate or an optional linker moiety, said linker moiety being either nucleic acid based or non-nucleic acid based.

[000208] In some embodiments, the chimeric polynucleotides of the invention have a sequence comprising Formula II:

$$[A_\eta]\text{-}ï_J^1\text{-}[B_0]$$

Formula II

[000209] wherein each A and B is independently any nucleoside;

[000210] n and o are, independently 15 to 1000; and

[000211] L^1 has the structure of Formula III:

$$\text{^-(R}^{1})_a\text{-(R}^{2})_b\text{-(R}^{3})_c\text{- R}^4\text{-(R}^{5})_d\text{-(R}^{6})_e\text{-(R}^{7})_f\text{—}\}$$

Formula III

[000212] wherein a, b, c, d, e, and f are each, independently, 0 or 1;

[000213] each of R^1, R^3, R^5, and R^7, is, independently, selected from optionally substituted Ci-C_6 alkylene, optionally substituted *Ci-Ce* heteroalkylene, O, S, and NR^8;

[000214] R^2 and R^6 are each, independently, selected from carbonyl, thiocarbonyl, sulfonyl, or phosphoryl;

[000215] R^4 is optionally substituted Ci-Cio alkylene, optionally substituted C2-$C1_0$ alkenylene, optionally substituted C2-$C1_0$ alkynylene, optionally substituted C2-C9 heterocyclylene, optionally substituted $_{C6}$-C12 arylene, optionally substituted C2-$C1_{00}$ polyethylene glycolene, or optionally substituted $_{C1}$-$C1_0$ heteroalkylene, or a bond linking $(R^1)_a$-$(R^2)_b$-$(R^3)_c$ to $(R^5)_d$-$(R^6)_e$-$(R^7)_f$, wherein if c, d, e, f, g, and h are 0, R^4 is not a bond; and

[000216] R^8 is hydrogen, optionally substituted C1-C4 alkyl, optionally substituted C2-C4 alkenyl, optionally substituted C2-C4 alkynyl, optionally substituted C2-C6 heterocyclyl, optionally substituted $_{C6}$-C12 aryl, or optionally substituted C1-C7 heteroalkyl;

[000217] wherein L^1 is attached to $[A_n]$ and $[B_0]$ at the sugar of one of the nucleosides (e.g., at the 3' position of a sugar of a nucleoside of $[A_n]$ and the 5' position of a sugar of a nucleoside of $[B_0]$ or at the 5' position of a sugar of a nucleoside of $[A_n]$ and the 3' position of a sugar of a nucleoside of $[B_0]$).

[000218] In other embodiments, the chimeric polynucleotides of the invention have a sequence comprising Formula II:

$$[A_\eta]\text{-ï}_\text{.}^1\text{-[B}_0\text{]}$$

Formula II

[000219] wherein each A and B is independently any nucleoside;

[000220] n and 0 are, independently 15 to 1000; and

[000221] L^1 is a bond or has the structure of Formula III:

$$\}\text{- (R}^{1})_a\text{-(R}^{2})b\text{-(R}^{3})c\text{-R}^{4}\text{-(R}^{5})d\text{-(R}^{6})e\text{-(R}^{7})_f\text{—}\}$$

Formula III

[000222] wherein a, b, c, d, e, and f are each, independently, 0 or 1;

[000223] each of R^1, R^3, R^5, and R^7, is, independently, selected from optionally substituted Ci-C_6 alkylene, optionally substituted C1-C6 heteroalkylene, O, S, and NR^8;

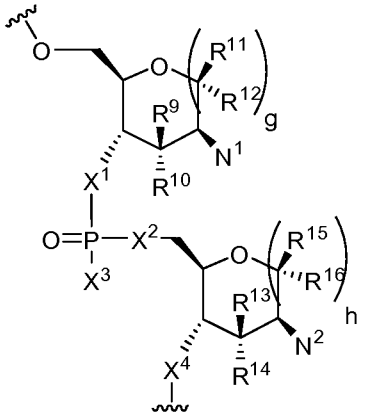
[000224] R^2 and R^6 are each, independently, selected from carbonyl, thiocarbonyl, sulfonyl, or phosphoryl;

[000225] R^4 is optionally substituted Ci-Cio alkylene, optionally substituted C2-$C1_0$ alkenylene, optionally substituted C2-$C1_0$ alkynylene, optionally substituted C2-C9 heterocyclylene, optionally substituted $_{C\,6}$-C12 arylene, optionally substituted C2-$C1_{00}$ polyethylene glycolene, or optionally substituted $_{C}$'$_1$-$C1_0$ heteroalkylene, or a bond linking $(R^1)_a$-$(R^2)_b$-$(R^3)_c$ to $(R^5)_d$-(R^6)e-$(R^7)_f$; and

[000226] R^8 is hydrogen, optionally substituted C1-C4 alkyl, optionally substituted C2-C4 alkenyl, optionally substituted C2-C4 alkynyl, optionally substituted C2-C6 heterocyclyl, optionally substituted $_{C\,6}$-C12 aryl, or optionally substituted C1-C7 heteroalkyl;

[000227] wherein L^1 is attached to $[A_n]$ and $[B_0]$ at the sugar of one of the nucleosides (e.g., at the 3' position of a sugar of a nucleoside of $[A_n]$ and the 5' position of a sugar of a nucleoside of $[B_0]$ or at the 5' position of a sugar of a nucleoside of $[A_n]$ and the 3' position of a sugar of a nucleoside of $[B_0]$);

[000228] wherein at least one of $[A_n]$ or $[B_0]$ comprises the structure of Formula IV:



Formula IV

[000229] wherein each of N^1 and N^2 is independently a nucleobase;

[000230] each of R^9, R^{10}, R^{11}, R^{12}, R^{13}, R^{14}, R^{15}, and R^{16} is, independently, H, halo, hydroxy, thiol, optionally substituted C1-C6 alkyl, optionally substituted C1-C6 heteroalkyl, optionally substituted C2-C6 heteroalkenyl, optionally substituted C2-C6 heteroalkynyl, optionally substituted amino, azido, or optionally substituted $_{C\,6}$-$C1_0$ aryl;

[000231] each of g and h is, independently, 0 or 1;

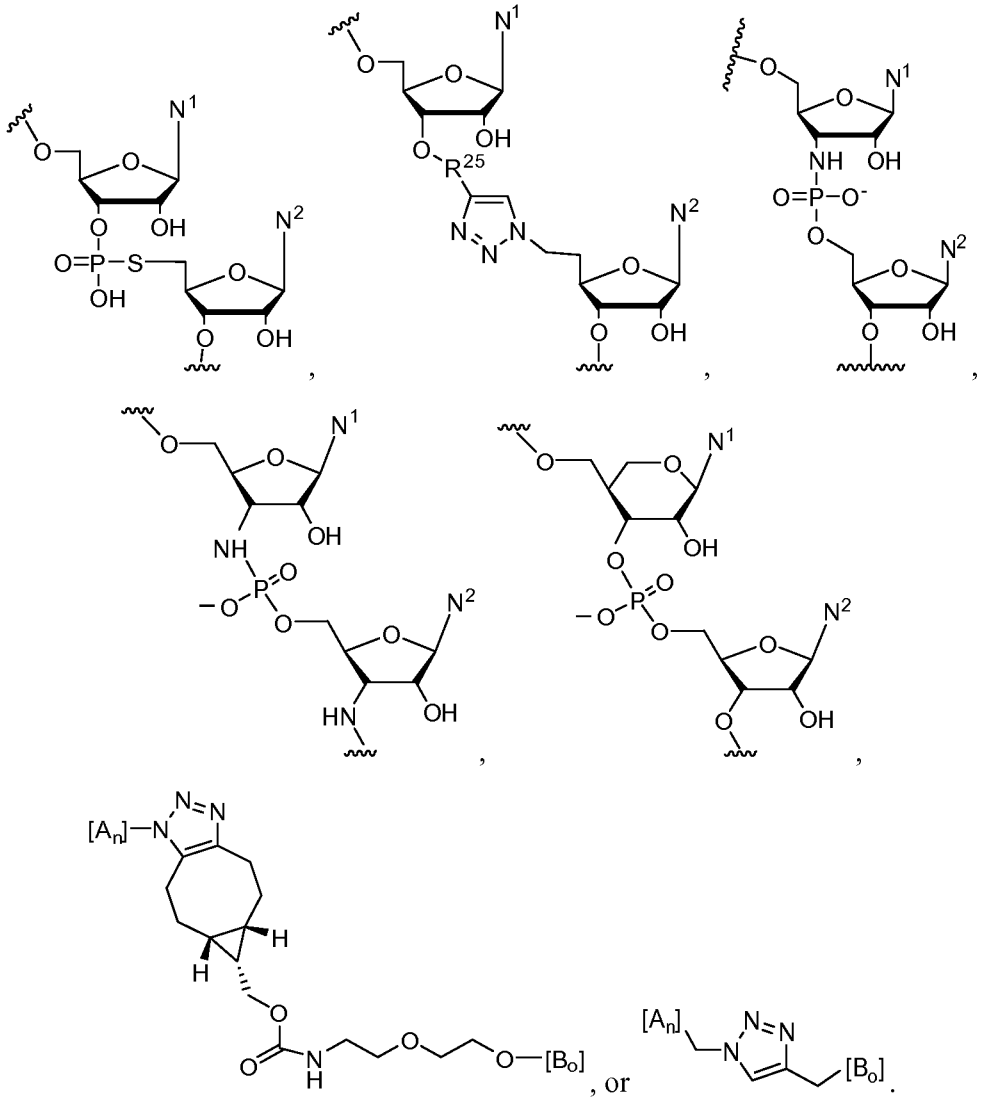
[000232] each X^1 and X^4 is, independently, O, NH, or S; and

[000233] each X^2 is independently O or S; and

[000234] each X^3 is OH or SH, or a salt thereof;

[000235] wherein at least one of X^1, X^2, or X^4 is NH or S.

[000236] For example, in some embodiments, the chimeric polynucleotides of the invention include the structure:



[000237] In other embodiments, the chimeric polynucleotides of the invention have a sequence comprising Formula II:

$$[A_n]\text{-}L^1\text{-}[B_o]$$

Formula II

[000238] wherein each A and B is independently any nucleoside;

[000239] n and o are, independently 15 to 1000; and

[000240] L^1 has the structure of Formula III:

$$\text{-}(R^1)_a\text{-}(R^2)_b\text{-}(R^3)_c\text{-}R^4\text{-}(R^5)_d\text{-}(R^6)_e\text{-}(R^7)_f\text{-}$$

Formula III

[000241] wherein a, b, c, d, e, and f are each, independently, 0 or 1;

[000242] each of R^1, R^3, R^5, and R^7, is, independently, selected from optionally substituted Ci-C_6 alkylene, optionally substituted *Ci-Ce* heteroalkylene, O, S, and NR^8;

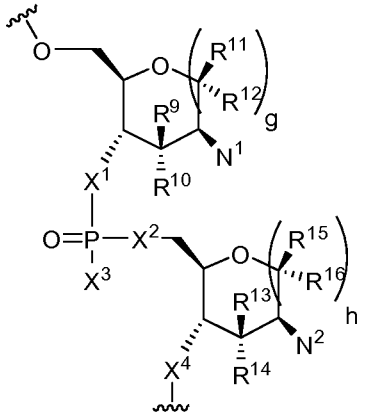
[000243] R^2 and R^6 are each, independently, selected from carbonyl, thiocarbonyl, sulfonyl, or phosphoryl;

[000244] R^4 is optionally substituted Ci-Cio alkylene, optionally substituted C2-$C1_0$ alkenylene, optionally substituted C2-$C1_0$ alkynylene, optionally substituted C2-C9 heterocyclylene, optionally substituted $_{C6}$-C12 arylene, optionally substituted C2-$C1_{00}$ polyethylene glycolene, or optionally substituted C_1-$C1_0$ heteroalkylene, or a bond linking $(R^1)_a$-$(R^2)_b$-$(R^3)_c$ to $(R^5)_d$-$(R^6)_e$-$(R^7)_f$, wherein if c, d, e, f, g, and h are 0, R^4 is not a bond; and

[000245] R^8 is hydrogen, optionally substituted C1-C4 alkyl, optionally substituted C2-C4 alkenyl, optionally substituted C2-C4 alkynyl, optionally substituted C2-C6 heterocyclyl, optionally substituted $_{C6}$-C12 aryl, or optionally substituted C1-C7 heteroalkyl;

[000246] wherein L^1 is attached to $[A_n]$ and $[B_0]$ at the sugar of one of the nucleosides (e.g., at the 3' position of a five-membered sugar ring or 4' position of a six membered sugar ring of a nucleoside of $[A_n]$ and the 5' position of a five-membered sugar ring or 6' position of of a six membered sugar ring of a nucleoside of $[B_0]$ or at the 5' position of a five-membered sugar ring or 6' position of of a six membered sugar ring of a nucleoside of $[A_n]$ and the 3' position of a five-membered sugar ring or 4' position of a six membered sugar ring of a nucleoside of $[B_0]$).

[000247] In some embodiments, at least one of $[A_n]$ and $[B_0]$ includes the structure of Formula IV:



Formula IV

[000248] wherein each of N^1 and N^2 is independently a nucleobase;

[000249] each of R^9, R^{10}, R^{11}, R^{12}, R^{13}, R^{14}, R^{15}, and R^{16} is, independently, H, halo, hydroxy, thiol, optionally substituted C1-C6 alkyl, optionally substituted C1-C6 heteroalkyl, optionally substituted C2-C6 heteroalkenyl, optionally substituted C2-C6 heteroalkynyl, optionally substituted amino, azido, or optionally substituted $_{C6}$-C10 aryl;

[000250] each of g and h is, independently, 0 or 1;

[000251] each X^1 and X^4 is, independently, O, NH, or S;

[000252] each X^2 is independently O or S; and

[000253] each X^3 is OH or SH, or a salt thereof.

[000254] In another aspect, the invention features a chimeric polynucleotide encoding a polypeptide, wherein the polynucleotide has a sequence including Formula II:

$$[A_n]\text{-}L^1\text{-}[B_0]$$

Formula II

[000255] wherein each A and B is independently any nucleoside;

[000256] n and o are, independently 15 to 1000; and

[000257] L^1 is a bond or has the structure of Formula III:

$$*\text{-}(R^1)_a\text{-}(R^2)_b\text{-}(R^3)_c\text{-}R^4\text{-}(R^5)_d\text{-}(R^6)_e\text{-}(R^7)_f\text{-}*$$

Formula III

[000258] wherein a, b, c, d, e, and f are each, independently, 0 or 1;

[000259] each of R^1, R^3, R^5, and R^7, is, independently, selected from optionally substituted C1-C6 alkylene, optionally substituted C1-C6 heteroalkylene, O, S, and NR^8;

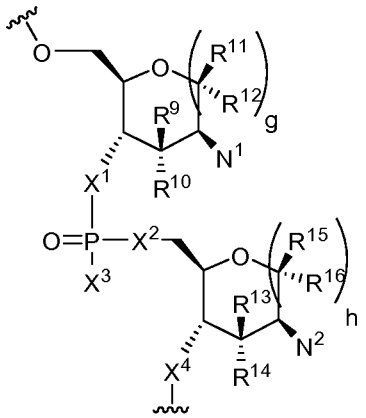
[000260] R^2 and R^6 are each, independently, selected from carbonyl, thiocarbonyl, sulfonyl, or phosphoryl;

[000261] R^4 is optionally substituted Ci-Cio alkylene, optionally substituted C2-$C1_0$ alkenylene, optionally substituted C2-$C1_0$ alkynylene, optionally substituted C2-C9 heterocyclylene, optionally substituted C_6-C12 arylene, optionally substituted C2-$C1_{00}$ polyethylene glycolene, or optionally substituted C_1-$C1_0$ heteroalkylene, or a bond linking $(R^1)_a$-$(R^2)_b$-$(R^3)_c$ to $(R^5)_d$-$(R^6)_e$-$(R^7)_f$; and

[000262] R^8 is hydrogen, optionally substituted C_1-C_4 alkyl, optionally substituted C2-C4 alkenyl, optionally substituted C2-C4 alkynyl, optionally substituted C2-C6 heterocyclyl, optionally substituted C_6-Ci2 aryl, or optionally substituted C1-C7 heteroalkyl;

[000263] wherein L^1 is attached to $[A_n]$ and $[B_0]$ at the sugar of one of the nucleosides (e.g., at the 3' position of a five-membered sugar ring or 4' position of a six membered sugar ring of a nucleoside of $[A_n]$ and the 5' position of a five-membered sugar ring or 6' position of of a six membered sugar ring of a nucleoside of $[B_0]$ or at the 5' position of a five-membered sugar ring or 6' position of of a six membered sugar ring of a nucleoside of $[A_n]$ and the 3' position of a five-membered sugar ring or 4' position of a six membered sugar ring of a nucleoside of $[B_0]$).

[000264] wherein at least one of $[A_n]$ or $[B_0]$ includes the structure of Formula IV:



Formula IV

[000265] wherein each of N^1 and N^2 is independently a nucleobase;

[000266] each of R^9, R^{10}, R^{11}, R^{12}, R^{13}, R^{14}, R^{15}, and R^{16} is, independently, H, halo, hydroxy, thiol, optionally substituted Ci-C_6 alkyl, optionally substituted Ci-C_6 heteroalkyl, optionally substituted C2-C6 heteroalkenyl, optionally substituted C2-C6 heteroalkynyl, optionally substituted amino, azido, or optionally substituted C_6-$C1_0$ aryl;

[000267] each of g and h is, independently, 0 or 1;

[000268] each X^1 and X^4 is, independently, O, NH, or S; and

[000269] each X^2 is independently O or S; and

[000270] each X^3 is OH or SH, or a salt thereof;

[000271] wherein at least one of X^1, X^2, or X^4 is NH or S.

[000272] In some embodiments, X^1 is NH. In other embodiments, X^4 is NH. In certain embodiments, X^2 is S.

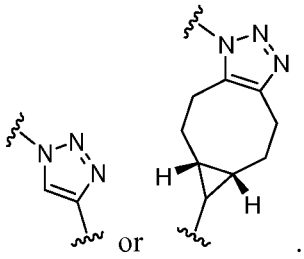
[000273] In some embodiments, the polynucleotide includes: (a) a coding region; (b) a 5' UTR including at least one Kozak sequence; (c) a 3' UTR; and (d) at least one 5' cap structure. In other embodiments, the polynucleotide further includes (e) a poly-A tail.

[000274] In some embodiments, one of the coding region, the 5' UTR including at least one Kozak sequence, the 3' UTR, the 5' cap structure, or the poly-A tail includes $[A_n]$-L^1-$[B_0]$.

[000275] In other embodiments, one of the coding region, the 5' UTR including at least one Kozak sequence, the 3' UTR, the 5' cap structure, or the poly-A tail includes $[A_n]$ and another of the coding region, the 5' UTR including at least one Kozak sequence, the 3' UTR, the 5' cap structure, or the poly-A tail includes $[B_0]$.

[000276] In certain embodiments, the polynucleotide includes at least one modified nucleoside (e.g., a nucleoside described herein).

[000277] In some embodiments, R^4 is optionally substituted C2-9 heterocyclylene, for example, the heterocycle may have the structure:

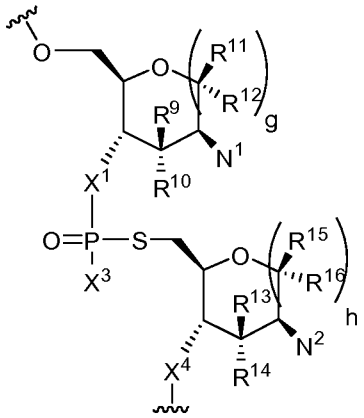


or .

[000278] In certain embodiments, L^1 is attached to $[A_n]$ at the 3' position of a five-membered sugar ring or 4' position of a six membered sugar ring of one of the nucleosides and to $[B_0]$ at the 5' position of a five-membered sugar ring or 6' position of of a six membered sugar ring of one of the nucleosides.

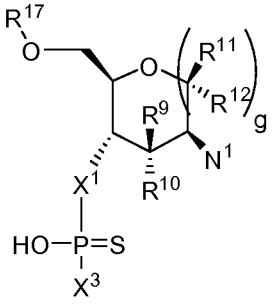
[000279] In some embodiments, the polynucleotide is circular.

[000280] In another aspect, the invention features a method of producing a composition including a chimeric polynucleotide encoding a polypeptide, wherein the polynucleotide includes the structure of Formula V:



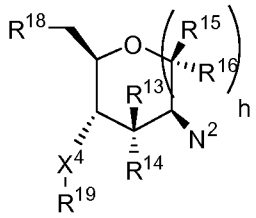
Formula V

[000281] This method includes reacting a compound having the structure of Formula VI:



Formula VI

with a compound having the structure of Formula VII:



Formula VII

[000282] wherein each of N^1 and N^2 is, independently, a nucleobase;

[000283] each of R^9, R^{10}, R^{11}, R^{12}, R^{13}, R^{14}, R^{15}, and R^{16} is, independently, H, halo, hydroxy, thiol, optionally substituted Ci-C_6 alkyl, optionally substituted Ci-C_6 heteroalkyl, optionally substituted C2-C6 heteroalkenyl, optionally substituted C2-C6 heteroalkynyl, optionally substituted amino, azido, or optionally substituted C_6-$C1_0$ aryl;

[000284] each of g and h is, independently, 0 or 1;

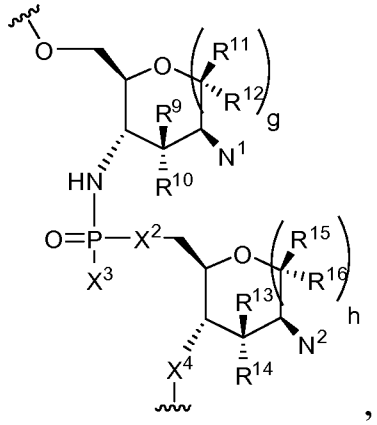
[000285] each X^1 and X^4 is, independently, O, NH, or S; and

[000286] each X^3 is independently OH or SH, or a salt thereof;

[000287] each of R^{17} and R^{19} is, independently, a region of linked nucleosides; and

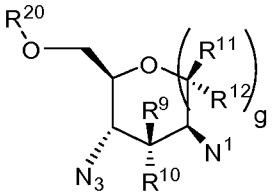
[000288] R^{18} is a halogen.

[000289] In another aspect, the invention features a method of producing a composition including a chimeric polynucleotide encoding a polypeptide, wherein the polynucleotide includes the structure of Formula VIII:



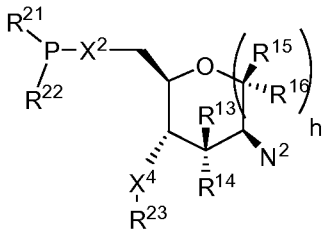
Formula VIII

[000290] This method includes reacting a compound having the structure of Formula IX:



Formula IX

with a compound having the structure of Formula X:



Formula X

[000291] wherein each of N^1 and N^2 is, independently, a nucleobase;

[000292] each of R^9, R^{10}, R^{11}, R^{12}, R^{13}, R^{14}, R^{15}, and R^{16} is, independently, H, halo, hydroxy, thiol, optionally substituted Ci-C_6 alkyl, optionally substituted Ci-C_6 heteroalkyl, optionally substituted $C_{2\text{-}C6}$ heteroalkenyl, optionally substituted $C_{2\text{-}C6}$ heteroalkynyl, optionally substituted amino, azido, or optionally substituted $_{C\,6}$-C10 aryl;

[000293] each of g and h is, independently, 0 or 1;

[000294] each X^4 is, independently, O, NH, or S; and

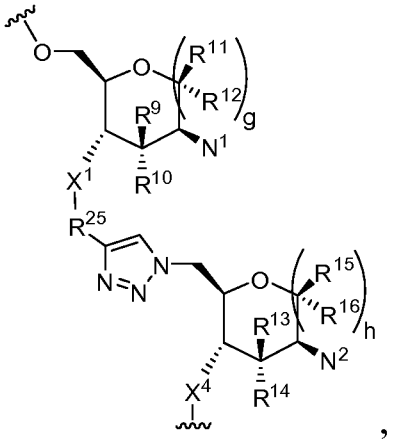
[000295] each X^2 is independently O or S;

[000296] each X^3 is independently OH, SH, or a salt thereof;

[000297] each of R^{20} and R^{23} is, independently, a region of linked nucleosides; and

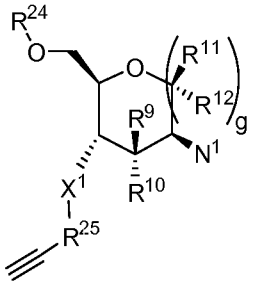
[000298] each of R^{21} and R^{22} is, independently, optionally substituted ***Ci-Ce*** alkoxy.

[000299] In another aspect, the invention features a method of producing a composition including a chimeric polynucleotide encoding a polypeptide, wherein the polynucleotide includes the structure of Formula XI:



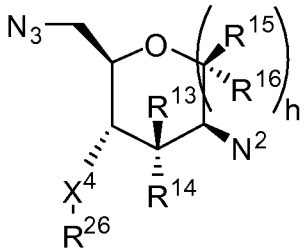
Formula XI

[000300] This method includes reacting a compound having the structure of Formula XII:



Formula XII

with a compound having the structure of Formula XIII:



Formula XIII

[000301] wherein each of N^1 and N^2 is, independently, a nucleobase;

[000302] each of R^9, R^{10}, R^{11}, R^{12}, R^{13}, R^{14}, R^{15}, and R^{16} is, independently, H, halo, hydroxy, thiol, optionally substituted Ci-C_6 alkyl, optionally substituted Ci-C_6 heteroalkyl, optionally substituted $C_{2\text{-}C6}$ heteroalkenyl, optionally substituted $C_{2\text{-}C6}$ heteroalkynyl, optionally substituted amino, azido, or optionally substituted $_{C\ 6}$-C_{10} aryl;

[000303] each of g and h is, independently, 0 or 1;

[000304] each X^4 is, independently, O, NH, or S; and

[000305] each X^2 is independently O or S;

[000306] each X^3 is independently OH, SH, or a salt thereof;

[000307] each of R^{24} and R^{26} is, independently, a region of linked nucleosides; and

[000308] R^{25} is optionally substituted $C_{1\text{-}C6}$ alkylene or optionally substituted $C_{1\text{-}C6}$ heteroalkylene or R^{25} and the alkynyl group together form optionally substituted cycloalkynyl.

[000309] In another aspect, the invention features a method of producing a composition including a chimeric polynucleotide encoding a polypeptide, wherein the polynucleotide has a sequence including Formula II:

$$[A_\eta]\text{-}L^1\text{-}[B_0],$$

Formula II

[000310] This method includes reacting a compound having the structure of Formula XIV

$$[A_n]\text{-}(R^1)_a\text{-}(R^2)_b\text{-}(R^3)_c\text{-}N_3$$

Formula XIV

with a compound having the structure of Formula XV:

$$R^{27}\text{-}(R^5)_d\text{-}(R^6)e\text{-}(R^7)_f\text{-}[B_0]$$

Formula XV

[000311] wherein each A and B is independently any nucleoside;

[000312] n and o are, independently 15 to 1000; and

[000313] L^1 has the structure of Formula III:

$$\{(R^1)a\text{-}(R^2)b\text{-}(R^3)c\text{-}R^4\text{-}(R^5)d\text{-}(R^6)e\text{-}(RVi\}$$

Formula III

[000314] wherein a, b, c, d, e, and f are each, independently, 0 or 1;

[000315] wherein each A and B is independently any nucleoside;

[000316] n and o are, independently 15 to 1000;

[000317] R^1, R^3, R^5, and R^7 each, independently, is selected from optionally substituted ***Ci-Ce*** alkylene, optionally substituted ***Ci-Ce*** heteroalkylene, O, S, and NR^8;

[000318] R^2 and R^6 are each, independently, selected from carbonyl, thiocarbonyl, sulfonyl, or phosphoryl;

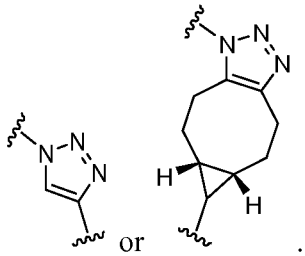
[000319] R^4 is an optionally substituted triazolene; and

[000320] R^8 is hydrogen, optionally substituted C1-C4 alkyl, optionally substituted C3-C4 alkenyl, optionally substituted C2-C4 alkynyl, optionally substituted C2-C6 heterocyclyl, optionally substituted C6-C12 aryl, or optionally substituted C1-C7 heteroalkyl; and

[000321] R^{27} is an optionally substituted C2-C3 alkynyl or an optionally substituted C_8-C_{12} cycloalkynyl,

[000322] wherein L^1 is attached to $[A_n]$ and $[B_0]$ at the sugar of one of the nucleosides.

[000323] In some embodiments, the optionally substituted triazolene has the structure:



or .

[000324] Figures 4 and 5 provide schematics of a series of chimeric polynucleotides illustrating various patterns of positional modifications based on Formula I as well as those having a blocked or structured 3' terminus.

[000325] Chimeric polynucleotides, including the parts or regions thereof, of the present invention may be classified as hemimers, gapmers, wingmers, or blockmers.

[000326] As used herein, a "hemimer" is chimeric polynucleotide comprising a region or part which comprises half of one pattern, percent, position or population of a chemical modification(s) and half of a second pattern, percent, position or population of a chemical modification(s). Chimeric polynucleotides of the present invention may also comprise hemimer subregions. In one embodiment, a part or region is 50% of one and 50% of another.

[000327] In one embodiment the entire chimeric polynucleotide can be 50% of one and 50% of the other. Any region or part of any chimeric polynucleotide of the invention may be a hemimer. Types of hemimers include pattern hemimers, population hemimers or position hemimers. By definition, hemimers are 50:50 percent hemimers.

[000328] As used herein, a "gapmer" is a chimeric polynucleotide having at least three parts or regions with a gap between the parts or regions. The "gap" can comprise a region of linked nucleosides or a single nucleoside which differs from the chimeric nature of the two parts or regions flanking it. The two parts or regions of a gapmer may be the same or different from each other.

[000329] As used herein, a "wingmer" is a chimeric polynucleotide having at least three parts or regions with a gap between the parts or regions. Unlike a gapmer, the two flanking parts or regions surrounding the gap in a wingmer are the same in degree or kind. Such similarity may be in the length of number of units of different modifications or in the number of modifications. The wings of a wingmer may be longer or shorter than the gap. The wing parts or regions may be 20, 30, 40, 50, 60 70, 80, 90 or 95% greater or shorter in length than the region which comprises the gap-

[000330] As used herein, a "blockmer" is a patterned polynucleotide where parts or regions are of equivalent size or number and type of modifications. Regions or subregions in a blockmer may be 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 6 1 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270,

271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 295, 296, 297, 298, 299, 300, 310, 320, 330, 340, 350, 360, 370, 380, 390, 400, 410, 420, 430, 440, 450, 460, 470, 480, 490 or 500, nuclesides long.

[000331] Chimeric polynucleotides, including the parts or regions thereof, of the present invention having a chemical modification pattern are referred to as "pattern chimeras." Pattern chimeras may also be referred to as blockmers. Pattern chimeras are those polynucleotides having a pattern of modifications within, across or among regions or parts.

[000332] Patterns of modifications within a part or region are those which start and stop within a defined region. Patterns of modifications across a part or region are those patterns which start in on part or region and end in another adjacent part or region. Patterns of modifications among parts or regions are those which begin and end in one part or region and are repeated in a different part or region, which is not necessarily adjacent to the first region or part.

[000333] The regions or subregions of pattern chimeras or blockmers may have simple alternating patterns such as ABAB[AB]n where each "A" and each "B" represent different chemical modifications (at at least one of the base, sugar or backbone linker), different types of chemical modifications (e.g., naturally occurring and non-naturally occurring), different percentages of modifications or different populations of modifications. The pattern may repeat n number of times where n=3-300. Further, each A or B can represent from 1-2500 units (e.g., nucleosides) in the pattern. Patterns may also be alternating multiples such as AABBAABB[AABB]n (an alternating double multiple) or AAABBBAAABBB[AAABBB]n (an alternating triple multiple) pattern. The pattern may repeat n number of times where n=3-300.

[000334] Different patterns may also be mixed together to form a second order pattern. For example, a single alternating pattern may be combined with a triple alternating pattern to form a second order alternating pattern A'B'. One example would be [ABABAB][AAABBBAAABBB] [ABABAB][AAABBBAAABBB] [ABABAB][AAABBBAAABBB], where [ABABAB] is A' and [AAABBBAAABBB] is B'. In like fashion, these patterns may be repeated n number of times, where n=3-300.

[000335] Patterns may include three or more different modifications to form an ABCABC[ABC]n pattern. These three component patterns may also be multiples, such as AABBCCAABBCC[AABBCC]n and may be designed as combinations with other patterns such as ABCABCAABBCCABCABCAABBCC, and may be higher order patterns.

[000336] Regions or subregions of position, percent, and population modifications need not reflect an equal contribution from each modification type. They may form series such as "1-2-3-4", "1-2-4-8", where each integer represents the number of units of a particular modification

type. Alternatively, they may be odd only, such as ' 1-3-3-1-3-1-5" or even only "2-4-2-4-6-4-8" or a mixture of both odd and even number of units such as "1-3-4-2-5-7-3-3-4".

[000337] Pattern chimeras may vary in their chemical modification by degree (such as those described above) or by kind (e.g., different modifications).

[000338] Chimeric polynucleotides, including the parts or regions thereof, of the present invention having at least one region with two or more different chemical modifications of two or more nucleoside members of the same nucleoside type (A, C, G, T, or U) are referred to as "positionally modified" chimeras. Positionally modified chimeras are also referred to herein as "selective placement" chimeras or "selective placement polynucleotides". As the name implies, selective placement refers to the design of polynucleotides which, unlike polynucleotides in the art where the modification to any A, C, G, T or U is the same by virtue of the method of synthesis, can have different modifications to the individual As, Cs, Gs, Ts or Us in a polynucleotide or region thereof. For example, in a positionally modified chimeric polynucleotide, there may be two or more different chemical modifications to any of the nucleoside types of As, Cs, Gs, Ts, or Us. There may also be combinations of two or more to any two or more of the same nucleoside type. For example, a positionally modified or selective placement chimeric polynucleotide may comprise 3 different modifications to the population of adenines in the molecule and also have 3 different modifications to the population of cytosines in the construct—all of which may have a unique, non-random, placement.

[000339] Chimeric polynucleotides, including the parts or regions thereof, of the present invention having a chemical modification percent are referred to as "percent chimeras." Percent chimeras may have regions or parts which comprise at least 1%, at least 2%, at least 5%, at least 8%, at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or at least 99% positional, pattern or population of modifications. Alternatively, the percent chimera may be completely modified as to modification position, pattern, or population. The percent of modification of a percent chimera may be split between naturally occurring and non-naturally occurring modifications.

[000340] Chimeric polynucleotides, including the parts or regions thereof, of the present invention having a chemical modification population are referred to as "population chimeras." A population chimera may comprise a region or part where nucleosides (their base, sugar or backbone linkage, or combination thereof) have a select population of modifications. Such modifications may be selected from functional populations such as modifications which induce, alter or modulate a phenotypic outcome. For example, a functional population may be a population or selection of chemical modifications which increase the level of a cytokine. Other

functional populations may individually or collectively function to decrease the level of one or more cytokines. Use of a selection of these like-function modifications in a chimeric polynucleotide would therefore constitute a "functional population chimera." As used herein, a "functional population chimera" may be one whose unique functional feature is defined by the population of modifications as described above or the term may apply to the overall function of the chimeric polynucleotide itself. For example, as a whole the chimeric polynucleotide may function in a different or superior way as compared to an unmodified or non-chimeric polynucleotide.

[000341] It should be noted that polynucleotides which have a uniform chemical modification of all of any of the same nucleoside type or a population of modifications produced by mere downward titration of the same starting modification in all of any of the same nucleoside type, or a measured percent of a chemical modification of all any of the same nucleoside type but with random incorporation, such as where all uridines are replaced by a uridine analog, e.g., pseudouridine, are not considred chimeric. Likewise, polynucleotides having a uniform chemical modification of two, three, or four of the same nucleoside type throughout the entire polynucleotide (such as all uridines and all cytosines, etc. are modified in the same way) are not considered chimeric polynucleotides. One example of a polynucleotide which is not chimeric is the canonical pseudouridine/5 -methyl cytosine modified polynucleotide of the prior art. These uniform polynucleotides are arrived at entirely via *in vitro* transcription (IVT) enzymatic synthesis; and due to the limitations of the synthesizing enzymes, they contain only one kind of modification at the occurrence of each of the same nucleoside type, i.e., adenosine (A), thymidine (T), guanosine (G), cytidine (C) or uradine (U), found in the polynucleotide. Such polynucleotides may be characterized as IVT polynucleotides.

[000342] The chimeric polynucleotides of the present invention may be structurally modified or chemically modified. When the chimeric polynucleotides of the present invention are chemically and/or structurally modified the polynucleotides may be referred to as "modified chimeric polynucleotides."

[000343] In some embodiments of the invention, the chimeric polynucleotides may encode two or more peptides or polypeptides of interest. Such peptides or polypeptides of interest include the heavy and light chains of antibodies, an enzyme and its substrate, a label and its binding molecule, a second messenger and its enzyme or the components of multimeric proteins or complexes.

[000344] The regions or parts of the chimeric polynucleotides of the present invention may be separated by a linker or spacer moiety. Such linkers or spaces may be nucleic acid based or non-nucleosidic.

[000345] In one embodiment, the chimeric polynucleotides of the present invention may include a sequence encoding a self-cleaving peptide described herein, such as, but not limited to, a 2A peptide. The polynucleotide sequence of the 2A peptide in the chimeric polynucleotide may be modified or codon optimized by the methods described herein and/or are known in the art.

[000346] Notwithstanding the foregoing, the chimeric polynucleotides of the present invention may comprise a region or part which is not positionally modified or not chimeric as defined herein.

[000347] For example, a region or part of a chimeric polynucleotide may be uniformly modified at one or more A, T, C, G, or U but according to the invention, the polynucleotides will not be uniformly modified throughout the entire region or part.

[000348] Regions or parts of chimeric polynucleotides may be from 15-1000 nucleosides in length and a polynucleotide may have from 2-100 different regions or patterns of regions as described herein.

[000349] In one embodiment, chimeric polynucleotides encode one or more polypeptides of interest. In another embodiment, the chimeric polynucleotides are substantially non-coding. In another embodiment, the chimeric polynucleotides have both coding and non-coding regions and parts.

[000350] Figure 4 illustrates the design of certain chimeric polynucleotides of the present invention when based on the scaffold of the polynucleotide of Figure 1. Shown in the figure are the regions or parts of the chimeric polynucleotides where patterned regions represent those regions which are positionally modified and open regions illustrate regions which may or may not be modified but which are, when modified, uniformly modified. Chimeric polynucleotides of the present invention may be completely positionally modified or partially positionally modified. They may also have subregions which may be of any pattern or design. Shown in Figure 2 are a chimeric subregion and a hemimer subregion.

[000351] In one embodiment, the shortest length of a region of the chimeric polynucleotide of the present invention encoding a peptide can be the length that is sufficient to encode for a dipeptide, a tripeptide, a tetrapeptide, a pentapeptide, a hexapeptide, a heptapeptide, an octapeptide, a nonapeptide, or a decapeptide. In another embodiment, the length may be sufficient to encode a peptide of 2-30 amino acids, e.g. 5-30, 10-30, 2-25, 5-25, 10-25, or 10-20

amino acids. The length may be sufficient to encode for a peptide of at least 11, 12, 13, 14, 15, 17, 20, 25 or 30 amino acids, or a peptide that is no longer than 40 amino acids, e.g. no longer than 35, 30, 25, 20, 17, 15, 14, 13, 12, 11 or 10 amino acids. Examples of dipeptides that the polynucleotide sequences can encode or include, but are not limited to, carnosine and anserine.

[000352] In one embodiment, the length of a region of the chimeric polynucleotide of the present invention encoding the peptide or polypeptide of interest is greater than about 30 nucleotides in length (e.g., at least or greater than about 35, 40, 45, 50, 55, 60, 70, 80, 90, 100, 120, 140, 160, 180, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1,000, 1,100, 1,200, 1,300, 1,400, 1,500, 1,600, 1,700, 1,800, 1,900, 2,000, 2,500, and 3,000, 4,000, 5,000, 6,000, 7,000, 8,000, 9,000, 10,000, 20,000, 30,000, 40,000, 50,000, 60,000, 70,000, 80,000, 90,000 or up to and including 100,000 nucleotides). As used herein, such a region may be referred to as a "coding region" or "region encoding."

[000353] In some embodiments, the chimeric polynucleotide includes from about 30 to about 100,000 nucleotides (e.g., from 30 to 50, from 30 to 100, from 30 to 250, from 30 to 500, from 30 to 1,000, from 30 to 1,500, from 30 to 3,000, from 30 to 5,000, from 30 to 7,000, from 30 to 10,000, from 30 to 25,000, from 30 to 50,000, from 30 to 70,000, from 100 to 250, from 100 to 500, from 100 to 1,000, from 100 to 1,500, from 100 to 3,000, from 100 to 5,000, from 100 to 7,000, from 100 to 10,000, from 100 to 25,000, from 100 to 50,000, from 100 to 70,000, from 100 to 100,000, from 500 to 1,000, from 500 to 1,500, from 500 to 2,000, from 500 to 3,000, from 500 to 5,000, from 500 to 7,000, from 500 to 10,000, from 500 to 25,000, from 500 to 50,000, from 500 to 70,000, from 500 to 100,000, from 1,000 to 1,500, from 1,000 to 2,000, from 1,000 to 3,000, from 1,000 to 5,000, from 1,000 to 7,000, from 1,000 to 10,000, from 1,000 to 25,000, from 1,000 to 50,000, from 1,000 to 70,000, from 1,000 to 100,000, from 1,500 to 3,000, from 1,500 to 5,000, from 1,500 to 7,000, from 1,500 to 10,000, from 1,500 to 25,000, from 1,500 to 50,000, from 1,500 to 70,000, from 1,500 to 100,000, from 2,000 to 3,000, from 2,000 to 5,000, from 2,000 to 7,000, from 2,000 to 10,000, from 2,000 to 25,000, from 2,000 to 50,000, from 2,000 to 70,000, and from 2,000 to 100,000).

[000354] According to the present invention, regions or subregions of the chimeric polynucleotides may also range independently from 15-1,000 nucleotides in length (e.g., greater than 30, 40, 45, 50, 55, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500, 550, 600, 650, 700, 750, 800, 850, 900 and 950 nucleotides or at least 30, 40, 45, 50, 55, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950 and 1,000 nucleotides).

[000355] According to the present invention, regions or subregions of chimeric polynucleotides may range from absent to 500 nucleotides in length (e.g., at least 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, or 500 nucleotides). Where the region is a polyA tail, the length may be determined in units of or as a function of polyA Binding Protein binding. In this embodiment, the polyA tail is long enough to bind at least 4 monomers of PolyA Binding Protein. PolyA Binding Protein monomers bind to stretches of approximately 38 nucleotides. As such, it has been observed that polyA tails of about 80 nucleotides to about 160 nucleotides are functional. The chimeric polynucleotides of the present invention which function as an mRNA need not comprise a polyA tail.

[000356] According to the present invention, chimeric polynucleotides which function as an mRNA may have a capping region. The capping region may comprise a single cap or a series of nucleotides forming the cap. In this embodiment the capping region may be from 1 to 10, e.g. 2-9, 3-8, 4-7, 1-5, 5-10, or at least 2, or 10 or fewer nucleotides in length. In some embodiments, the cap is absent.

[000357] The present invention contemplates chimeric polynucleotides which are circular or cyclic. As the name implies circular polynucleotides are circular in nature meaning that the termini are joined in some fashion, whether by ligation, covalent bond, common association with the same protein or other molecule or complex or by hybridization. Any of the circular polynucleotides as taught in, for example, co-pending International Application No. PCT/US2014/053904, filed September 3, 2014 (Attorney Docket No. M051.20), the contents of each of which are incorporated herein by reference in their entirety, may be made chimeric according to the present invention.

[000358] Chimeric polynucleotides, formulations and compositions comprising chimeric polynucleotides, and methods of making, using and administering chimeric polynucleotides are also described in co-pending International Application No. PCT/US20 14/053907, filed September 3, 2014 (Attorney Docket No. M057.20); each of which is incorporated by reference in its entirety.

Circular Polynucleotide Architecture

[000359] The present invention contemplates polynucleotides which are circular or cyclic. As the name implies circular polynucleotides are circular in nature meaning that the termini are joined in some fashion, whether by ligation, covalent bond, common association with the same protein or other molecule or complex or by hybridization. Any of the circular polynucleotides as taught in, for example, co-pending International Publication No. WO2015034925, (Attorney

Docket No. M05 1.20), the contents of each of which are incorporated herein by reference in their entirety.

[000360] Circular polynucleotides of the present invention may be designed according to the circular RNA construct scaffolds shown in Figures 6A-6G. These figures are also described in co-pending International Application No. WO2015034925, (Attorney Docket No. M051.20), the contents of each of which are incorporated herein by reference in their entirety. Such polynucleotides may be referred to as cicular polynucleotides or circular constructs.

[000361] The circular polynucleotides or circPs of the present invention which encode at least one peptide or polypeptide of interest are known as circular RNAs or circRNA. As used herein, "circular RNA" or "circRNA" means a circular polynucleotide that can encode at least one peptide or polypeptide of interest. The circPs of the present invention which comprise at least one sensor sequence and do not encode a peptide or polypeptide of interest are known as circular sponges or circSP. As used herein, "circular sponges," "circular polynucleotide sponges" or "circSP" means a circular polynucleotide which comprises at least one sensor sequence and does not encode a polypeptide of interest. As used herein, "sensor sequence" means a receptor or pseudo-receptor for endogenous nucleic acid binding molecules. Non-limiting examples of sensor sequences include, microRNA binding sites, microRNA seed sequences, microRNA binding sites without the seed sequence, transcription factor binding sites and artificial binding sites engineered to act as pseudo-receptors and portions and fragments thereof.

[000362] The circPs of the present invention which comprise at least one sensor sequence and encode at least one peptide or polypeptide of interest are known as circular RNA sponges or circRNA-SP. As used herein, "circular RNA sponges" or "circRNA-SP" means a circular polynucleotide which comprises at least one sensor sequence and at least one region encoding at least one peptide or polypeptide of interest.

[000363] Figures 6A-6G show a representative circular construct **200** of the circular polynucleotides of the present invention. As used herein, the term "circular construct" refers to a circular polynucleotide transcript which may act substantially similar to and have properties of a RNA molecule. In one embodiment the circular construct acts as an mRNA. If the circular construct encodes one or more peptides or polypeptides of interest (e.g., a circRNA or circRNA-SP) then the polynucleotide transcript retains sufficient structural and/or chemical features to allow the polypeptide of interest encoded therein to be translated. Circular constructs may be polynucleotides of the invention. When structurally or chemically modified, the construct may be referred to as a modified circP, modified circSP, modified circRNA or modified circRNA-SP.

[000364] Turning to Figure 6A, the circular construct **200** here contains a first region of linked nucleotides **202** that is flanked by a first flanking region **204** and a second flanking region **206.** As used herein, the "first region" may be referred to as a "coding region," a "non-coding region" or "region encoding" or simply the "first region." In one embodiment, this first region may comprise nucleotides such as, but is not limited to, encoding at least one peptide or polypeptide of interest and/or nucleotides encoding a sensor region. The peptide or polypeptide of interest may comprise at its 5' terminus one or more signal peptide sequences encoded by a signal peptide sequence region **203.** The first flanking region **204** may comprise a region of linked nucleosides or portion thereof which may act similarly to an untranslated region (UTR) in a mRNA and/or DNA sequence. The first flanking region may also comprise a region of polarity **208.** The region of polarity **208** may include an IRES sequence or portion thereof. As a non-limiting example, when linearlized this region may be split to have a first portion be on the 5' terminus of the first region **202** and second portion be on the 3' terminus of the first region **202.** The second flanking region **206** may comprise a tailing sequence region **210** and may comprise a region of linked nucleotides or portion thereof **212** which may act similarly to a UTR in an mRNA and/or DNA.

[000365] Bridging the 5' terminus of the first region **202** and the first flanking region **204** is a first operational region **205.** In one embodiment, this operational region may comprise a start codon. The operational region may alternatively comprise any translation initiation sequence or signal including a start codon.

[000366] Bridging the 3' terminus of the first region **202** and the second flanking region **206** is a second operational region **207.** Traditionally this operational region comprises a stop codon. The operational region may alternatively comprise any translation initiation sequence or signal including a stop codon. According to the present invention, multiple serial stop codons may also be used. In one embodiment, the operation region of the present invention may comprise two stop codons. The first stop codon may be "TGA" or "UGA" and the second stop codon may be selected from the group consisting of "TAA," "TGA," "TAG," "UAA," "UGA" or "UAG."

[000367] Turning to Figure 6B, at least one non-nucleic acid moiety **201** may be used to prepare a circular construct **200** where the non-nucleic acid moiety **201** is used to bring the first flanking region **204** near the second flanking region **206.** Non-limiting examples of non-nucleic acid moieties which may be used in the present invention are described herein. The circular construct **200** may comprise more than one non-nucleic acid moiety wherein the additional non-nucleic acid moieties may be heterologous or homologous to the first non-nucleic acid moiety.

[000368] Turning to Figure 6C, the first region of linked nucleosides **202** may comprise a spacer region **214.** This spacer region **214** may be used to separate the first region of linked nucleosides **202** so that the circular construct can include more than one open reading frame, non-coding region or an open reading frame and a non-coding region.

[000369] Turning to Figure 6D, the second flanking region **206** may comprise one or more sensor regions **216** in the 3'UTR **212.** These sensor sequences as discussed herein operate as pseudo-receptors (or binding sites) for ligands of the local microenvironment of the circular construct. For example, microRNA bindng sites or miRNA seeds may be used as sensors such that they function as pseudoreceptors for any microRNAs present in the environment of the circular polynucleotide. As shown in Figure 6D, the one or more sensor regions **216** may be separated by a spacer region **214.**

[000370] As shown in Figure 6E, a circular construct **200,** which includes one or more sensor regions **216,** may also include a spacer region **214** in the first region of linked nucleosides **202.** As discussed above for Figure 6B, this spacer region **214** may be used to separate the first region of linked nucleosides **202** so that the circular construct can include more than one open reading frame and/or more than one non-coding region.

[000371] Turning to Figure 6F, a circular construct **200** may be a non-coding construct known as a circSP comprising at least one non-coding region such as, but not limited to, a sensor region **216.** Each of the sensor regions **216** may include, but are not limited to, a miR sequence, a miR seed, a miR binding site and/or a miR sequence without the seed.

[000372] Turning to Figure 6G, at least one non-nucleic acid moiety **201** may be used to prepare a circular construct **200** which is a non-coding construct. The circular construct **200** which is a non-coding construct may comprise more than one non-nucleic acid moiety wherein the additional non-nucleic acid moieties may be heterologous or homologous to the first non-nucleic acid moiety.

[000373] Circular polynucleotides, formulations and compositions comprising circular polynucleotides, and methods of making, using and administering circular polynucleotides are also described in co-pending International Patent Publication No. WO2015034925, the contents of which is incorporated by reference in its entirety.

Multimers of Polynucleotides

[000374] According to the present invention, multiple distinct polynucleotides such as chimeric polynucleotides and/or IVT polynucleotides may be linked together through the 3'-end using nucleotides which are modified at the 3'-terminus. Chemical conjugation may be used to control the stoichiometry of delivery into cells. For example, the glyoxylate cycle enzymes, isocitrate

lyase and malate synthase, may be supplied into cells at a 1:1 ratio to alter cellular fatty acid metabolism. This ratio may be controlled by chemically linking chimeric polynucleotides and/or IVT polynucleotides using a 3'-azido terminated nucleotide on one polynucleotides species and a C5-ethynyl or alkynyl-containing nucleotide on the opposite polynucleotide species. The modified nucleotide is added post-transcriptionally using terminal transferase (New England Biolabs, Ipswich, MA) according to the manufacturer's protocol. After the addition of the 3'-modified nucleotide, the two polynucleotides species may be combined in an aqueous solution, in the presence or absence of copper, to form a new covalent linkage via a click chemistry mechanism as described in the literature.

[000375] In another example, more than two polynucleotides such as chimeric polynucleotides and/or IVT polynucleotides may be linked together using a functionalized linker molecule. For example, a functionalized saccharide molecule may be chemically modified to contain multiple chemical reactive groups (SH-, NH_2-, N_3, etc…) to react with the cognate moiety on a 3'-functionalized mRNA molecule (i.e., a 3'-maleimide ester, 3'-NHS-ester, alkynyl). The number of reactive groups on the modified saccharide can be controlled in a stoichiometric fashion to directly control the stoichiometric ratio of conjugated chimeric polynucleotides and/or IVT polynucleotides.

[000376] In one embodiment, the chimeric polynucleotides and/or IVT polynucleotides may be linked together in a pattern. The pattern may be a simple alternating pattern such as $CD[CD]_X$ where each "C" and each "D" represent a chimeric polynucleotide, IVT polynucleotide, different chimeric polynucleotides or different IVT polynucleotides. The pattern may repeat x number of times, where x= 1-300. Paterns may also be alternating multiples such as $CCDD[CCDD]_x$ (an alternating double multiple) or $CCCDDD[CCCDDD]_x$ (an alternating triple multiple) pattern. The alternating double multiple or alternating triple multiple may repeat x number of times, where x= 1-300.

Conjugates and Combinations of Polynucleotides

[000377] In order to further enhance protein production, polynucleotides of the present invention can be designed to be conjugated to other polynucleotides, dyes, intercalating agents *(e.g.* acridines), cross-linkers *(e.g.* psoralene, mitomycin C), porphyrins (TPPC4, texaphyrin, Sapphyrin), polycyclic aromatic hydrocarbons *(e.g.,* phenazine, dihydrophenazine), artificial endonucleases *(e.g.* EDTA), alkylating agents, phosphate, amino, mercapto, PEG *(e.g.,* PEG-40K), MPEG, [MPEG]2, polyamino, alkyl, substituted alkyl, radiolabeled markers, enzymes, haptens *(e.g.* biotin), transport/absorption facilitators *(e.g.,* aspirin, vitamin E, folic acid), synthetic ribonucleases, proteins, e.g., glycoproteins, or peptides, e.g., molecules having a

specific affinity for a co-ligand, or antibodies *e.g.,* an antibody, that binds to a specified cell type such as a cancer cell, endothelial cell, or bone cell, hormones and hormone receptors, non-peptidic species, such as lipids, lectins, carbohydrates, vitamins, cofactors, or a drug.

[000378] Conjugation may result in increased stability and/or half life and may be particularly useful in targeting the polynucleotides to specific sites in the cell, tissue or organism.

[000379] According to the present invention, the polynucleotides may be administered with, conjugated to or further encode one or more of RNAi agents, siRNAs, shRNAs, miRNAs, miRNA binding sites, antisense RNAs, ribozymes, catalytic DNA, tRNA, RNAs that induce triple helix formation, aptamers or vectors, and the like.

Bifunctional Polynucleotides

[000380] In one embodiment of the invention are bifunctional polynucleotides (e.g., bifunctional IVT polynucleotides, bifunctional chimeric polynucleotides or bifunctional circular polynucleotides). As the name implies, bifunctional polynucleotides are those having or capable of at least two functions. These molecules may also by convention be referred to as multi-functional. Bifunctional polynucleotides are described in paragraphs [000176] - [000178] of copending International Publication No. WO20 1503 8892, the contents of which are herein incorporated by reference in its entirety.

Noncoding Polynucleotides

[000381] As described herein, provided are polynucleotides having sequences that are partially or substantially not translatable, e.g., having a noncoding region. As one non-limiting example, the noncoding region may be the first region of the IVT polynucleotide or the circular polynucleotide. Alternatively, the noncoding region may be a region other than the first region. As another non-limiting example, the noncoding region may be the A, B and/or C region of the chimeric polynucleotide.

[000382] Such molecules are generally not translated, but can exert an effect on protein production by one or more of binding to and sequestering one or more translational machinery components such as a ribosomal protein or a transfer RNA (tRNA), thereby effectively reducing protein expression in the cell or modulating one or more pathways or cascades in a cell which in turn alters protein levels. The polynucleotide may contain or encode one or more long noncoding RNA (IncRNA, or lincRNA) or portion thereof, a small nucleolar RNA (sno-RNA), micro RNA (miRNA), small interfering RNA (siRNA) or Piwi-interacting RNA (piRNA). Examples of such IncRNA molecules and RNAi constructs designed to target such IncRNA any of which may be encoded in the polynucleotides are taught in International Publication, WO2012/018881 A2, the contents of which are incorporated herein by reference in their entirety.

Polypeptides of Interest

[000383] Polynucleotides of the present invention may encode one or more peptides or polypeptides of interest. They may also affect the levels, signaling or function of one or more peptides or polypeptides. Polypeptides of interest, according to the present invention include any of those taught in, for example, those listed in Table 6 of International Publication Nos. WO2013151666, WO2013151668, WO2013151663, WO2013151669, WO2013151670, WO2013151664, WO2013151665, WO2013151736; Tables 6 and 7 International Publication No. WO2013151672; Tables 6, 178 and 179 of International Publication No. WO2013151671; Tables 6, 185 and 186 of International Publication No WO2013151667; the contents of each of which are herein incorporated by reference in their entireties.

[000384] According to the present invention, the polynucleotide may be designed to encode one or more polypeptides of interest or fragments thereof. Such polypeptide of interest may include, but is not limited to, whole polypeptides, a plurality of polypeptides or fragments of polypeptides, which independently may be encoded by one or more regions or parts or the whole of a polynucleotide. As used herein, the term "polypeptides of interest" refer to any polypeptide which is selected to be encoded within, or whose function is affected by, the polynucleotides of the present invention.

[000385] As used herein, "polypeptide" means a polymer of amino acid residues (natural or unnatural) linked together most often by peptide bonds. The term, as used herein, refers to proteins, polypeptides, and peptides of any size, structure, or function. In some instances the polypeptide encoded is smaller than about 50 amino acids and the polypeptide is then termed a peptide. If the polypeptide is a peptide, it will be at least about 2, 3, 4, or at least 5 amino acid residues long. Thus, polypeptides include gene products, naturally occurring polypeptides, synthetic polypeptides, homologs, orthologs, paralogs, fragments and other equivalents, variants, and analogs of the foregoing. A polypeptide may be a single molecule or may be a multi-molecular complex such as a dimer, trimer or tetramer. They may also comprise single chain or multichain polypeptides such as antibodies or insulin and may be associated or linked. Most commonly disulfide linkages are found in multichain polypeptides. The term polypeptide may also apply to amino acid polymers in which one or more amino acid residues are an artificial chemical analogue of a corresponding naturally occurring amino acid.

[000386] The term "polypeptide variant" refers to molecules which differ in their amino acid sequence from a native or reference sequence. The amino acid sequence variants may possess substitutions, deletions, and/or insertions at certain positions within the amino acid sequence, as compared to a native or reference sequence. Ordinarily, variants will possess at least about 50%

identity (homology) to a native or reference sequence, and preferably, they will be at least about 80%, more preferably at least about 90% identical (homologous) to a native or reference sequence.

[000387] In some embodiments "variant mimics" are provided. As used herein, the term "variant mimic" is one which contains one or more amino acids which would mimic an activated sequence. For example, glutamate may serve as a mimic for phosphoro-threonine and/or phosphoro-serine. Alternatively, variant mimics may result in deactivation or in an inactivated product containing the mimic, e.g., phenylalanine may act as an inactivating substitution for tyrosine; or alanine may act as an inactivating substitution for serine.

[000388] "Homology" as it applies to amino acid sequences is defined as the percentage of residues in the candidate amino acid sequence that are identical with the residues in the amino acid sequence of a second sequence after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent homology. Methods and computer programs for the alignment are well known in the art. It is understood that homology depends on a calculation of percent identity but may differ in value due to gaps and penalties introduced in the calculation.

[000389] By "homologs" as it applies to polypeptide sequences means the corresponding sequence of other species having substantial identity to a second sequence of a second species.

[000390] "Analogs" is meant to include polypeptide variants which differ by one or more amino acid alterations, e.g., substitutions, additions or deletions of amino acid residues that still maintain one or more of the properties of the parent or starting polypeptide.

[000391] The present invention contemplates several types of compositions which are polypeptide based including variants and derivatives. These include substitutional, insertional, deletion and covalent variants and derivatives. The term "derivative" is used synonymously with the term "variant" but generally refers to a molecule that has been modified and/or changed in any way relative to a reference molecule or starting molecule.

[000392] As such, polynucleotides encoding peptides or polypeptides containing substitutions, insertions and/or additions, deletions and covalent modifications with respect to reference sequences, in particular the polypeptide sequences disclosed herein, are included within the scope of this invention. For example, sequence tags or amino acids, such as one or more lysines, can be added to the peptide sequences of the invention (e.g., at the N-terminal or C-terminal ends). Sequence tags can be used for peptide purification or localization. Lysines can be used to increase peptide solubility or to allow for biotinylation. Alternatively, amino acid residues located at the carboxy and amino terminal regions of the amino acid sequence of a peptide or protein may optionally be deleted providing for truncated sequences. Certain amino acids (e.g.,

C-terminal or N-terminal residues) may alternatively be deleted depending on the use of the sequence, as for example, expression of the sequence as part of a larger sequence which is soluble, or linked to a solid support.

[000393] "Substitutional variants" when referring to polypeptides are those that have at least one amino acid residue in a native or starting sequence removed and a different amino acid inserted in its place at the same position. The substitutions may be single, where only one amino acid in the molecule has been substituted, or they may be multiple, where two or more amino acids have been substituted in the same molecule.

[000394] As used herein the term "conservative amino acid substitution" refers to the substitution of an amino acid that is normally present in the sequence with a different amino acid of similar size, charge, or polarity. Examples of conservative substitutions include the substitution of a non-polar (hydrophobic) residue such as isoleucine, valine and leucine for another non-polar residue. Likewise, examples of conservative substitutions include the substitution of one polar (hydrophilic) residue for another such as between arginine and lysine, between glutamine and asparagine, and between glycine and serine. Additionally, the substitution of a basic residue such as lysine, arginine or histidine for another, or the substitution of one acidic residue such as aspartic acid or glutamic acid for another acidic residue are additional examples of conservative substitutions. Examples of non-conservative substitutions include the substitution of a non-polar (hydrophobic) amino acid residue such as isoleucine, valine, leucine, alanine, methionine for a polar (hydrophilic) residue such as cysteine, glutamine, glutamic acid or lysine and/or a polar residue for a non-polar residue.

[000395] "Insertional variants" when referring to polypeptides are those with one or more amino acids inserted immediately adjacent to an amino acid at a particular position in a native or starting sequence. "Immediately adjacent" to an amino acid means connected to either the alpha-carboxy or alpha-amino functional group of the amino acid.

[000396] "Deletional variants" when referring to polypeptides are those with one or more amino acids in the native or starting amino acid sequence removed. Ordinarily, deletional variants will have one or more amino acids deleted in a particular region of the molecule.

[000397] "Covalent derivatives" when referring to polypeptides include modifications of a native or starting protein with an organic proteinaceous or non-proteinaceous derivatizing agent, and/or post-translational modifications. Covalent modifications are traditionally introduced by reacting targeted amino acid residues of the protein with an organic derivatizing agent that is capable of reacting with selected side-chains or terminal residues, or by harnessing mechanisms of post-translational modifications that function in selected recombinant host cells. The resultant

covalent derivatives are useful in programs directed at identifying residues important for biological activity, for immunoassays, or for the preparation of anti-protein antibodies for immunoaffinity purification of the recombinant glycoprotein. Such modifications are within the ordinary skill in the art and are performed without undue experimentation.

[000398] Certain post-translational modifications are the result of the action of recombinant host cells on the expressed polypeptide. Glutaminyl and asparaginyl residues are frequently post-translationally deamidated to the corresponding glutamyl and aspartyl residues. Alternatively, these residues are deamidated under mildly acidic conditions. Either form of these residues may be present in the polypeptides produced in accordance with the present invention.

[000399] Other post-translational modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the alpha-amino groups of lysine, arginine, and histidine side chains (T. E. Creighton, Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)).

[000400] "Features" when referring to polypeptides are defined as distinct amino acid sequence-based components of a molecule. Features of the polypeptides encoded by the polynucleotides of the present invention include surface manifestations, local conformational shape, folds, loops, half-loops, domains, half-domains, sites, termini or any combination thereof.

[000401] As used herein when referring to polypeptides the term "surface manifestation" refers to a polypeptide based component of a protein appearing on an outermost surface.

[000402] As used herein when referring to polypeptides the term "local conformational shape" means a polypeptide based structural manifestation of a protein which is located within a definable space of the protein.

[000403] As used herein when referring to polypeptides the term "fold" refers to the resultant conformation of an amino acid sequence upon energy minimization. A fold may occur at the secondary or tertiary level of the folding process. Examples of secondary level folds include beta sheets and alpha helices. Examples of tertiary folds include domains and regions formed due to aggregation or separation of energetic forces. Regions formed in this way include hydrophobic and hydrophilic pockets, and the like.

[000404] As used herein the term "turn" as it relates to protein conformation means a bend which alters the direction of the backbone of a peptide or polypeptide and may involve one, two, three or more amino acid residues.

[000405] As used herein when referring to polypeptides the term "loop" refers to a structural feature of a polypeptide which may serve to reverse the direction of the backbone of a peptide or polypeptide. Where the loop is found in a polypeptide and only alters the direction of the

backbone, it may comprise four or more amino acid residues. Oliva et al. have identified at least 5 classes of protein loops (J. Mol Biol 266 (4): 814-830; 1997). Loops may be open or closed. Closed loops or "cyclic" loops may comprise 2, 3, 4, 5, 6, 7, 8, 9, 10 or more amino acids between the bridging moieties. Such bridging moieties may comprise a cysteine-cysteine bridge (Cys-Cys) typical in polypeptides having disulfide bridges or alternatively bridging moieties may be non-protein based such as the dibromozylyl agents used herein.

[000406] As used herein when referring to polypeptides the term "half-loop" refers to a portion of an identified loop having at least half the number of amino acid resides as the loop from which it is derived. It is understood that loops may not always contain an even number of amino acid residues. Therefore, in those cases where a loop contains or is identified to comprise an odd number of amino acids, a half-loop of the odd-numbered loop will comprise the whole number portion or next whole number portion of the loop (number of amino acids of the loop/2+/-0.5 amino acids). For example, a loop identified as a 7 amino acid loop could produce half-loops of 3 amino acids or 4 amino acids (7/2=3.5+/-0.5 being 3 or 4).

[000407] As used herein when referring to polypeptides the term "domain" refers to a motif of a polypeptide having one or more identifiable structural or functional characteristics or properties (e.g., binding capacity, serving as a site for protein-protein interactions).

[000408] As used herein when referring to polypeptides the term "half-domain" means a portion of an identified domain having at least half the number of amino acid resides as the domain from which it is derived. It is understood that domains may not always contain an even number of amino acid residues. Therefore, in those cases where a domain contains or is identified to comprise an odd number of amino acids, a half-domain of the odd-numbered domain will comprise the whole number portion or next whole number portion of the domain (number of amino acids of the domain/2+/-0.5 amino acids). For example, a domain identified as a 7 amino acid domain could produce half-domains of 3 amino acids or 4 amino acids (7/2=3.5+/-0.5 being 3 or 4). It is also understood that sub-domains may be identified within domains or half-domains, these subdomains possessing less than all of the structural or functional properties identified in the domains or half domains from which they were derived. It is also understood that the amino acids that comprise any of the domain types herein need not be contiguous along the backbone of the polypeptide (i.e., nonadjacent amino acids may fold structurally to produce a domain, half-domain or subdomain).

[000409] As used herein when referring to polypeptides the terms "site" as it pertains to amino acid based embodiments is used synonymously with "amino acid residue" and "amino acid side chain." A site represents a position within a peptide or polypeptide that may be modified,

manipulated, altered, derivatized or varied within the polypeptide based molecules of the present invention.

[000410] As used herein the terms "termini" or "terminus" when referring to polypeptides refers to an extremity of a peptide or polypeptide. Such extremity is not limited only to the first or final site of the peptide or polypeptide but may include additional amino acids in the terminal regions. The polypeptide based molecules of the present invention may be characterized as having both an N-terminus (terminated by an amino acid with a free amino group (NH2)) and a C-terminus (terminated by an amino acid with a free carboxyl group (COOH)). Proteins of the invention are in some cases made up of multiple polypeptide chains brought together by disulfide bonds or by non-covalent forces (multimers, oligomers). These sorts of proteins will have multiple N- and C-termini. Alternatively, the termini of the polypeptides may be modified such that they begin or end, as the case may be, with a non-polypeptide based moiety such as an organic conjugate.

[000411] Once any of the features have been identified or defined as a desired component of a polypeptide to be encoded by the polynucleotide of the invention, any of several manipulations and/or modifications of these features may be performed by moving, swapping, inverting, deleting, randomizing or duplicating. Furthermore, it is understood that manipulation of features may result in the same outcome as a modification to the molecules of the invention. For example, a manipulation which involved deleting a domain would result in the alteration of the length of a molecule just as modification of a nucleic acid to encode less than a full length molecule would.

[000412] Modifications and manipulations can be accomplished by methods known in the art such as, but not limited to, site directed mutagenesis or *apriori* incorporation during chemical synthesis. The resulting modified molecules may then be tested for activity using *in vitro* or *in vivo* assays such as those described herein or any other suitable screening assay known in the art.

[000413] According to the present invention, the polypeptides may comprise a consensus sequence which is discovered through rounds of experimentation. As used herein a "consensus" sequence is a single sequence which represents a collective population of sequences allowing for variability at one or more sites.

[000414] As recognized by those skilled in the art, protein fragments, functional protein domains, and homologous proteins are also considered to be within the scope of polypeptides of interest of this invention. For example, provided herein is any protein fragment (meaning a polypeptide sequence at least one amino acid residue shorter than a reference polypeptide sequence but otherwise identical) of a reference protein 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 or

greater than 100 amino acids in length. In another example, any protein that includes a stretch of about 20, about 30, about 40, about 50, or about 100 amino acids which are about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, about 95%, or about 100% identical to any of the sequences described herein can be utilized in accordance with the invention. In certain embodiments, a polypeptide to be utilized in accordance with the invention includes 2, 3, 4, 5, 6, 7, 8, 9, 10, or more mutations as shown in any of the sequences provided or referenced herein.

Types of Polypeptides of Interest

[000415] The polynucleotides of the present invention may be designed to encode polypeptides of interest selected from any of several target categories including, but not limited to, biologies, antibodies, vaccines, therapeutic proteins or peptides, cell penetrating peptides, secreted proteins, plasma membrane proteins, cytoplasmic or cytoskeletal proteins, intracellular membrane bound proteins, nuclear proteins, proteins associated with human disease, targeting moieties or those proteins encoded by the human genome for which no therapeutic indication has been identified but which nonetheless have utility in areas of research and discovery.

[000416] In one embodiment, polynucleotides may encode variant polypeptides which have a certain identity with a reference polypeptide sequence. As used herein, a "reference polypeptide sequence" refers to a starting polypeptide sequence. Reference sequences may be wild type sequences or any sequence to which reference is made in the design of another sequence. A "reference polypeptide sequence" may, e.g., be any one of those polypeptides disclosed in Table 6 and 7 of U.S. Provisional Patent Application Nos. 61/681,720, 61/737,213, 61/681,742; Table 6 of International Publication Nos. WO2013151666, WO2013151668, WO2013151663, WO2013151669, WO2013151670, WO2013151664, WO2013151665, WO2013151736; Tables 6 and 7 International Publication No. WO2013151672; Tables 6, 178 and 179 of International Publication No. WO2013151671; Tables 6, 185 and 186 of International Publication No WO2013 15 1667; the contents of each of which are herein incorporated by reference in their entireties.

[000417] Reference molecules (polypeptides or polynucleotides) may share a certain identity with the designed molecules (polypeptides or polynucleotides). The term "identity" as known in the art, refers to a relationship between the sequences of two or more peptides, polypeptides or polynucleotides, as determined by comparing the sequences. In the art, identity also means the degree of sequence relatedness between them as determined by the number of matches between strings of two or more amino acid residues or nucleosides. Identity measures the percent of identical matches between the smaller of two or more sequences with gap alignments (if any) addressed by a particular mathematical model or computer program (i.e., "algorithms"). Identity

of related peptides can be readily calculated by known methods. Such methods include, but are not limited to, those described in Computational Molecular Biology, Lesk, A. M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D. W., ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part 1, Griffin, A. M., and Griffin, H. G., eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M. Stockton Press, New York, 1991; and Carillo et al, SIAM J. Applied Math. 48, 1073 (1988).

[000418] In some embodiments, the encoded polypeptide variant may have the same or a similar activity as the reference polypeptide. Alternatively, the variant may have an altered activity (e.g., increased or decreased) relative to a reference polypeptide. Generally, variants of a particular polynucleotide or polypeptide of the invention will have at least about 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% but less than 100% sequence identity to that particular reference polynucleotide or polypeptide as determined by sequence alignment programs and parameters described herein and known to those skilled in the art. Such tools for alignment include those of the BLAST suite (Stephen F. Altschul, Thomas L. Madden, Alejandro A. Schaffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402.) Other tools are described herein, specifically in the definition of "Identity."

[000419] Default parameters in the BLAST algorithm include, for example, an expect threshold of 10, Word size of 28, Match/Mismatch Scores 1, -2, Gap costs Linear. Any filter can be applied as well as a selection for species specific repeats, e.g., Homo sapiens.

Polynucleotide Regions

[000420] In some embodiments, polynucleotides may be designed to comprise regions, subregions or parts which function in a similar manner as known regions or parts of other nucleic acid based molecules. Such regions include those polynucleotide regions discussed herein as well as noncoding regions. Noncoding regions may be at the level of a single nucleoside such as the case when the region is or incorporates one or more cytotoxic nucleosides.

Cytotoxic Nucleosides

[000421] In one embodiment, the polynucleotides of the present invention may incorporate one or more cytotoxic nucleosides. For example, cytotoxic nucleosides may be incorporated into polynucleotides such as bifunctional modified RNAs or mRNAs. Cytotoxic nucleosides are

described in paragraphs [000223] - [000227] of copending International Publication No. WO2015038892, the contents of which are herein incorporated by reference in its entirety.

Polynucleotides having Untranslated Regions (UTRs)

[000422] The polynucleotides of the present invention may comprise one or more regions or parts which act or function as an untranslated region. Where polynucleotides are designed to encode at least one polypeptide of interest, the polynucleotides may comprise one or more of these untranslated regions.

[000423] By definition, wild type untranslated regions (UTRs) of a gene are transcribed but not translated. In mRNA, the 5'UTR starts at the transcription start site and continues to the start codon but does not include the start codon; whereas, the 3'UTR starts immediately following the stop codon and continues until the transcriptional termination signal. There is growing body of evidence about the regulatory roles played by the UTRs in terms of stability of the nucleic acid molecule and translation. The regulatory features of a UTR can be incorporated into the polynucleotides of the present invention to, among other things, enhance the stability of the molecule. The specific features can also be incorporated to ensure controlled down-regulation of the transcript in case they are misdirected to undesired organs sites. The untranslated regions may be incorporated into a vector system which can produce mRNA and/or be delivered to a cell, tissue and/or organism to produce a polypeptide of interest.

[000424] Nucleotides may be mutated, replaced and/or removed from the 5' (or 3') UTRs. For example, one or more nucleotides upstream of the start codon may be replaced with another nucleotide. The nucleotide or nucletides to be replaced may be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, 55, 60 or more than 60 nucleotides upstream of the start codon. As another example, one or more nucleotides upstream of the start codon may be removed from the UTR.

[000425] In one embodiment, at least one purine upstream of the start codon may be replaced with a pyrimidine. The purine to be replaced may be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, 55, 60 or more than 60 nucleotides upstream of the start codon. As a non-limiting example, an adenine which is three nucleotides upstream of the start codon may be replaced with a thymine. As another non-limiting example, an adenine which is nine nucleotides upstream of the start codon may be replaced with a thymine.

[000426] In one embodiment, at least one nucleotide upstream of the start codon may be removed from the UTR. In one aspect, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, 55, 60 or more than 60 nucleotides upstream of the

start codon may be removed from the UTR of the polynucleotides described herein. As a non-limiting example, the 9 nucleotides upstream of the start codon may be removed from the UTR (See e.g., 5UTR-038 described in Table 2). As another non-limiting example, the 21 nucleotides upstream of the start codon may be removed from the UTR (See e.g., 5UTR-040 described in Table 2).

[000427] In one embodiment, a 5'UTR of the polynucleotide comprising a kozak sequence may comprise at least one substitution. As a non-limiting example the kozak sequence prior to substitution may be GCCACC and after substitution it is GCCTCC.

[000428] In one embodiment, the 5'UTR of the polynucleotides described herein may not include a kozak sequence (See e.g. 5UTR-040 described in Table 2).

5' UTR and Translation Initiation

[000429] Natural 5'UTRs bear features which play roles in translation initiation. They harbor signatures like Kozak sequences which are commonly known to be involved in the process by which the ribosome initiates translation of many genes. Kozak sequences have the consensus CCR(A/G)CCAUGG, where R is a purine (adenine or guanine) three bases upstream of the start codon (AUG), which is followed by another 'G'. 5'UTR also have been known to form secondary structures which are involved in elongation factor binding.

[000430] 5'UTR secondary structures involved in elongation factor binding can interact with other RNA binding molecules in the 5'UTR or 3'UTR to regulate gene expression. For example, the elongation factor EIF4A2 binding to a secondarily structured element in the 5'UTR is necessary for microRNA mediated repression (Meijer HA et al, Science, 2013, 340, 82-85, herein incorporated by reference in its entirety). The different secondary structures in the 5'UTR can be incorporated into the flanking region to either stabilize or selectively destalized mRNAs in specific tissues or cells.

[000431] By engineering the features typically found in abundantly expressed genes of specific target organs, one can enhance the stability and protein production of the polynucleotides of the invention. For example, introduction of 5' UTR of liver-expressed mRNA, such as albumin, serum amyloid A, Apolipoprotein A/B/E, transferrin, alpha fetoprotein, erythropoietin, or Factor VIII, could be used to enhance expression of a nucleic acid molecule, such as polynucleotides, in hepatic cell lines or liver. Likewise, use of 5' UTR from other tissue-specific mRNA to improve expression in that tissue is possible for muscle (MyoD, Myosin, Myoglobin, Myogenin, Herculin), for endothelial cells (Tie-1, CD36), for myeloid cells (C/EBP, AML1, G-CSF, GM-CSF, CD1 lb, MSR, Fr-1, i-NOS), for leukocytes (CD45, CD 18), for adipose tissue (CD36, GLUT4, ACRP30, adiponectin) and for lung epithelial cells (SP-A/B/C/D). Untranslated regions

useful in the design and manufacture of polynucleotides include, but are not limited, to those disclosed in co-pending, co-owned International Patent Publication No. WO2014164253 (Attorney Docket Number M42.20), the contents of which are incorporated herein by reference in its entirety.

[000432] Other non-UTR sequences may also be used as regions or subregions within the polynucleotides. For example, introns or portions of introns sequences may be incorporated into regions of the polynucleotides of the invention. Incorporation of intronic sequences may increase protein production as well as polynucleotide levels.

[000433] Combinations of features may be included in flanking regions and may be contained within other features. For example, the ORF may be flanked by a 5' UTR which may contain a strong Kozak translational initiation signal and/or a 3' UTR which may include an oligo(dT) sequence for templated addition of a poly-A tail. 5'UTR may comprise a first polynucleotide fragment and a second polynucleotide fragment from the same and/or different genes such as the 5'UTRs described in US Patent Application Publication No. 20100293625, herein incorporated by reference in its entirety.

[000434] Co-pending, co-owned International Patent Publication No. WO2014164253 (Attorney Docket Number M42.20), provides a listing of exemplary UTRs which may be utilized in the polynucleotide of the present invention as flanking regions. Variants of 5' or 3' UTRs may be utilized wherein one or more nucleotides are added or removed to the termini, including A, T, C or G.

[000435] It should be understood that any UTR from any gene may be incorporated into the regions of the polynucleotide. Furthermore, multiple wild-type UTRs of any known gene may be utilized. It is also within the scope of the present invention to provide artificial UTRs which are not variants of wild type regions. These UTRs or portions thereof may be placed in the same orientation as in the transcript from which they were selected or may be altered in orientation or location. Hence a 5' or 3' UTR may be inverted, shortened, lengthened, made with one or more other 5' UTRs or 3' UTRs. As used herein, the term "altered" as it relates to a UTR sequence, means that the UTR has been changed in some way in relation to a reference sequence. For example, a 3' or 5' UTR may be altered relative to a wild type or native UTR by the change in orientation or location as taught above or may be altered by the inclusion of additional nucleotides, deletion of nucleotides, swapping or transposition of nucleotides. Any of these changes producing an "altered" UTR (whether 3' or 5') comprise a variant UTR.

[000436] In one embodiment, a double, triple or quadruple UTR such as a 5′ or 3' UTR may be used. As used herein, a "double" UTR is one in which two copies of the same UTR are encoded

either in series or substantially in series. For example, a double beta-globin 3' UTR may be used as described in US Patent publication 20100129877, the contents of which are incorporated herein by reference in its entirety.

[000437] It is also within the scope of the present invention to have patterned UTRs. As used herein "patterned UTRs" are those UTRs which reflect a repeating or alternating pattern, such as ABABAB or AABBAABBAABB or ABCABCABC or variants thereof repeated once, twice, or more than 3 times. In these patterns, each letter, A, B, or C represent a different UTR at the nucleotide level.

[000438] In one embodiment, flanking regions are selected from a family of transcripts whose proteins share a common function, structure, feature of property. For example, polypeptides of interest may belong to a family of proteins which are expressed in a particular cell, tissue or at some time during development. The UTRs from any of these genes may be swapped for any other UTR of the same or different family of proteins to create a new polynucleotide. As used herein, a "family of proteins" is used in the broadest sense to refer to a group of two or more polypeptides of interest which share at least one function, structure, feature, localization, origin, or expression pattern.

[000439] In one embodiment, flanking regions may be heterologous.

[000440] In one embodiment, the 5' untranslated region may be derived from a different species than the 3' untranslated region.

[000441] The untranslated region may also include translation enhancer elements (TEE). As a non-limiting example, the TEE may include those described in US Application No. 20090226470, herein incorporated by reference in its entirety, and those known in the art.

5 'UTR and Histone Stem Loops

[000442] In one embodiment, the polynucleotides may include a nucleic acid sequence which is derived from the 5'UTR of a 5'-terminal oligopyrimidine (TOP) gene and at least one histone stem loop. Non-limiting examples of nucleic acid sequences which are derived from the 5'UTR of a TOP gene are taught in International Patent Publication No. WO2013 143699, the contents of which are herein incorporated by reference in its entirety.

5 'UTR and GTX gene sequences

[000443] In one embodiment, at least one fragment of the IRES sequences from a GTX gene may be included in the 5'UTR. As a non-limiting example, the fragment may be an 18 nucleotide sequence from the IRES of the GTX gene. While not wishing to be bound by theory, the addition of at least one fragment of the IRES sequence from the GTX gene in the 5'UTR may assist in the ribosome docking to the 5'UTR which may increase protein expression. As

another non-limiting example, an 18 nucleotide sequence fragment from the IRES sequence of a GTX gene may be tandemly repeated in the 5'UTR of a polynucleotide described herein. The 18 nucleotide sequence may be repeated in the 5'UTR at least one, at least twice, at least three times, at least four times, at least five times, at least six times, at least seven times, at least eight times, at least nine times or more than ten times

[000444] In one embodiment, a polynucleotide may include at least one 18 nucleotide fragment of the IRES sequences from a GTX gene in the 5'UTR. In another embodiment, a polynucleotide may include at least five 18 nucleotide fragments of the IRES sequences from a GTX gene in the 5'UTR. In one embodiment the 18 nucleotide fragment may be AATTCTGACATCCGGCGG (SEQ ID NO: 3) or a fragment or variant thereof.

[000445] In one embodiment, a polynucleotide may include at least one 18 nucleotide fragment of the IRES sequences from a GTX gene in the 5'UTR in order to increase expression of the protein encoded by the polynucleotide.

[000446] In one embodiment, a polynucleotide may include at least one fragment of the IRES sequences from a GTX gene may be included in the 5'UTR where the at least one fragment of the IRES sequence from the GTX gene include at least one chemical modification. As a non-limiting example, the at least one chemical modification may be 5-methylcytosine.

[000447] In one embodiment, a polynucleotide may include at least one fragment of the IRES sequences from a GTX gene and at least one translation enhancer element sequence or fragment thereof in the 5'UTR.

5 'UTR and Purines at the Start Site for Translation

[000448] In one embodiment, the polynucleotides described herein comprise at least one purine residue (adenine or guanine) at the start site for translation of the polynucleotide. In another embodiment, the polynucleotides described herein comprise at least two consecutive purine residues (adenine or guanine) at the start site for translation of the polynucleotide.

[000449] In one embodiment, the polynucleotides described herein comprise at least one purine residue (adenine or guanine) at the T7 start site for translation of the polynucleotide. In another embodiment, the polynucleotides described herein comprise at least two consecutive purine residues (adenine or guanine) at the T7 start site for translation of the polynucleotide.

[000450] In one embodiment, the polynucleotides described herein comprise three consecutive guanine (G) residues at the start site for translation. In another embodiment, the polynucleotides described herein comprise two consecutive guanine (G) residues at the start site for translation. In yet another embodiment, the polynucleotides described herein comprise one guanine (G)

residues at the start site for translation. In yet another embodiment, the polynucleotides described herein do not comprise a guanine (G) residue at the start site for translation.

[000451] In one embodiment, the polynucleotides described herein comprise three consecutive guanine (G) residues at the T7 start site for translation. In another embodiment, the polynucleotides described herein comprise two consecutive guanine (G) residues at the T7 start site for translation. In yet another embodiment, the polynucleotides described herein comprise one guanine (G) residues at the T7 start site for translation. In yet another embodiment, the polynucleotides described herein do not comprise a guanine (G) residue at the T7 start site for translation.

[000452] In one embodiment, the polynucleotides described herein comprise at least one pyrimidine residue (cytosine, thymine or uracil) at the start site for translation of the polynucleotide. In another embodiment, the polynucleotides described herein comprise at least two consecutive pyrimidine residues (cytosine, thymine or uracil) at the start site for translation of the polynucleotide.

[000453] In one embodiment, the polynucleotides described herein comprise at least one pyrimidine residue (cytosine, thymine or uracil) at the T7 start site for translation of the polynucleotide. In another embodiment, the polynucleotides described herein comprise at least two consecutive pyrimidine residues (cytosine, thymine or uracil) at the T7 start site for translation of the polynucleotide.

[000454] In one embodiment, the polynucleotides described herein comprise three consecutive cytosine (C) residues at the start site for translation. In another embodiment, the polynucleotides described herein comprise two consecutive cytosine (C) residues at the start site for translation. In yet another embodiment, the polynucleotides described herein comprise one cytosine (C) residues at the start site for translation. In yet another embodiment, the polynucleotides described herein do not comprise a cytosine (C) residue at the start site for translation.

[000455] In one embodiment, the polynucleotides described herein comprise three consecutive cytosine (C) residues at the T7 start site for translation. In another embodiment, the polynucleotides described herein comprise two consecutive cytosine (C) residues at the T7 start site for translation. In yet another embodiment, the polynucleotides described herein comprise one cytosine (C) residues at the T7 start site for translation. In yet another embodiment, the polynucleotides described herein do not comprise a cytosine (C) residue at the T7 start site for translation.

[000456] In one embodiment, the polynucleotides described herein comprise three consecutive thymine (T) residues at the start site for translation. In another embodiment, the polynucleotides

described herein comprise two consecutive thymine (T) residues at the start site for translation. In yet another embodiment, the polynucleotides described herein comprise one thymine (T) residues at the start site for translation. In yet another embodiment, the polynucleotides described herein do not comprise a thymine (T) residue at the start site for translation.

[000457] In one embodiment, the polynucleotides described herein comprise three consecutive thymine (T) residues at the T7 start site for translation. In another embodiment, the polynucleotides described herein comprise two consecutive thymine (T) residues at the T7 start site for translation. In yet another embodiment, the polynucleotides described herein comprise one thymine (T) residues at the T7 start site for translation. In yet another embodiment, the polynucleotides described herein do not comprise a thymine (T) residue at the T7 start site for translation.

[000458] In one embodiment, the polynucleotides described herein comprise three consecutive uracil (U) residues at the start site for translation. In another embodiment, the polynucleotides described herein comprise two consecutive uracil (U) residues at the start site for translation. In yet another embodiment, the polynucleotides described herein comprise one uracil (U) residues at the start site for translation. In yet another embodiment, the polynucleotides described herein do not comprise an uracil (U) residue at the start site for translation.

[000459] In one embodiment, the polynucleotides described herein comprise three consecutive uracil (U) residues at the T7 start site for translation. In another embodiment, the polynucleotides described herein comprise two consecutive uracil (U) residues at the T7 start site for translation. In yet another embodiment, the polynucleotides described herein comprise one uracil (U) residues at the T7 start site for translation. In yet another embodiment, the polynucleotides described herein do not comprise an uracil (U) residue at the T7 start site for translation.

[000460] In one embodiment, the polynucleotides described herein do not comprise a guanine (G), cytosine (C), thymine (T) or uracil (U) residue at the start site for translation.

5 'UTR, 3 'UTR and Translation Enhancer Elements (TEEs)

[000461] In one embodiment, the 5'UTR of the polynucleotides may include at least one translational enhancer polynucleotide, translation enhancer element, translational enhancer elements (collectively referred to as "TEE"s). As a non-limiting example, the TEE may be located between the transcription promoter and the start codon. The polynucleotides with at least one TEE in the 5'UTR may include a cap at the 5'UTR. Further, at least one TEE may be located in the 5'UTR of polynucleotides undergoing cap-dependent or cap-independent translation.

[000462] The term "translational enhancer element" or "translation enhancer element" (herein collectively referred to as "TEE") refers to sequences that increase the amount of polypeptide or protein produced from a nucleic acid. TEEs are described in paragraphs [001 16] - [00140] of copending International Publication No. WO2014081507, the contents of which are herein in corproated by reference in its entirety.

Heterologous 5 'UTRs

[000463] A 5' UTR may be provided as a flanking region to the polynucleotides of the invention. 5'UTR may be homologous or heterologous to the coding region found in the polynucleotides of the invention. Multiple 5' UTRs may be included in the flanking region and may be the same or of different sequences. Any portion of the flanking regions, including none, may be codon optimized and any may independently contain one or more different structural or chemical modifications, before and/or after codon optimization.

[000464] Shown in Lengthy Table 2 1 in US Provisional Application No 61/775,509, filed March 9, 2013, entitled Heterologous Untranslated Regions for mRNA, in Table 2, Table 2 1 and in Table 22 in US Provisional Application No 61/829,372, filed May 3 1, 2013, entitled Heterologous Untranslated Regions for mRNA, and in Table 2, Table 2 1 and in Table 22 in International Patent Application No PCT/US2014/021522, filed March 7, 2014, entitled Heterologous Untranslated Regions for mRNA the contents of each of which is herein incorporated by reference in its entirety, is a listing of the start and stop site of the polynucleotides of the invention. In Table 2 1 each 5'UTR (5'UTR-005 to 5'UTR 6851 1) is identified by its start and stop site relative to its native or wild type (homologous) transcript (ENST; the identifier used in the ENSEMBL database).

[000465] Additional 5'UTR which may be used with the polynucleotides of the invention are shown in the present disclosure in Table 2.

[000466] To alter one or more properties of the polynucleotides, primary constructs or mmRNA of the invention, 5'UTRs which are heterologous to the coding region of the polynucleotides of the invention are engineered into compounds of the invention. The polynucleotides are then administered to cells, tissue or organisms and outcomes such as protein level, localization and/or half life are measured to evaluate the beneficial effects the heterologous 5'UTR may have on the polynucleotides of the invention. Variants of the 5' UTRs may be utilized wherein one or more nucleotides are added or removed to the termini, including A, T, C or G. 5'UTRs may also be codon-optimized or modified in any manner described herein.

5 'UTR and Riboswitches

[000467] Riboswitches are commonly found in the 5' UTR of mRNA and comprise an aptamer domain and an expression platform. While not wishing to be bound by theory, riboswitches exert regulatory control over a transcript in a *cis*-fashion by directly binding a small molecule ligand (Garst et al. Cold Spring Harb Perspect Biol 201 l;3:a003533, 1-13, the contents of which are herein incorporated by reference in its entirety). The aptamer domain recognizes the effector molecule and the expression platform contains a structural switch that interfaces with the transcriptional or translational machinery. The overlap between the aptamer domain and the expression platform is called the switching sequence which regulates the folding of RNA into either the on or off state of the mRNA (see Figure IB described in Garst et al. Cold Spring Harb Perspect Biol 201 l;3:a003533, 1-13, the contents of which are herein incorporated by reference in its entirety). As a non-limiting example, the riboswitch may be any of the riboswitches described in Table 1 Garst et al. Cold Spring Harb Perspect Biol 201 l;3:a003533, 1-13, the contents of which are herein incorporated by reference in its entirety. As another non-limiting example, the riboswitch may be a synthetic RNA switch which can direct expression machinery.

[000468] In one embodiment, the polynucleotides described herein may comprise at least one riboswitch or fragment or variant thereof, which may be located an untranslated region of the polynucleotide. As a non-limiting example, at least one riboswitch may be located in the 5' untranslated region of the polynucleotide. As another non-limiting example, at least one riboswitch may be located in the 3' untranslated region of the polynucleotide.

[000469] In one embodiment, the polynucleotides described herein may comprise at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, at least 20 or more than 20 riboswitches.

[000470] In one embodiment, the order of the riboswitches in the polynucleotides described herein may be altered in order to form a branched or rod structure (see e.g., Figure 6A in Garst et al. Cold Spring Harb Perspect Biol 201 l;3:a003533, 1-13, the contents of which are herein incorporated by reference in its entirety).

[000471] In one embodiment, the polynucleotides described herein may comprise at least two riboswitches in order to form a branched structure in the 5' untranslated region of the polynucleotide. In another embodiment, the polynucleotides described herein may comprise at least four riboswitches in order to form two branched structures in the 5' untranslated region of the polynucleotide.

[000472] In one embodiment, the polynucleotides described herein may comprise at least two riboswitches in order to form a rod structure in the 5' untranslated region of the polynucleotide.

In another embodiment, the polynucleotides described herein may comprise at least four riboswitches in order to form a rod structure in the 5' untranslated region of the polynucleotide.

3' UTR and the AU Rich Elements

[000473] Natural or wild type 3' UTRs are known to have stretches of Adenosines and Uridines embedded in them. These AU rich signatures are particularly prevalent in genes with high rates of turnover. Based on their sequence features and functional properties, the AU rich elements (AREs) can be separated into three classes (Chen et al, 1995): Class I AREs contain several dispersed copies of an AUUUA motif within U-rich regions. C-Myc and MyoD contain class I AREs. Class II AREs possess two or more overlapping UUAUUUA(U/A)(U/A) nonamers. Molecules containing this type of AREs include GM-CSF and TNF-a. Class III ARES are less well defined. These U rich regions do not contain an AUUUA motif. c-Jun and Myogenin are two well-studied examples of this class. Most proteins binding to the AREs are known to destabilize the messenger, whereas members of the ELAV family, most notably HuR, have been documented to increase the stability of mRNA. HuR binds to AREs of all the three classes. Engineering the HuR specific binding sites into the 3' UTR of nucleic acid molecules will lead to HuR binding and thus, stabilization of the message *in vivo.*

[000474] Introduction, removal or modification of 3' UTR AU rich elements (AREs) can be used to modulate the stability of polynucleotides of the invention. When engineering specific polynucleotides, one or more copies of an ARE can be introduced to make polynucleotides of the invention less stable and thereby curtail translation and decrease production of the resultant protein. Likewise, AREs can be identified and removed or mutated to increase the intracellular stability and thus increase translation and production of the resultant protein. Transfection experiments can be conducted in relevant cell lines, using polynucleotides of the invention and protein production can be assayed at various time points post-transfection. For example, cells can be transfected with different ARE-engineering molecules and by using an ELISA kit to the relevant protein and assaying protein produced at 6 hour, 12 hour, 24 hour, 48 hour, and 7 days post-transfection.

Untranslated Regions and microRNA Binding Sites

[000475] microRNAs (or miRNA) are 19-25 nucleotide long noncoding RNAs that bind to the 3'UTR of nucleic acid molecules and down-regulate gene expression either by reducing nucleic acid molecule stability or by inhibiting translation. The polynucleotides of the invention may comprise one or more microRNA target sequences, microRNA seqences, or microRNA seeds. Such sequences may correspond to any known microRNA such as those taught in US Publication US2005/0261218 and US Publication US2005/0059005, the contents of which are

incorporated herein by reference in their entirety. As a non-limiting embodiment, known microRNAs, their sequences and seed sequences in human genome are listed in Table 11 of US Patent Publication No. US20140147454, the contents of which are herein incorporated by reference in its entirety. The miR sequence which may be used with the polynucleotides described herein may be any of SEQ ID NO: 171-1 191 or 2213-3233 listed in Table 11 of US Patent Publication No. US20140147454, the contents of which are herein incorporated by reference in its entirety. The miR binding site (miR BS) sequence which may be used with the polynucleotides described herein may be any of SEQ ID NO: 1192-2212 or 3234-4254 listed in Table 11 of US Patent Publication No. US20140147454, the contents of which are herein incorporated by reference in its entirety.

[000476] microRNAs are differentially expressed in different tissues and cells as described in Table 12 of US Patent Publication No. US20140147454, the contents of which are herein incorporated by reference in its entirety.

[000477] microRNAs enriched in specific types of immune cells are listed in Table 1 of US Provisional Application No. 62/025,985, the contents of which are herein incorporated by reference in its entirety below. As a non-limiting example, microRNAs enriched in specific types of immune cells are described in Table 13 of US Patent Publication No. US20140147454, the contents of which are herein incorporated by reference in its entirety. Furthermore, novel miroRNAs are discovered in the immune cells in the art through micro-array hybridization and microtome analysis (Jima DD et al, Blood, 2010, 116:el 18-el27; Vaz C et al, BMC Genomics, 2010, 11,288, the content of each of which is incorporated herein by reference in its entirety).

[000478] A microRNA sequence comprises a "seed" region, i.e., a sequence in the region of positions 2-8 of the mature microRNA, which sequence has perfect Watson-Crick complementarity to the miRNA target sequence. A microRNA seed may comprise positions 2-8 or 2-7 of the mature microRNA. In some embodiments, a microRNA seed may comprise 7 nucleotides (e.g., nucleotides 2-8 of the mature microRNA), wherein the seed-complementary site in the corresponding miRNA target is flanked by an adenine (A) opposed to microRNA position 1. In some embodiments, a microRNA seed may comprise 6 nucleotides (e.g., nucleotides 2-7 of the mature microRNA), wherein the seed-complementary site in the corresponding miRNA target is flanked byan adenine (A) opposed to microRNA position 1. See for example, Grimson A, Farh KK, Johnston WK, Garrett-Engele P, Lim LP, Bartel DP; Mol Cell. 2007 Jul 6;27(1):91-105; each of which is herein incorporated by reference in their entirety. The bases of the microRNA seed have complete complementarity with the target sequence. By engineering microRNA target sequences into the polynucleotides (e.g., in a 3'UTR like region or

other region) of the invention one can target the molecule for degradation or reduced translation, provided the microRNA in question is available. This process will reduce the hazard of off target effects upon nucleic acid molecule delivery. Identification of microRNA, microRNA target regions, and their expression patterns and role in biology have been reported (Bonauer et al, Curr Drug Targets 2010 11:943-949; Anand and Cheresh Curr Opin Hematol 201 1 18: 171-176; Contreras and Rao Leukemia 2012 26:404-413 (201 1 Dec 20. doi: 10. 1038/leu.201 1.356); Bartel Cell 2009 136:215-233; Landgraf et al, Cell, 2007 129:1401-1414; each of which is herein incorporated by reference in its entirety).

[000479] microRNAs are are differentially expressed in different tissues and cells, and often associated with different types of dieases (e.g.cancer cells). The decision of removal or insertion of microRNA binding sites, or any combination, is dependent on microRNA expression patterns and their profilings in cancer cells. Various microRNAs and the tissue, the associated disease and biological function are described in Table 12 of International Patent Application No. PCT/US 13/62943 (Attorney Docket No. M39.21), the contents of which are herein incorporated by reference in its entirety.

[000480] Examples of tissues where microRNA are known to regulate mRNA, and thereby protein expression, include, but are not limited to, liver (miR-122), muscle (miR-133, miR-206, miR-208), endothelial cells (miR-17-92, miR-126), myeloid cells (miR-142-3p, miR-142-5p, miR-16, miR-21, miR-223, miR-24, miR-27), adipose tissue (let-7, miR-30c), heart (miR-ld, miR-149), kidney (miR-192, miR-194, miR-204), and lung epithelial cells (let-7, miR-133, miR-126). MicroRNA can also regulate complex biological processes such as angiogenesis (miR-132) (Anand and Cheresh Curr Opin Hematol 201 1 18:171-176; herein incorporated by reference in its entirety).

[000481] MicroRNAs may also be enriched in specific types of immune cells. A non-exhaustive listing of the microRNAs enriched in immune cells is described in Table 13 of International Patent Application No. PCT/US 13/62943 (Attorney Docket No. M39.21), the contents of which are herein incorporated by reference in its entirety. Furthermore, novel miroRNAs are discovered in the immune cells in the art through micro-array hybridization and microtome analysis (Jima DD et al, Blood, 2010, 116:el 18-el27; Vaz C et al, BMC Genomics, 2010, 11,288, the content of each of which is incorporated herein by reference in its entirety).

[000482] In one embodiment, polynucleotides of the invention would not only encode a polypeptide but also a microRNA sequence or a sensor sequences. Sensor sequences include, for example, microRNA binding sites, transcription factor binding sites, structured mRNA sequences and/or motifs, artificial binding sites engineered to act as pseudo-receptors for

endogenous nucleic acid binding molecules. Non-limiting examples, of polynucleotides comprising at least one sensor sequence are described in co-pending and co-owned U.S. Provisional Patent Application Nos. US 61/753,661, US 61/754,159, US61/781.097, US 61/829,334, US 61/839,893, US 61/842,733, US 61/857,304, and International Patent Application No. PCT/US13/62531, filed September 30, 2013, entitled Signal-Sensor Polynucleotides for the Alteration of Cellular Phenotypes, the contents of each of which are herein incorporated by reference in its entirety.

[000483] In one embodiment, microRNA (miRNA) profiling of the target cells or tissues is conducted to determine the presence or absence of miRNA in the cells or tissues.

[000484] For example, if the polynucleotide and is not intended to be delivered to the liver but ends up there, then miR-122, a microRNA abundant in liver, can inhibit the expression of the gene of interest if one or multiple target sites of miR-122 are engineered into the 3' UTR region of the polynucleotides. Introduction of one or multiple binding sites for different microRNA can be engineered to further decrease the longevity, stability, and protein translation of polynucleotides.

[000485] As used herein, the term "microRNA site" refers to a microRNA target site or a microRNA recognition site, or any nucleotide sequence to which a microRNA binds or associates. It should be understood that "binding" may follow traditional Watson-Crick hybridization rules or may reflect any stable association of the microRNA with the target sequence at or adjacent to the microRNA site.

[000486] Conversely, for the purposes of the polynucleotides of the present invention, microRNA binding sites can be engineered out of (i.e. removed from) sequences in which they occur, e.g., in order to increase protein expression in specific tissues. For example, miR-122 binding sites may be removed to improve protein expression in the liver. Regulation of expression in multiple tissues can be accomplished through introduction or removal or one or several microRNA binding sites.

[000487] In one embodiment, the polynucleotides of the present invention may include at least one miRNA-binding site in the 3'UTR in order to direct cytotoxic or cytoprotective mRNA therapeutics to specific cells such as, but not limited to, normal and/or cancerous cells (e.g., HEP3B or SNU449).

[000488] In another embodiment, the polynucleotides of the present invention may include three miRNA-binding sites in the 3'UTR in order to direct cytotoxic or cytoprotective mRNA therapeutics to specific cells such as, but not limited to, normal and/or cancerous cells (e.g., HEP3B or SNU449).

[000489] Expression profiles, microRNA and cell lines useful in the present invention include those taught in for example, in International Patent Publication Nos. WO20141 13089 (Attorney Docket Number M37) and WO2014081507 (Attorney Docket Number M39), the contents of each of which are incorporated by reference in their entirety.

[000490] In the polynucleotides of the present invention, binding sites for microRNAs that are involved in such processes may be removed or introduced, in order to tailor the expression of the polynucleotides expression to biologically relevant cell types or to the context of relevant biological processes. A listing of microRNA, miR sequences and miR binding sites is listed in Table 9 of U.S. Provisional Application No. 61/753,661 filed January 17, 2013, in Table 9 of U.S. Provisional Application No. 61/754,159 filed January 18, 2013, and in Table 7 of U.S. Provisional Application No. 61/758,921 filed January 31, 2013, each of which are herein incorporated by reference in their entireties.

[000491] Examples of use of microRNA to drive tissue or disease-specific gene expression are listed (Getner and Naldini, Tissue Antigens. 2012, 80:393-403; herein incorporated by reference in its entirety). In addition, microRNA seed sites can be incorporated into mRNA to decrease expression in certain cells which results in a biological improvement. An example of this is incorporation of miR-142 sites into a UGTlAl-expressing lentiviral vector. The presence of miR-142 seed sites reduced expression in hematopoietic cells, and as a consequence reduced expression in antigen-presenting cells, leading to the absence of an immune response against the virally expressed UGT1A1 (Schmitt et al, Gastroenterology 2010; 139:999-1007; Gonzalez-Asequinolaza et al. Gastroenterology 2010, 139:726-729; both herein incorporated by reference in its entirety) . Incorporation of miR-142 sites into polynucleotides such as modified mRNA could not only reduce expression of the encoded protein in hematopoietic cells, but could also reduce or abolish immune responses to the mRNA-encoded protein. Incorporation of miR-142 seed sites (one or multiple) into mRNA would be important in the case of treatment of patients with complete protein deficiencies (UGT1A1 type I, LDLR-deficient patients, CRIM-negative Pompe patients, etc.).

[000492] Specifically, microRNAs are known to be differentially expressed in immune cells (also called hematopoietic cells), such as antigen presenting cells (APCs) (e.g. dendritic cells and macrophages), macrophages, monocytes, B lymphocytes, T lymphocytes, granuocytes, natural killer cells, etc. Immune cell specific microRNAs are involved in immunogenicity, autoimmunity, the immune -response to infection, inflammation, as well as unwanted immune response after gene therapy and tissue/organ transplantation. Immune cells specific microRNAs also regulate many aspects of development, proliferation, differentiation and apoptosis of

hematopoietic cells (immune cells). For example, miR-142 and miR-146 are exclusively expressed in the immune cells, particularly abundant in myeloid dendritic cells. It was demonstrated in the art that the immune response to exogenous nucleic acid molecules was shut-off by adding miR-142 binding sites to the 3'UTR of the delivered gene construct, enabling more stable gene transfer in tissues and cells. miR-142 efficiently degrades the exogenous mRNA in antigen presenting cells and suppresses cytotoxic elimination of transduced cells (Annoni A et al, blood, 2009, 114, 5152-5161; Brown BD, et al, Nat med. 2006, 12(5), 585-591; Brown BD, et al, blood, 2007, 110(13): 4144-4152, each of which is herein incorporated by reference in its entirety).

[000493] An antigen-mediated immune response can refer to an immune response triggered by foreign antigens, which, when entering an organism, are processed by the antigen presenting cells and displayed on the surface of the antigen presenting cells. T cells can recognize the presented antigen and induce a cytotoxic elimination of cells that express the antigen.

[000494] Introducing the miR-142 binding site into the 3'-UTR of a polynucleotide of the present invention can selectively repress the gene expression in the antigen presenting cells through miR-142 mediated mRNA degradation, limiting antigen presentation in APCs (e.g. dendritic cells) and thereby preventing antigen-mediated immune response after the delivery of the polynucleotides. The polynucleotides are therefore stably expressed in target tissues or cells without triggering cytotoxic elimination.

[000495] In one embodiment, microRNAs binding sites that are known to be expressed in immune cells, in particular, the antigen presenting cells, can be engineered into the polynucleotide to suppress the expression of the sensor-signal polynucleotide in APCs through microRNA mediated RNA degradation, subduing the antigen-mediated immune response, while the expression of the polynucleotide is maintained in non-immune cells where the immune cell specific microRNAs are not expressed. For example, to prevent the immunogenic reaction caused by a liver specific protein expression, the miR-122 binding site can be removed and the miR-142 (and/or mirR-146) binding sites can be engineered into the 3-UTR of the polynucleotide.

[000496] To further drive the selective degradation and suppression of mRNA in APCs and macrophage, the polynucleotide may include another negative regulatory element in the 3-UTR, either alone or in combination with mir-142 and/or mir-146 binding sites. As a non-limiting example, one regulatory element is the Constitutive Decay Elements (CDEs).

[000497] Immune cells specific microRNAs include, but are not limited to, hsa-let-7a-2-3p, hsa-let-7a-3p, hsa-7a-5p, hsa-let-7c, hsa-let-7e-3p, hsa-let-7e-5p, hsa-let-7g-3p, hsa-let-7g-5p,

hsa-let-7i-3p, hsa-let-7i-5p, miR-10a-3p, miR-10a-5p, miR-1 184, hsa-let-7f-l—3p, hsa-let-7f-2—5p, hsa-let-7f-5p, miR-125b-l-3p, miR-125b-2-3p, miR-125b-5p, miR-1279, miR-130a-3p, miR-130a-5p, miR-132-3p, miR-132-5p, miR-142-3p, miR-142-5p, miR-143-3p, miR-143-5p, miR-146a-3p, miR-146a-5p, miR-146b-3p, miR-146b-5p, miR-147a, miR-147b, miR-148a-5p, miR-148a-3p, miR-150-3p, miR-150-5p, miR-15lb, miR-155-3p, miR-155-5p, miR-15a-3p, miR-15a-5p, miR-15b-5p, miR-15b-3p, miR-16-l-3p, miR-16-2-3p, miR-16-5p, miR-17-5p, miR-181a-3p, miR-181a-5p, miR-181a-2-3p, miR-182-3p, miR-182-5p, miR-197-3p, miR-197-5p, miR-21-5p, miR-21-3p, miR-214-3p, miR-214-5p, miR-223-3p, miR-223-5p, miR-221-3p, miR-221-5p, miR-23b-3p, miR-23b-5p, miR-24-l-5p,miR-24-2-5p, miR-24-3p, miR-26a-l-3p, miR-26a-2-3p, miR-26a-5p, miR-26b-3p, miR-26b-5p, miR-27a-3p, miR-27a-5p, miR-27b-3p,miR-27b-5p, miR-28-3p, miR-28-5p, miR-2909, miR-29a-3p, miR-29a-5p, miR-29b-l-5p, miR-29b-2-5p, miR-29c-3p, miR-29c-5p,, miR-30e-3p, miR-30e-5p, miR-331-5p, miR-339-3p, miR-339-5p, miR-345-3p, miR-345-5p, miR-346, miR-34a-3p, miR-34a-5p, , miR-363-3p, miR-363-5p, miR-372, miR-377-3p, miR-377-5p, miR-493-3p, miR-493-5p, miR-542, miR-548b-5p, miR548c-5p, miR-548i, miR-548j, miR-548n, miR-574-3p, miR-598, miR-718, miR-935, miR-99a-3p, miR-99a-5p, miR-99b-3p and miR-99b-5p. microRNAs that are enriched in specific types of immune cells are listed in Table 13 of US Patent Application No. 14/043,927 (Attorney Docket No. M039. 11), filed on October 2, 2013, the contents of which are herein incorporated by reference in its entirety. Furthermore, novel miroRNAs are discovered in the immune cells in the art through micro-array hybridization and microtome analysis (Jima DD et al, Blood, 2010, 116:ell8-el27; Vaz C et al, BMC Genomics, 2010, 11,288, the content of each of which is incorporated herein by reference in its entirety).

[000498] MicroRNAs that are known to be expressed in the liver include, but are not limited to, miR-107, miR-122-3p, miR-122-5p, miR-1228-3p, miR-1228-5p, miR-1249, miR-129-5p, miR-1303, miR-151a-3p, miR-151a-5p, miR-152, miR-194-3p, miR-194-5p, miR-199a-3p, miR-199a-5p, miR-199b-3p, miR-199b-5p, miR-296-5p, miR-557, miR-581, miR-939-3p, miR-939-5p. MicroRNA binding sites from any liver specific microRNA can be introduced to or removed from the polynucleotides to regulate the expression of the polynucleotides in the liver. Liver specific microRNAs binding sites can be engineered alone or further in combination with immune cells (e.g. APCs) microRNA binding sites in order to prevent immune reaction against protein expression in the liver.

[000499] In one embodiment, the polynucleotides described herein comprise at least one miR sequence known to be expressed in the liver. As a non-limiting example, the polynucleotides described herein may include at least one miR-122 sequence or fragment thereof. The miR-122

sequence may include the seed sequence or it may be without the seed sequence. As a non-limiting example, the polynucleotides described herein may include at least one miR-122 sequence or fragment thereof in the 3'UTR.

[000500] MicroRNAs that are known to be expressed in the lung include, but are not limited to, let-7a-2-3p, let-7a-3p, let-7a-5p, miR-126-3p, miR-126-5p, miR-127-3p, miR-127-5p, miR-130a-3p, miR-130a-5p, miR-130b-3p, miR-130b-5p, miR-133a, miR-133b, miR-134, miR-1 8a-3p, miR-18a-5p, miR-18b-3p, miR-18b-5p, miR-24-l-5p, miR-24-2-5p, miR-24-3p, miR-296-3p, miR-296-5p, miR-32-3p, miR-337-3p, miR-337-5p, miR-381-3p, miR-381-5p and mir-21. MicroRNA binding sites from any lung specific microRNA can be introduced to or removed from the polynucleotide to regulate the expression of the polynucleotide in the lung. Lung specific microRNAs binding sites can be engineered alone or further in combination with immune cells (e.g. APCs) microRNA binding sites in order to prevent an immune reaction against protein expression in the lung.

[000501] In one embodiment, the polynucleotides described herein comprise at least one miR sequence known to be expressed in the lung. As a non-limiting example, the polynucleotides described herein may include at least one miR-21 sequence or fragment thereof. The miR-21 sequence may include the seed sequence or it may be without the seed sequence. As a non-limiting example, the polynucleotides described herein may include at least one miR-2 1 sequence or fragment thereof in the 3'UTR.

[000502] MicroRNAs that are known to be expressed in the heart include, but are not limited to, miR-1, miR-133a, miR-133b, miR-149-3p, miR-149-5p, miR-186-3p, miR-186-5p, miR-208a, miR-208b, miR-210, miR-296-3p, miR-320, miR-451a, miR-451b, miR-499a-3p, miR-499a-5p, miR-499b-3p, miR-499b-5p, miR-744-3p, miR-744-5p, miR-92b-3p and miR-92b-5p. MicroRNA binding sites from any heart specific microRNA can be introduced to or removed from the polynucleotides to regulate the expression of the polynucleotides in the heart. Heart specific microRNAs binding sites can be engineered alone or further in combination with immune cells (e.g. APCs) microRNA binding sites to prevent an immune reaction against protein expression in the heart.

[000503] MicroRNAs that are known to be expressed in the nervous system include, but are not limited to, miR-124-5p, miR-125a-3p, miR-125a-5p, miR-1 25b- l-3p, miR-125b-2-3p, miR-125b-5p, miR-1271-3p, miR-1271-5p, miR-128, miR-132-5p, miR-135a-3p, miR-135a-5p, miR-135b-3p, miR-135b-5p, miR-137, miR-139-5p, miR-139-3p, miR-149-3p, miR-149-5p, miR-153, miR-181c-3p, miR-181c-5p, miR-183-3p, miR-183-5p, miR-190a, miR-190b, miR-212-3p, miR-212-5p, miR-219-l-3p, miR-219-2-3p, miR-23a-3p, miR-23a-5p,miR-30a-5p, miR-30b-3p,

miR-30b-5p, miR-30c-l-3p, miR-30c-2-3p, miR-30c-5p, miR-30d-3p, miR-30d-5p, miR-329, miR-342-3p, miR-3665, miR-3666, miR-380-3p, miR-380-5p, miR-383, miR-410, miR-425-3p, miR-425-5p, miR-454-3p, miR-454-5p, miR-483, miR-510, miR-516a-3p, miR-548b-5p, miR-548c-5p, miR-571, miR-7-l-3p, miR-7-2-3p, miR-7-5p, miR-802, miR-922, miR-9-3p, miR-9-5p, miR-132-3p and miR-132-5p. MicroRNAs enriched in the nervous system further include those specifically expressed in neurons, including, but not limited to, miR-132-3p, miR-132-5p, miR-148b-3p, miR-148b-5p, miR-151a-3p, miR-151a-5p, miR-212-3p, miR-212-5p, miR-320b, miR-320e, miR-323a-3p, miR-323a-5p, miR-324-5p, miR-325, miR-326, miR-328, miR-922 and those specifically expressed in glial cells, including, but not limited to, miR-1250, miR-219-1-3p, miR-219-2-3p, miR-219-5p, miR-23a-3p, miR-23a-5p, miR-3065-3p, miR-3065-5p, miR-30e-3p, miR-30e-5p, miR-32-5p, miR-338-5p, miR-657. MicroRNA binding sites from any CNS specific microRNA can be introduced to or removed from the polynucleotides to regulate the expression of the polynucleotide in the nervous system. Nervous system specific microRNAs binding sites can be engineered alone or further in combination with immune cells (e.g. APCs) microRNA binding sites in order to prevent immune reaction against protein expression in the nervous system.

[000504] In one embodiment, the polynucleotides described herein comprise at least one miR sequence known to be expressed in tissue associated with the central nervous system or in the central nervous system. As a non-limiting example, the polynucleotides described herein may include at least one miR seuqence or fragment thereof such as miR-132-3p, miR-132-5p, miR-124-5p, miR-125a-3p, miR-125a-5p, miR-125b-l-3p, miR-125b-2-3p and miR-125b-5p. The miR sequence may include the seed sequence or it may be without the seed sequence. As a non-limiting example, the polynucleotides described herein may include at least one miR sequence or fragment thereof that can target the central nervous system in the 3'UTR.

[000505] MicroRNAs that are known to be expressed in the pancreas include, but are not limited to, miR-105-3p, miR-105-5p, miR-184, miR-195-3p, miR-195-5p, miR-196a-3p, miR-196a-5p, miR-214-3p, miR-214-5p, miR-216a-3p, miR-216a-5p, miR-30a-3p, miR-33a-3p, miR-33a-5p, miR-375, miR-7-l-3p, miR-7-2-3p, miR-493-3p, miR-493-5p and miR-944. MicroRNA binding sites from any pancreas specific microRNA can be introduced to or removed from the polynucleotide to regulate the expression of the polynucleotide in the pancreas. Pancreas specific microRNAs binding sites can be engineered alone or further in combination with immune cells (e.g. APCs) microRNA binding sites in order to prevent an immune reaction against protein expression in the pancreas.

[000506] MicroRNAs that are known to be expressed in the kidney further include, but are not limited to, miR-122-3p, miR-145-5p, miR-17-5p, miR-192-3p, miR-192-5p, miR-194-3p, miR-194-5p, miR-20a-3p, miR-20a-5p, miR-204-3p, miR-204-5p, miR-210, miR-216a-3p, miR-216a-5p, miR-296-3p, miR-30a-3p, miR-30a-5p, miR-30b-3p, miR-30b-5p, miR-30c-l-3p, miR-30c-2-3p, miR30c-5p, miR-324-3p, miR-335-3p, miR-335-5p, miR-363-3p, miR-363-5p and miR-562. MicroRNA binding sites from any kidney specific microRNA can be introduced to or removed from the polynucleotide to regulate the expression of the polynucleotide in the kidney. Kidney specific microRNAs binding sites can be engineered alone or further in combination with immune cells (e.g. APCs) microRNA binding sites to prevent an immune reaction against protein expression in the kidney.

[000507] MicroRNAs that are known to be expressed in the muscle further include, but are not limited to, let-7g-3p, let-7g-5p, miR-1, miR-1286, miR-133a, miR-133b, miR-140-3p, miR-143-3p, miR-143-5p, miR-145-3p, miR-145-5p, miR-188-3p, miR-188-5p, miR-206, miR-208a, miR-208b, miR-25-3p, miR-25-5p, and miR-1 MicroRNA binding sites from any muscle specific microRNA can be introduced to or removed from the polynucleotide to regulate the expression of the polynucleotide in the muscle. Muscle specific microRNAs binding sites can be engineered alone or further in combination with immune cells (e.g. APCs) microRNA binding sites to prevent an immune reaction against protein expression in the muscle.

[000508] In one embodiment, the polynucleotides described herein comprise at least one miR sequence known to be expressed in muscle tissue. As a non-limiting example, the polynucleotides described herein may include at least one miR sequence or fragment thereof such as miR-133a, miR-133b, miR-1 and miR-206. The miR sequence may include the seed sequence or it may be without the seed sequence. As a non-limiting example, the polynucleotides described herein may include at least one miR sequence or fragment thereof that can target the muscle tissue in the 3'UTR.

[000509] MicroRNAs are differentially expressed in different types of cells, such as endothelial cells, epithelial cells and adipocytes. For example, microRNAs that are expressed in endothelial cells include, but are not limited to, let-7b-3p, let-7b-5p, miR-100-3p, miR-100-5p, miR-101-3p, miR-101-5p, miR-126-3p, miR-126-5p, miR-1236-3p, miR-1236-5p, miR-130a-3p, miR-130a-5p, miR-17-5p, miR-17-3p, miR-18a-3p, miR-1 8a-5p, , miR-19a-3p, miR-19a-5p, miR- 19b- 1-5p, miR-19b-2-5p, miR-19b-3p, miR-20a-3p, miR-20a-5p, miR-217, miR-210, miR-21-3p, miR-21-5p, miR-221-3p, miR-221-5p, miR-222-3p, miR-222-5p, miR-23a-3p, miR-23a-5p, miR-296-5p, miR-361-3p, miR-361-5p, miR-421, miR-424-3p, miR-424-5p, miR-513a-5p, miR-92a-l-5p, miR-92a-2-5p, miR-92a-3p, miR-92b-3p and miR-92b-5p. Many novel microRNAs are

discovered in endothelial cells from deep-sequencing analysis (Voellenkle C et al, RNA, 2012, 18, 472-484, herein incorporated by reference in its entirety) microRNA binding sites from any endothelial cell specific microRNA can be introduced to or removed from the polynucleotide to modulate the expression of the polynucleotide in the endothelial cells in various conditions.

[000510] For further example, microRNAs that are expressed in epithelial cells include, but are not limited to, let-7b-3p, let-7b-5p, miR-1246, miR-200a-3p, miR-200a-5p, miR-200b-3p, miR-200b-5p, miR-200c-3p, miR-200c-5p, miR-338-3p, miR-429, miR-451a, miR-451b, miR-494, miR-802 and miR-34a, miR-34b-5p , miR-34c-5p, miR-449a, miR-449b-3p, miR-449b-5p specific in respiratory ciliated epithelial cells; let-7 family, miR-133a, miR-133b, miR-126 specific in lung epithelial cells; miR-382-3p, miR-382-5p specific in renal epithelial cells and miR-762 specific in corneal epithelial cells. MicroRNA binding sites from any epithelial cell specific MicroRNA can be introduced to or removed from the polynucleotide to modulate the expression of the polynucleotide in the epithelial cells in various conditions.

[000511] In addition, a large group of microRNAs are enriched in embryonic stem cells, controlling stem cell self-renewal as well as the development and/or differentiation of various cell lineages, such as neural cells, cardiac, hematopoietic cells, skin cells, osteogenic cells and muscle cells (Kuppusamy KT et al, Curr. Mol Med, 2013, 13(5), 757-764; Vidigal JA and Ventura A, Semin Cancer Biol. 2012, 22(5-6), 428-436; Goff LA et al, PLoS One, 2009, 4:e7192; Morin RD et al, Genome Res,2008,18, 610-621; Yoo JK et al, Stem Cells Dev. 2012, 21(1 1), 2049-2057, each of which is herein incorporated by reference in its entirety). MicroRNAs abundant in embryonic stem cells include, but are not limited to, let-7a-2-3p, let-a-3p, let-7a-5p, let7d-3p, let-7d-5p, miR-103a-2-3p, miR-103a-5p, miR-106b-3p, miR-106b-5p, miR-1246, miR-1275, miR-138-l-3p, miR-138-2-3p, miR-138-5p, miR-154-3p, miR-154-5p, miR-200c-3p, miR-200c-5p, miR-290, miR-301a-3p, miR-301a-5p, miR-302a-3p, miR-302a-5p, miR-302b-3p, miR-302b-5p, miR-302c-3p, miR-302c-5p, miR-302d-3p, miR-302d-5p, miR-302e, miR-367-3p, miR-367-5p, miR-369-3p, miR-369-5p, miR-370, miR-371, miR-373, miR-380-5p, miR-423-3p, miR-423-5p, miR-486-5p, miR-520c-3p, miR-548e, miR-548f, miR-548g-3p, miR-548g-5p, miR-548i, miR-548k, miR-5481, miR-548m, miR-548n, miR-548o-3p, miR-548o-5p, miR-548p, miR-664a-3p, miR-664a-5p, miR-664b-3p, miR-664b-5p, miR-766-3p, miR-766-5p, miR-885-3p, miR-885-5p,miR-93-3p, miR-93-5p, miR-941,miR-96-3p, miR-96-5p, miR-99b-3p and miR-99b-5p. Many predicted novel microRNAs are discovered by deep sequencing in human embryonic stem cells (Morin RD et al, Genome Res,2008,18, 610-621; Goff LA et al, PLoS One, 2009, 4:e7192; Bar M et al, Stem cells, 2008, 26, 2496-2505, the content of each of which is incorporated herein by references in its entirety).

[000512] In one embodiment, the binding sites of embryonic stem cell specific microRNAs can be included in or removed from the 3-UTR of the polynucleotide to modulate the development and/or differentiation of embryonic stem cells, to inhibit the senescence of stem cells in a degenerative condition (e.g. degenerative diseases), or to stimulate the senescence and apoptosis of stem cells in a disease condition (e.g. cancer stem cells).

[000513] In one embodiment, the polynucleotides described herein comprise at least one miR sequence known to be expressed in the spleen. As a non-limiting example, the polynucleotides described herein may include at least one miR sequence or fragment thereof such as miR-142-3p. The miR sequence may include the seed sequence or it may be without the seed sequence. As a non-limiting example, the polynucleotides described herein may include at least one miR sequence or fragment thereof that can target the tissue of the spleen in the 3'UTR.

[000514] In one embodiment, the polynucleotides described herein comprise at least one miR sequence known to be expressed in the endothelium. As a non-limiting example, the polynucleotides described herein may include at least one miR sequence or fragment thereof such as miR-126. The miR sequence may include the seed sequence or it may be without the seed sequence. As a non-limiting example, the polynucleotides described herein may include at least one miR sequence or fragment thereof that can target the tissue of the endothelium in the 3'UTR.

[000515] In one embodiment, the polynucleotides described herein comprise at least one miR sequence known to be expressed in ovarian tissue. As a non-limiting example, the polynucleotides described herein may include at least one miR sequence or fragment thereof such as miR-484. The miR sequence may include the seed sequence or it may be without the seed sequence. As a non-limiting example, the polynucleotides described herein may include at least one miR sequence or fragment thereof that can target ovarian tissue in the 3'UTR.

[000516] In one embodiment, the polynucleotides described herein comprise at least one miR sequence known to be expressed in colorectal tissue. As a non-limiting example, the polynucleotides described herein may include at least one miR sequence or fragment thereof such as miR-17. The miR sequence may include the seed sequence or it may be without the seed sequence. As a non-limiting example, the polynucleotides described herein may include at least one miR sequence or fragment thereof that can target colorectal tissue in the 3'UTR.

[000517] In one embodiment, the polynucleotides described herein comprise at least one miR sequence known to be expressed in prostate tissue. As a non-limiting example, the polynucleotides described herein may include at least one miR sequence or fragment thereof such as miR-34a. The miR sequence may include the seed sequence or it may be without the

seed sequence. As a non-limiting example, the polynucleotides described herein may include at least one miR sequence or fragment thereof that can target prostate tissue in the 3'UTR.

[000518] Many microRNA expression studies are conducted in the art to profile the differential expression of microRNAs in various cancer cells /tissues and other diseases. Some microRNAs are abnormally over-expressed in certain cancer cells and others are under-expressed. For example, microRNAs are differentially expressed in cancer cells (WO2008/154098, US2013/0059015, US2013/0042333, WO201 1/157294); cancer stem cells (US20 12/0053224); pancreatic cancers and diseases (US2009/0131348, US201 1/0171646, US2010/0286232, US8389210); asthma and inflammation (US8415096); prostate cancer (US2013/0053264); hepatocellular carcinoma (WO2012/151212, US20 12/0329672, WO2008/054828, US8252538); lung cancer cells (WO201 1/076143, WO2013/033640, WO2009/070653, US2010/0323357); cutaneous T cell lymphoma (WO2013/01 1378); colorectal cancer cells (WO201 1/0281756, WO201 1/076142); cancer positive lympho nodes (WO2009/1 00430, US2009/0263803); nasopharyngeal carcinoma (EP2 112235); chronic obstructive pulmonary disease (US20 12/0264626, US2013/0053263); thyroid cancer (WO2013/066678); ovarian cancer cells (US2012/0309645, WO201 1/095623); breast cancer cells (WO2008/1 54098, WO2007/081740, US20 12/02 14699), leukemia and lymphoma (WO2008/073915, US2009/0092974, US2012/03 16081, US2012/0283310, WO2010/018563, the content of each of which is incorporated herein by reference in their entirety.)

[000519] As a non-limiting example, microRNA sites that are over-expressed in certain cancer and/or tumor cells can be removed from the 3-UTR of the polynucleotide encoding the polypeptide of interest, restoring the expression suppressed by the over-expressed microRNAs in cancer cells, thus ameliorating the corresponsive biological function, for instance, transcription stimulation and/or repression, cell cycle arrest, apoptosis and cell death. Normal cells and tissues, wherein microRNAs expression is not up-regulated, will remain unaffected.

[000520] MicroRNA can also regulate complex biological processes such as angiogenesis (miR-132) (Anand and Cheresh Curr Opin Hematol 2011 18:171-176). In the polynucleotides of the invention, binding sites for microRNAs that are involved in such processes may be removed or introduced, in order to tailor the expression of the polynucleotides expression to biologically relevant cell types or to the context of relevant biological processes. In this context, the mRNA are defined as auxotrophic mRNA.

[000521] MicroRNA gene regulation may be influenced by the sequence surrounding the microRNA such as, but not limited to, the species of the surrounding sequence, the type of sequence (e.g., heterologous, homologous and artificial), regulatory elements in the surrounding

sequence and/or structural elements in the surrounding sequence. The microRNA may be influenced by the 5'UTR and/or the 3'UTR. As a non-limiting example, a non-human 3'UTR may increase the regulatory effect of the microRNA sequence on the expression of a polypeptide of interest compared to a human 3'UTR of the same sequence type.

[000522] In one embodiment, other regulatory elements and/or structural elements of the 5'-UTR can influence microRNA mediated gene regulation. One example of a regulatory element and/or structural element is a structured IRES (Internal Ribosome Entry Site) in the 5'UTR, which is necessary for the binding of translational elongation factors to initiate protein translation. EIF4A2 binding to this secondarily structured element in the 5'UTR is necessary for microRNA mediated gene expression (Meijer HA et al, Science, 2013, 340, 82-85, herein incorporated by reference in its entirety). The polynucleotides of the invention can further be modified to include this structured 5'-UTR in order to enhance microRNA mediated gene regulation.

[000523] At least one microRNA site can be engineered into the 3' UTR of the polynucleotides of the present invention. In this context, at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, at least ten or more microRNA sites may be engineered into the 3' UTR of the ribonucleic acids of the present invention. In one embodiment, the microRNA sites incorporated into the polynucleotides may be the same or may be different microRNA sites. In another embodiment, the microRNA sites incorporated into the polynucleotides may target the same or different tissues in the body. As a non-limiting example, through the introduction of tissue-, cell-type-, or disease-specific microRNA binding sites in the 3' UTR of polynucleotides, the degree of expression in specific cell types (e.g. hepatocytes, myeloid cells, endothelial cells, cancer cells, etc.) can be reduced.

[000524] In one embodiment, a microRNA site can be engineered near the 5' terminus of the 3'UTR, about halfway between the 5' terminus and 3'terminus of the 3'UTR and/or near the 3'terminus of the 3'UTR. As a non-limiting example, a microRNA site may be engineered near the 5' terminus of the 3'UTR and about halfway between the 5' terminus and 3'terminus of the 3'UTR. As another non-limiting example, a microRNA site may be engineered near the 3'terminus of the 3'UTR and about halfway between the 5' terminus and 3'terminus of the 3'UTR. As yet another non-limiting example, a microRNA site may be engineered near the 5' terminus of the 3'UTR and near the 3' terminus of the 3'UTR.

[000525] In another embodiment, a 3'UTR can comprise 4 microRNA sites. The microRNA sites may be complete microRNA binding sites, microRNA seed sequences and/or microRNA binding site sequences without the seed sequence.

[000526] In one embodiment, a polynucleotide of the invention may be engineered to include at least one microRNA in order to dampen the antigen presentation by antigen presenting cells. The microRNA may be the complete microRNA sequence, the microRNA seed sequence, the microRNA sequence without the seed or a combination thereof. As a non-limiting example, the microRNA incorporated into the nucleic acid may be specific to the hematopoietic system. As another non-limiting example, the microRNA incorporated into the nucleic acid of the invention to dampen antigen presentation is miR-142-3p.

[000527] In one embodiment, a polynucleotide may be engineered to include microRNA sites which are expressed in different tissues of a subject. As a non-limiting example, a polynucleotide of the present invention may be engineered to include miR-192 and miR-122 to regulate expression of the polynucleotide in the liver and kidneys of a subject. In another embodiment, a polynucleotide may be engineered to include more than one microRNA sites for the same tissue. For example, a polynucleotide of the present invention may be engineered to include miR-17-92 and miR-126 to regulate expression of the polynucleotide in endothelial cells of a subject.

[000528] In one embodiment, the therapeutic window and or differential expression associated with the target polypeptide encoded by the polynucleotide invention may be altered. For example, polynucleotides may be designed whereby a death signal is more highly expressed in cancer cells (or a survival signal in a normal cell) by virtue of the miRNA signature of those cells. Where a cancer cell expresses a lower level of a particular miRNA, the polynucleotide encoding the binding site for that miRNA (or miRNAs) would be more highly expressed. Hence, the target polypeptide encoded by the polynucleotide is selected as a protein which triggers or induces cell death. Neigboring noncancer cells, harboring a higher expression of the same miRNA would be less affected by the encoded death signal as the polynucleotide would be expressed at a lower level due to the affects of the miRNA binding to the binding site or "sensor" encoded in the 3'UTR. Conversely, cell survival or cytoprotective signals may be delivered to tissues containing cancer and non cancerous cells where a miRNA has a higher expression in the cancer cells—the result being a lower survival signal to the cancer cell and a larger survival signature to the normal cell. Multiple polynucleotides may be designed and administered having different signals according to the previous paradigm.

[000529] In one embodiment, the polynucleotides of the present invention comprise a 3' UTR and at least one miR sequence located in the 3' UTR. The miR sequence may be located anywhere in the 3' UTR such as, but not limited to, at the beginning of the 3' UTR, near the 5' end of the poly-A tailing region, in the middle of the 3' UTR, halfway between the 5' end and the 3'end of the 3' UTR, at the end of the 3' UTR and/or at the 3' end of the 3' UTR.

[000530] In one embodiment, the polynucleotides of the present invention comprise a 3' UTR and more than one miR sequences located in the 3' UTR. As a non-limiting example, the 3'UTR may comprise two miR sequences. As another non-limiting example, the 3'UTR may comprise three miR sequences. As yet another non-limiting example, the 3'UTR may comprise four miR sequences.

[000531] In one embodiment, the expression of a nucleic acid may be controlled by incorporating at least one sensor sequence in the nucleic acid and formulating the nucleic acid. As a non-limiting example, a nucleic acid may be targeted to an orthotopic tumor by having a nucleic acid incorporating a miR-122 binding site and formulated in a lipid nanoparticle comprising the cationic lipid DLin-KC2-DMA.

[000532] Through an understanding of the expression patterns of microRNA in different cell types, polynucleotides can be engineered for more targeted expression in specific cell types or only under specific biological conditions. Through introduction of tissue-specific microRNA binding sites, polynucleotides could be designed that would be optimal for protein expression in a tissue or in the context of a biological condition.

[000533] Transfection experiments can be conducted in relevant cell lines, using polynucleotides and protein production can be assayed at various time points post-transfection. For example, cells can be transfected with different microRNA binding site- polynucleotides and by using an ELISA kit to the relevant protein and assaying protein produced at 6 hr, 12 hr, 24 hr, 48 hr, 72 hr and 7 days post-transfection. *In vivo* experiments can also be conducted using microRNA-binding site-engineered molecules to examine changes in tissue-specific expression of formulated polynucleotides.

[000534] Non-limiting examples of cell lines which may be useful in these investigations include those from ATCC (Manassas, VA) including MRC-5, A549, T84, NCI-H2126 [H2126], NCI-H1688 [H1688], WI-38, WI-38 VA-13 subline 2RA, WI-26 VA4, C3A [HepG2/C3A, derivative of Hep G2 (ATCC HB-8065)], THLE-3, H69AR, NCI-H292 [H292], CFPAC-l, NTERA-2 cl.Dl [NT2/D1], DMS 79, DMS 53, DMS 153, DMS 114, MSTO-21 1H, SW 1573 [SW-1573, SW1573], SW 1271 [SW-1271, SW1271], SHP-77, SNU-398, SNU-449, SNU-182, SNU-475, SNU-387, SNU-423, NL20, NL20-TA [NL20T-A], THLE-2, HBE135-E6E7, HCC827, HCC4006, NCI-H23 [H23], NCI-H1299, NCI-H187 [H187], NCI-H358 [H-358, H358], NCI-H378 [H378], NCI-H522 [H522], NCI-H526 [H526], NCI-H727 [H727], NCI-H810 [H810], NCI-H889 [H889], NCI-H1155 [HI 155], NCI-H1404 [H1404], NCI-N87 [N87], NCI-H196 [H196], NCI-H21 1 [H211], NCI-H220 [H220], NCI-H250 [H250], NCI-H524 [H524], NCI-H647 [H647], NCI-H650 [H650], NCI-H71 1 [H71 1], NCI-H719 [H719], NCI-

H740 [H740], NCI-H748 [H748], NCI-H774 [H774], NCI-H838 [H838], NCI-H841 [H841], NCI-H847 [H847], NCI-H865 [H865], NCI-H920 [H920], NCI-H1048 [H1048], NCI-H1092 [H1092], NCI-H1 105 [HI 105], NCI-H1 184 [HI 184], NCI-H1238 [H1238], NCI-H1341 [H1341], NCI-H1385 [H1385], NCI-H1417 [H1417], NCI-H1435 [H1435], NCI-H1436 [H1436], NCI-H1437 [H1437], NCI-H1522 [H1522], NCI-H1563 [H1563], NCI-H1568 [H1568], NCI-H1573 [H1573], NCI-H1581 [H1581], NCI-H1618 [H1618], NCI-H1623 [H1623], NCI-H1650 [H-1650, H1650], NCI-H1651 [H1651], NCI-H1666 [H-1666, H1666], NCI-H1672 [H 1672], NCI-H 1693 [H1693], NCI-H1694 [H1694], NCI-H1703 [H1703], NCI-H1734 [H-1734, H1734], NCI-H1755 [H1755], NCI-H1755 [H1755], NCI-H1770 [H1770], NCI-H1793 [H1793], NCI-H1836 [H1836], NCI-H1838 [H1838], NCI-H1869 [H1869], NCI-H1876 [H1876], NCI-H1882 [H1882], NCI-H1915 [H1915], NCI-H1930 [H1930], NCI-H1944 [H1944], NCI-H1975 [H-1975, H1975], NCI-H1993 [H1993], NCI-H2023 [H2023], NCI-H2029 [H2029], NCI-H2030 [H2030], NCI-H2066 [H2066], NCI-H2073 [H2073], NCI-H2081 [H2081], NCI-H2085 [H2085], NCI-H2087 [H2087], NCI-H2106 [H2106], NCI-H2110 [H2110], NCI-H2135 [H2135], NCI-H2141 [H2141], NCI-H2171 [H2171], NCI-H2172 [H2172], NCI-H2195 [H2195], NCI-H2196 [H2196], NCI-H2198 [H2198], NCI-H2227 [H2227], NCI-H2228 [H2228], NCI-H2286 [H2286], NCI-H2291 [H2291], NCI-H2330 [H2330], NCI-H2342 [H2342], NCI-H2347 [H2347], NCI-H2405 [H2405], NCI-H2444 [H2444], UMC-1 1, NCI-H64 [H64], NCI-H735 [H735], NCI-H735 [H735], NCI-H1963 [H1963], NCI-H2107 [H2107], NCI-H2108 [H2108], NCI-H2122 [H2122], Hs 573.T, Hs 573.Lu, PLC/PRF/5, BEAS-2B, Hep G2, Tera-1, Tera-2, NCI-H69 [H69], NCI-H128 [H128], ChaGo-K-1, NCI-H446 [H446], NCI-H209 [H209], NCI-H 146 [H146], NCI-H441 [H441], NCI-H82 [H82], NCI-H460 [H460], NCI-H596 [H596], NCI-H676B [H676B], NCI-H345 [H345], NCI-H820 [H820], NCI-H520 [H520], NCI-H661 [H661], NCI-H510A [H510A, NCI-H5 10], SK-HEP-1, A-427, Calu-1, Calu-3, Calu-6, SK-LU-1, SK-MES-1, SW 900 [SW-900, SW900], Malme-3M, and Capan-1.

[000535] In some embodiments, polynucleotides can be designed to incorporate microRNA binding region sites that either have 100% identity to known seed sequences or have less than 100% identity to seed sequences. The seed sequence can be partially mutated to decrease microRNA binding affinity and as such result in reduced downmodulation of that mRNA transcript. In essence, the degree of match or mis-match between the target mRNA and the microRNA seed can act as a rheostat to more finely tune the ability of the microRNA to modulate protein expression. In addition, mutation in the non-seed region of a microRNA binding site may also impact the ability of microRNA to modulate protein expression.

[000536] In one embodiment, a miR sequence may be incorporated into the loop of a stem loop.

[000537] In another embodiment, a miR seed sequence may be incorporated in the loop of a stem loop and a miR binding site may be incorporated into the 5' or 3' stem of the stem loop.

[000538] In one embodiment, a TEE may be incorporated on the 5'end of the stem of a stem loop and a miR seed may be incorporated into the stem of the stem loop. In another embodiment, a TEE may be incorporated on the 5'end of the stem of a stem loop, a miR seed may be incorporated into the stem of the stem loop and a miR binding site may be incorporated into the 3'end of the stem or the sequence after the stem loop. The miR seed and the miR binding site may be for the same and/or different miR sequences.

[000539] In one embodiment, the incorporation of a miR sequence and/or a TEE sequence changes the shape of the stem loop region which may increase and/or descrease translation. (see e.g, Kedde et al. A Pumilio-induced RNA structure switch in p27-3'UTR controls miR-221 and miR-22 accessibility. Nature Cell Biology. 2010, herein incorporated by reference in its entirety).

[000540] In one embodiment, the incorporation of a miR sequence and/or a TEE sequence changes the shape of the stem loop region which may increase and/or descrease translation. (see e.g, Kedde et al. A Pumilio-induced RNA structure switch in p27-3'UTR controls miR-221 and miR-22 accessibility. Nature Cell Biology. 2010, herein incorporated by reference in its entirety).

[000541] In one embodiment, the 5'UTR may comprise at least one microRNA sequence. The microRNA sequence may be, but is not limited to, a 19 or 22 nucleotide sequence and/or a microRNA sequence without the seed.

[000542] In one embodiment the microRNA sequence in the 5'UTR may be used to stabilize the polynucleotides described herein.

[000543] In another embodiment, a microRNA sequence in the 5'UTR may be used to decrease the accessibility of the site of translation initiation such as, but not limited to a start codon. Matsuda et al (PLoS One. 2010 1l(5):el5057; herein incorporated by reference in its entirety) used antisense locked nucleic acid (LNA) oligonucleotides and exon-junctino complexes (EJCs) around a start codon (-4 to +37 where the A of the AUG codons is +1) in order to decrease the accessibility to the first start codon (AUG). Matsuda showed that altering the sequence around the start codon with an LNA or EJC the efficiency, length and structural stability of the polynucleotides is affected. The polynucleotides of the present invention may comprise a microRNA sequence, instead of the LNA or EJC sequence described by Matsuda et al, near the

site of translation initiation in order to decrease the accessibility to the site of translation initiation. The site of translation initiation may be prior to, after or within the microRNA sequence. As a non-limiting example, the site of translation initiation may be located within a microRNA sequence such as a seed sequence or binding site. As another non-limiting example, the site of translation initiation may be located within a miR-122 sequence such as the seed sequence or the mir-122 binding site.

[000544] In one embodiment, the polynucleotides of the present invention may include at least one microRNA in order to dampen the antigen presentation by antigen presenting cells. The microRNA may be the complete microRNA sequence, the microRNA seed sequence, the microRNA sequence without the seed or a combination thereof. As a non-limiting example, the microRNA incorporated into the polynucleotides of the present invention may be specific to the hematopoietic system. As another non-limiting example, the microRNA incorporated into the nucleic acids or mRNA of the present invention to dampen antigen presentation is miR-142-3p.

[000545] In one embodiment, the polynucleotides of the present invention may include at least one microRNA in order to dampen expression of the encoded polypeptide in a cell of interest. As a non-limiting example, the polynucleotides of the present invention may include at least one miR-122 binding site in order to dampen expression of an encoded polypeptide of interest in the liver. As another non-limiting example, the polynucleotides of the present invention may include at least one miR-142-3p binding site, miR-142-3p seed sequence, miR-142-3p binding site without the seed, miR-142-5p binding site, miR-142-5p seed sequence, miR-142-5p binding site without the seed, miR-146 binding site, miR-146 seed sequence and/or miR-146 binding site without the seed sequence.

[000546] In one embodiment, the polynucleotides of the present invention may comprise at least one miR sequence to dampen expression of the encoded polypeptide in muscle. As a non-limiting example, the polynucleotides of the present invention may comprise a miR-133 sequence, fragment or variant thereof. As another non-limiting example, the polynucleotides of the present invention may comprise a miR-206 sequence, fragment or variant thereof. As yet another non-limiting example, the polynucleotides of the present invention may comprise a miR-1 sequence, fragment or variant thereof.

[000547] In one embodiment, the polynucleotides of the present invention may comprise at least one miR sequence to dampen expression of the encoded polypeptide in endotherlium. As a non-limiting example, the polynucleotides of the present invention may comprise a miR-126 sequence, fragment or variant thereof.

[000548] In one embodiment, the polynucleotides of the present invention may comprise at least one miR sequence to dampen expression of the encoded polypeptide in the central nervous system (CNS). As a non-limiting example, the polynucleotides of the present invention may comprise a miR-132 sequence, fragment or variant thereof. As another non-limiting example, the polynucleotides of the present invention may comprise a miR-125 sequence, fragment or variant thereof. As yet another non-limiting example, the polynucleotides of the present invention may comprise a miR-124 sequence, fragment or variant thereof.

[000549] In one embodiment, the polynucleotides of the present invention may comprise at least one miR sequence which is a hematopoietic lineage specific miR sequence or fragment or variant thereof. As a non-limiting example, the hematopietic lineage specific miR sequence is miR-142-3p or a fragment thereof.

[000550] In one embodiment, the polynucleotides of the present invention may comprise at least one microRNA binding site in the 3'UTR in order to selectively degrade mRNA therapeutics in the immune cells to subdue unwanted immunogenic reactions caused by therapeutic delivery. As a non-limiting example, the microRNA binding site may make the polynucleotides more unstable in antigen presenting cells. Non-limiting examples of these microRNAs include mir-142-5p, mir-142-3p, mir-146a-5p and mir-146-3p.

[000551] In one embodiment, the polynucleotides of the present invention comprises at least one microRNA sequence in a region of the polynucleotides which may interact with a RNA binding protein.

5 'UTR, miR binding sites, translation enhancement and translational specificity

[000552] In one embodiment, the polynucleotides described herein comprise at least one microRNA binding site in the 5'UTR in order to enhance translation of the polynucleotide. As a non-limiting example, the polynucleotides described herein may comprise at least one miR-10a sequence or fragment thereof.

[000553] In one embodiment, the polynucleotides described herein comprise at least one microRNA binding site in the 5'UTR in order to reduce translational repression of the ribosomal protein mRNAs during amino acid starvation (see e.g., Orom et al. Mol Cell (2008) 30, 160-471; the contents of which are herein incorporated by reference in its entirety).

[000554] In one embodiment, the polynucleotides described herein comprise at least one sequence for miR-10a or miR-10b or a fragment thereof in the 5'UTR.

[000555] In one embodiment, the polynucleotides described herein comprise at least oen miR sequence to initiate translation of the polynucleotide in a specific tissue.

[000556] In one embodiment, the polynucleotides described herein comprise at least one sequence in the 5'UTR in order to slow down translation of the polynucleotide in order to improve the changes of proper folding of the encoded polypeptide. In another embodiment, the polynucleotides described herein comprise at least one sequence in the 5'UTR in order to slow translation of the polynucleotide in order to reduce errors in the translation process.

[000557] In one embodiment, the polynucleotides described herein comprise at least one sequence in the 5'UTR to slow translation in tissues where expression of the encoded polypeptide is not desired.

3 'UTR and Hetero-miRs

[000558] 3'UTRs of the polynucleotides described herein may comprise at least two miR sequences which are not the same. The miR sequences may down-regulate expression of the polynucleotide in the same tissue and/or organ or miR sequences may down-regulate the expression of the polynucleotide in different tissues and/or organs. When an UTR of the polynucleotides described herein comprise at least two miR sequences which are not the same sequence these miR sequences are known as hetero-miRs.

[000559] In one embodiment, the polynucleotides described herein comprise at least two different miR sequences in the 3'UTR. Each miR sequence may down-regulate expression of the polynucleotide in a different organ and/or tissue. As a non-limiting example, the 3'UTR of the polynucleotides described herein may comprise at least one miR sequence to down-regulate expression of the polynucleotide in organ A and at least one miR sequence to down-regulate expression of the polynucleotide in organ B. As another non-limiting example, the 3'UTR of the polynucleotides described herein may comprise at least one miR sequence to down-regulate expression of the polynucleotide in organ A and at least one miR sequence to down-regulate expression of the polynucleotide in organ B.

[000560] In one embodiment, the polynucleotides described herein comprise at least one miR-122 sequence and at least one miR-142 sequence in the 3'UTR.

[000561] In one embodiment, the polynucleotides described herein may comprise at least two different miR sequences which can reduce or suppress protein expression in the same cell type.

[000562] In one embodiment, the polynucleotides described herein comprise at least two different miR sequences in the 3'UTR which can reduce or suppress protein expression in the same cell type. Each miR sequence may down-regulate expression of the polynucleotide in the same tissue.

[000563] In one embodiment, the polynucleotides described herein comprise at least a miR-142-3p sequence and a miR-142-5p sequence or variant thereof in the 3'UTR which can reduce or suppress protein expression in the same cell type.

3 'UTR and Albumin Variants

[000564] 3' UTRs of the polynucleotides described herein may comprise a nucleic acid sequence which is derived from the 3' UTR of an albumin gene or from a variant of the 3'UTR of the albumin gene. 3'UTRs and albumin variants are described in paragraphs [000256] - [000257] in International Publication No. WO2015038892, the contents of which are herein incorporated by reference in its entirety.

3 ' UTR and Triple Helices

[000565] In one embodiment, polynucleotides of the present invention may include a triple helix on the 3' end of the polynucleotides. The 3' end of the polynucleotides of the present invention may include a triple helix alone or in combination with a Poly-A tail.

[000566] In one embodiment, the polynucleotides of the present invention may comprise at least a first and a second U-rich region, a conserved stem loop region between the first and second region and an A-rich region. The first and second U-rich region and the A-rich region may associate to form a triple helix on the 3' end of the nucleic acid. This triple helix may stabilize the polynucleotides, enhance the translational efficiency of the polynucleotides and/or protect the 3' end from degradation. Exemplary triple helices include, but are not limited to, the triple helix sequence of metastasis-associated lung adenocarcinoma transcript 1 (MALAT1), MEN -β and polyadenylated nuclear (PAN) RNA (See Wilusz et al, Genes & Development 2012 26:2392-2407; herein incorporated by reference in its entirety). In one embodiment, the 3' end of the polynucleotides of the present invention comprises a first U-rich region, a second U-rich region and an A-rich region. As a non-limiting example, the first U-rich region is SEQ ID: 4 as described in U.S. Provisional Application No. 62/025,985, the second U-rich region is SEQ ID NO: 5 or 6 as described in U.S. Provisional Application No. 62/025,985 and the A-rich region has SEQ ID NO: 7 as described in U.S. Provisional Application No. 62/025,985, the contents of which is herein incorporated by reference in its entirety. In another embodiment, the 3' end of the polynucleotides of the present invention comprises a triple helix formation structure comprising a first U-rich region, a conserved region, a second U-rich region and an A-rich region.

[000567] In one embodiment, the triple helix may be formed from the cleavage of a MALAT 1 sequence prior to the cloverleaf structure. While not meaning to be bound by theory, MALAT 1 is a long non-coding RNA which, when cleaved, forms a triple helix and a tRNA-like cloverleaf

structure. The MALATl transcript then localizes to nuclear speckles and the tRNA-like cloverleaf localizes to the cytoplasm (Wilusz et al. Cell 2008 135(5): 919-932; the contents of which is herein incorporated by reference in its entirety).

[000568] As a non-limiting example, the terminal end of the polynucleotides of the present invention comprising the MALATl sequence can then form a triple helix structure, after RNaseP cleavage from the cloverleaf structure, which stabilizes the nucleic acid (Peart et al. *Non-mRNA 3 'endformation: how the other half lives;* WIREs RNA 2013; the contents of which is herein incorporated by reference in its entirety).

[000569] In one embodiment, the polynucleotides described herein comprise a MALATl sequence. In another embodiment, the polynucleotides may be polyadenylated. In yet another embodiment, the polynucleotides is not polyadenylated but has an increased resistance to degradation compared to unmodified nucleic acids or mRNA.

[000570] In one embodiment, the polynucleotides of the present invention may comprise a MALATl sequence in the second flanking region (e.g., the 3'UTR). As a non-limiting example, the MALAT 1 sequence may be human or mouse.

[000571] In another embodiment, the cloverleaf structure of the MALATl sequence may also undergo processing by RNaseZ and CCA adding enzyme to form a tRNA-like structure called mascRNA (MALAT 1-associated small cytoplasmic RNA). As a non-limiting example, the mascRNA may encode a protein or a fragment thereof and/or may comprise a microRNA sequence. The mascRNA may comprise at least one chemical modification described herein.

[000572] In one embodiment, the polynucleotides of the invention may be comprise a hybrid nucleic acid including an RNA molecule that lacks a poly-A tail. In a non-limiting example, the polynucleotides lacking a poly-A tail may be linked to a 3' terminal sequence, which in some instances has a triple helical structure, and that functions to stabilize the RNA, as taught in International Patent Publication No. WO20 14062801 or may be produced using the vector constructs described in WO2014062801, the contents of which is herein incorporated by reference in its entirety.

Other regulatory elements in 3 'UTR

[000573] In addition to microRNA binding sites, other regulatory sequences in the 3'-UTR of natural mRNA, which regulate mRNA stability and translation in different tissues and cells, can be removed or introduced into polynucleotides. Such cis-regulatory elements may include, but are not limited to, Cis- RNP (Ribonucleoprotein)/RBP (RNA binding protein) regulatory elements, AU-rich element (AUE), structured stem-loop, constitutive decay elements (CDEs), GC-richness and other structured mRNA motifs (Parker BJ et al, Genome Research, 201 1, 21,

1929-1943, which is herein incorporated by reference in its entirety). For example, CDEs are a class of regulatory motifs that mediate mRNA degradation through their interaction with Roquin proteins. In particular, CDEs are found in many mRNAs that encode regulators of development and inflammation to limit cytokine production in macrophage (Leppek K et al, 2013, Cell, 153, 869-881, which is herein incorporated by reference in its entirety).

[000574] In one embodiment, a particular CDE can be introduced to the polynucleotides when the degradation of polypeptides in a cell or tissue is desired. A particular CDE can also be removed from the nucleic acids or mRNA to maintain a more stable mRNA in a cell or tissue for sustaining protein expression.

3 ' UTR and Viral Sequences

[000575] Additional viral sequences such as, but not limited to, the translation enhancer sequence of the barley yellow dwarf virus (BYDV-PAV), the Jaagsiekte sheep retrovirus (JSRV) and/or the Enzootic nasal tumor virus (See e.g., International Pub. No. WO2012129648; herein incorporated by reference in its entirety) can be engineered and inserted in the polynucleotides of the invention and can stimulate the translation of the construct *in vitro* and *in vivo.* Transfection experiments can be conducted in relevant cell lines at and protein production can be assayed by ELISA at 12hr, 24hr, 48hr, 72 hr and day 7 post-transfection.

Length of UTR

[000576] In one embodiment, the polynucleotides described herein may include a 5'UTR and/or a 3'UTR. The polynucleotide may further include a tailing region such as, but not limited to, a polyA tail, and/or a capping region.

[000577] In one embodiment, the polynucleotides described herein may include a 5'UTR and do not include a 3'UTR. The polynucleotide may further include a tailing region such as, but not limited to, a polyA tail.

[000578] In one embodiment, the polynucleotides described herein may include a 3'UTR and do not include a 5'UTR. The polynucleotide may further include a tailing region such as, but not limited to, a polyA tail.

[000579] In one embodiment, the polynucleotides described herein may include a 5'UTR of at least one nucleotide. The 5'UTR may be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100 or more than 100 nucleotides in length. As a non-limiting example, the 5'UTR may be 3 - 13 nucleotides in length. As another non-limiting example, the 5'UTR may

be 10 - 12 nucleotides in length. As yet another non-limiting example, the 5'UTR may be 13 nucleotides in length. As yet another non-limiting example, the 5'UTR may be 42 - 47 nucleotides in length.

[000580] In one embodiment, the polynucleotides described herein may include a 5'UTR that does not invoke circularization of the polynucleotide. The 5'UTR that does not invoke circularlization may be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100 or more than 100 nucleotides in length. As a non-limiting example, the 5'UTR that does not invoke circularlization may be 3 - 13 nucleotides in length. As another non-limiting example, the 5'UTR that does not invoke circularlization may be 10 - 12 nucleotides in length. As yet another non-limiting example, the 5'UTR that does not invoke circularlization may be 13 nucleotides in length. As yet another non-limiting example, the 5'UTR that does not invoke circularlization may be 42 - 47 nucleotides in length.

[000581] In one embodiment, the polynucleotides described herein may include a 5'UTR that has a length sufficient to have the ribosome associate with the polynucleotide and begin the translation of the polynucleotide. The 5'UTR may be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100 or more than 100 nucleotides in length. As a non-limiting example, the 5'UTR may be 3 - 13 nucleotides in length. As another non-limiting example, the 5'UTR may be 10 - 12 nucleotides in length. As yet another non-limiting example, the 5'UTR may be 13 nucleotides in length. As yet another non-limiting example, the 5'UTR may be 42 - 47 nucleotides in length.

[000582] In one embodiment, the polynucleotides described herein may include a 5'UTR that is approximately 47 nucleotides in length and a 3'UTR that is approximately 110 nucleotides in length.

[000583] In one embodiment, the polynucleotides described herein may include a 5'UTR that is approximately 13 nucleotides in length and a 3'UTR that is approximately 31 nucleotides in length.

[000584] In one embodiment, the polynucleotides described herein do not include a sequence of nucleotides which may function as a 5'UTR.

[000585] In one embodiment, the polynucleotides described herein do not include a sequence of nucleotides which may function as a 3'UTR.

[000586] In one embodiment, the polynucleotides described herein may include a 3'UTR. The 3'UTR may be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125 or more than 125 nucleotides in length. As a non-limiting example, the 3'UTR may be 30 nucleotides in length. As another non-limiting example, the 3'UTR may be 31 nucleotides in length. As another non-limiting example, the 3'UTR may be 110 nucleotides in length. As another non-limiting example, the 3'UTR may be 119 nucleotides in length.

RNA Motifs for RNA Binding Proteins (RBPs)

[000587] In one embodiment, the polynucleotides described herein may encode at least one RNA binding protein and/or fragment thereof. RNA binding proteins and RNA motifs for RNA binding proteins are described i paragraphs [00201] - [00215] and Example 23 of co-pending International Patent Publication No. WO2014081507 (Attorney Docket No. M039.21), the contents of which are herein incorporated by reference in its entirety. As a non-limiting example, Table 26 in Example 23 of co-pending International Patent Publication No. WO2014081507 (Attorney Docket No. M039.21), the contents of each of which are herein incorporated by reference in its entirety, describe RNA binding proteins and related nucleic acid and protein sequences.

Stem Loop

[000588] In one embodiment, the polynucleotides of the present invention may include a stem loop such as, but not limited to, a histone stem loop. Stem loops are described in paragraphs [00230] - [00241] of copending International Patent Publication No. WO2014081507, the contents of which are herein incorporated by reference in its entirety. The stem loop may be a nucleotide sequence that is about 25 or about 26 nucleotides in length such as, but not limited to, SEQ ID NOs: 7-17 as described in International Patent Publication No. WO2013 103659, herein incorporated by reference in its entirety. The histone stem loop may be located 3' relative to the coding region (e.g., at the 3' terminus of the coding region). As a non-limiting example, the stem loop may be located at the 3' end of a polynucleotide described herein.

Regions having a 5' Cap

[000589] The 5' cap structure of a natural mRNA is involved in nuclear export, increasing mRNA stability and binds the mRNA Cap Binding Protein (CBP), which is responsible for mRNA stability in the cell and translation competency through the association of CBP with poly(A) binding protein to form the mature cyclic mRNA species. The cap further assists the removal of 5' proximal introns removal during mRNA splicing.

[000590] Endogenous mRNA molecules may be 5'-end capped generating a 5'-ppp-5'-triphosphate linkage between a terminal guanosine cap residue and the 5'-terminal transcribed sense nucleotide of the mRNA molecule. This 5'-guanylate cap may then be methylated to generate an N7-methyl-guanylate residue. The ribose sugars of the terminal and/or anteterminal transcribed nucleotides of the 5' end of the mRNA may optionally also be 2'-0-methylated. 5'-decapping through hydrolysis and cleavage of the guanylate cap structure may target a nucleic acid molecule, such as an mRNA molecule, for degradation.

[000591] In some embodiments, polynucleotides may be designed to incorporate a cap moiety. Modifications to the polynucleotides of the present invention may generate a non-hydrolyzable cap structure preventing decapping and thus increasing mRNA half-life. Because cap structure hydrolysis requires cleavage of 5'-ppp-5' phosphorodiester linkages, modified nucleotides may be used during the capping reaction. For example, a Vaccinia Capping Enzyme from New England Biolabs (Ipswich, MA) may be used with a-thio-guanosine nucleotides according to the manufacturer's instructions to create a phosphorothioate linkage in the 5'-ppp-5' cap. Additional modified guanosine nucleotides may be used such as a-methyl-phosphonate and seleno-phosphate nucleotides.

[000592] Additional modifications include, but are not limited to, 2'-0-methylation of the ribose sugars of 5'-terminal and/or 5'-anteterminal nucleotides of the polynucleotide (as mentioned above) on the 2'-hydroxyl group of the sugar ring. Multiple distinct 5'-cap structures can be used to generate the 5'-cap of a nucleic acid molecule, such as a polynucleotide which functions as an mRNA molecule.

[000593] Cap analogs, which herein are also referred to as synthetic cap analogs, chemical caps, chemical cap analogs, or structural or functional cap analogs, differ from natural (i.e. endogenous, wild-type or physiological) 5'-caps in their chemical structure, while retaining cap function. Cap analogs may be chemically (i.e. non-enzymatically) or enzymatically synthesized and/or linked to the polynucleotides of the invention.

[000594] For example, the Anti-Reverse Cap Analog (ARCA) cap contains two guanines linked by a 5'-5'-triphosphate group, wherein one guanine contains an N7 methyl group as well as a 3'-0-methyl group (i.e., N7,3'-0-dimethyl-guanosine-5'-triphosphate-5'-guanosine (m^7G-

3'mppp-G; which may equivaliently be designated 3' 0-Me-m7G(5')ppp(5')G). The 3'-0 atom of the other, unmodified, guanine becomes linked to the 5'-terminal nucleotide of the capped polynucleotide. The N7- and 3'-0-methlyated guanine provides the terminal moiety of the capped polynucleotide.

[000595] Another exemplary cap is mCAP, which is similar to ARCA but has a 2'-0-methyl group on guanosine (i.e., N7,2'-0-dimethyl-guanosine-5'-triphosphate-5'-guanosine, m^7Gm-ppp-G).

[000596] In one embodiment, the cap is a dinucleotide cap analog. As a non-limiting example, the dinucleotide cap analog may be modified at different phosphate positions with a boranophosphate group or a phophoroselenoate group such as the dinucleotide cap analogs described in US Patent No. US 8,519,110, the contents of which are herein incorporated by reference in its entirety.

[000597] In another embodiment, the cap is a cap analog is a N7-(4-chlorophenoxyethyl) substituted dicucleotide form of a cap analog known in the art and/or described herein. Non-limiting examples of a N7-(4-chlorophenoxyethyl) substituted dicucleotide form of a cap analog include aN7-(4-chlorophenoxyethyl)-G(5')ppp(5')G and a N7-(4-chlorophenoxyethyl)-m $^{3'-}$°G(5')ppp(5')G cap analog (See e.g., the various cap analogs and the methods of synthesizing cap analogs described in Kore et al. Bioorganic & Medicinal Chemistry 2013 21:4570-4574; the contents of which are herein incorporated by reference in its entirety). In another embodiment, a cap analog of the present invention is a 4-chloro/bromophenoxyethyl analog.

[000598] While cap analogs allow for the concomitant capping of a polynucleotide or a region thereof, in an in vitro transcription reaction, up to 20% of transcripts can remain uncapped. This, as well as the structural differences of a cap analog from an endogenous 5'-cap structures of nucleic acids produced by the endogenous, cellular transcription machinery, may lead to reduced translational competency and reduced cellular stability.

[000599] Polynucleotides of the invention may also be capped post-manufacture (whether IVT or chemical synthesis), using enzymes, in order to generate more authentic 5'-cap structures. As used herein, the phrase "more authentic" refers to a feature that closely mirrors or mimics, either structurally or functionally, an endogenous or wild type feature. That is, a "more authentic" feature is better representative of an endogenous, wild-type, natural or physiological cellular function and/or structure as compared to synthetic features or analogs, etc., of the prior art, or which outperforms the corresponding endogenous, wild-type, natural or physiological feature in one or more respects. Non-limiting examples of more authentic 5'cap structures of the present invention are those which, among other things, have enhanced binding of cap binding proteins,

increased half life, reduced susceptibility to 5' endonucleases and/or reduced 5'decapping, as compared to synthetic 5'cap structures known in the art (or to a wild-type, natural or physiological 5'cap structure). For example, recombinant Vaccinia Virus Capping Enzyme and recombinant 2'-0-methyltransferase enzyme can create a canonical 5'-5'-triphosphate linkage between the 5'-terminal nucleotide of a polynucleotide and a guanine cap nucleotide wherein the cap guanine contains an N7 methylation and the 5'-terminal nucleotide of the mRNA contains a 2'-0-methyl. Such a structure is termed the Capl structure. This cap results in a higher translational-competency and cellular stability and a reduced activation of cellular pro-inflammatory cytokines, as compared, e.g., to other 5'cap analog structures known in the art. Cap structures include, but are not limited to, 7mG(5')ppp(5')N,pN2p (cap 0), 7mG(5')ppp(5')NlmpNp (cap 1), and 7mG(5')-ppp(5')MmpN2mp (cap 2).

[000600] As a non-limiting example, capping polynucleotides post-manufacture may be more efficient as nearly 100% of the polynucleotides may be capped. This is in contrast to -80% when a cap analog is linked to a polynucleotide in the course of an in vitro transcription reaction.

[000601] According to the present invention, 5' terminal caps may include endogenous caps or cap analogs. According to the present invention, a 5' terminal cap may comprise a guanine analog. Useful guanine analogs include, but are not limited to, inosine, Nl-methyl-guanosine, 2'fluoro-guanosine, 7-deaza-guanosine, 8-oxo-guanosine, 2-amino-guanosine, LNA-guanosine, and 2-azido-guanosine.

[000602] In one embodiment, the polynucleotides described herein may contain a modified 5'cap. A modification on the 5'cap may increase the stability of polynucleotide, increase the half-life of the polynucleotide, and could increase the polynucleotide translational efficiency. The modified 5'cap may include, but is not limited to, one or more of the following modifications: modification at the 2' and/or 3' position of a capped guanosine triphosphate (GTP), a replacement of the sugar ring oxygen (that produced the carbocyclic ring) with a methylene moiety (CH_2), a modification at the triphosphate bridge moiety of the cap structure, or a modification at the nucleobase (G) moiety.

[000603] In one embodiment, the polynucleotides described herein may contain a 5'cap such as, but not limited to, CAP-001 to CAP-225, described in International Patent Publication No. WO2014081507 (Attorney Docket No. M039.21), the contents of which are herein incorporated by reference in its entirety.

[000604] In another non-limiting example, of the modified capping structure substrates CAP-112 - CAP-225 could be added in the presence of vaccinia capping enzyme with a component to

create enzymatic activity such as, but not limited to, S-adenosylmethionine (AdoMet), to form a modified cap for the polynucleotides described herein.

[000605] In one embodiment, the replacement of the sugar ring oxygen (that produced the carbocyclic ring) with a methylene moiety (CI¾) could create greater stability to the C-N bond against phosphorylases as the C-N bond is resistant to acid or enzymatic hydrolysis. The methylene moiety may also increase the stability of the triphosphate bridge moiety and thus increasing the stability of the polynucleotide. As a non-limiting example, the cap substrate structure for cap dependent translation may have the structure such as, but not limited to, CAP-014 and CAP-015 and/or the cap substrate structure for vaccinia mRNA capping enzyme such as, but not limited to, CAP-123 and CAP-124. In another example, CAP-112 - CAP-122 and/or CAP-125 - CAP-225, can be modified by replacing the sugar ring oxygen (that produced the carbocyclic ring) with a methylene moiety (CH_2).

[000606] In another embodiment, the triphophosphate bridge may be modified by the replacement of at least one oxygen with sulfur (thio), a borane (BH_3) moiety, a methyl group, an ethyl group, a methoxy group and/or combinations thereof. This modification could increase the stability of the mRNA towards decapping enzymes. As a non-limiting example, the cap substrate structure for cap dependent translation may have the structure such as, but not limited to, CAP-016 - CAP-021 and/or the cap substrate structure for vaccinia mRNA capping enzyme such as, but not limited to, CAP-125 - CAP-130. In another example, CAP-003 - CAP-015, CAP-022 - CAP-124 and/or CAP-131 - CAP-225, can be modified on the triphosphate bridge by replacing at least one of the triphosphate bridge oxygens with sulfur (thio), a borane (BH_3) moiety, a methyl group, an ethyl group, a methoxy group and/or combinations thereof.

[000607] In one embodiment, CAP-001 - 134 and/or CAP-136 - CAP-225 may be modified to be a thioguanosine analog similar to CAP-135. The thioguanosine analog may comprise additional modifications such as, but not limited to, a modification at the triphosphate moiety (e.g., thio, BH_3, CH_3, C_2H_5, OCH_3, S and S with OCH_3), a modification at the 2' and/or 3' positions of 6-thio guanosine as described herein and/or a replacement of the sugar ring oxygen (that produced the carbocyclic ring) as described herein.

[000608] In one embodiment, CAP-001 - 121 and/or CAP-123 - CAP-225 may be modified to be a modified 5'cap similar to CAP-122. The modified 5'cap may comprise additional modifications such as, but not limited to, a modification at the triphosphate moiety (e.g., thio, BH_3, CH_3, $C2H5$, OCH_3, S and S with OCH_3), a modification at the 2' and/or 3' positions of 6-thio guanosine as described herein and/or a replacement of the sugar ring oxygen (that produced the carbocyclic ring) as described herein.

[000609] In one embodiment, the 5'cap modification may be the attachment of biotin or conjufation at the 2' or 3' position of a GTP.

[000610] In another embodiment, the 5' cap modification may include a CF_2 modified triphosphate moiety.

IRES Sequences

[000611] Further, provided are polynucleotides which may contain an internal ribosome entry site (IRES). First identified as a feature Picorna virus RNA, IRES plays an important role in initiating protein synthesis in absence of the 5' cap structure. An IRES may act as the sole ribosome binding site, or may serve as one of multiple ribosome binding sites of an mRNA. Polynucleotides containing more than one functional ribosome binding site may encode several peptides or polypeptides that are translated independently by the ribosomes ("multicistronic nucleic acid molecules"). When polynucleotides are provided with an IRES, further optionally provided is a second translatable region. Examples of IRES sequences that can be used according to the invention include without limitation, those from picornaviruses (e.g. FMDV), pest viruses (CFFV), polio viruses (PV), encephalomyocarditis viruses (ECMV), foot-and-mouth disease viruses (FMDV), hepatitis C viruses (HCV), classical swine fever viruses (CSFV), murine leukemia virus (MLV), simian immune deficiency viruses (SIV) or cricket paralysis viruses (CrPV).

[000612] In one embodiment, the polynucleotides described herein may comprise an IRES, fragment or variant thereof. In one embodiment, the polynucleotide may comprise an IRES sequence or fragment thereof which comprises at least one point mutation.

Tailing Regions

Poly-A tails

[000613] During RNA processing, a long chain of adenine nucleotides (poly-A tail) may be added to a polynucleotide such as an mRNA molecule in order to increase stability. Immediately after transcription, the 3' end of the transcript may be cleaved to free a 3' hydroxyl. Then poly-A polymerase adds a chain of adenine nucleotides to the RNA. The process, called polyadenylation, adds a poly-A tail that can be between, for example, approximately 80 to approximately 250 residues long, including approximately 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240 or 250 residues long. As a non-limiting example, the poly-A tail of polynucleotides of the present invention may be approximately 160 nucleotides in length. As another non-limiting example, the poly-A tail of the polynucleotides of the present invention may be approximately 140 nucleotides in length. As yet another non-limiting

example, the poly-A tail of the polynucleotides of the present invention may be approximately 80 nucleotides in length.

[000614] PolyA tails may also be added after the construct is exported from the nucleus.

[000615] According to the present invention, terminal groups on the poly A tail may be incorporated for stabilization. Polynucleotides of the present invention may incude des-3' hydroxyl tails. They may also include structural moieties or 2'-Omethyl modifications as taught by Junjie Li, et al. (Current Biology, Vol. 15, 1501-1507, August 23, 2005, the contents of which are incorporated herein by reference in its entirety).

[000616] The polynucleotides of the present invention may be desiged to encode transcripts with alternative polyA tail structures including histone mRNA. According to Norbury, "Terminal uridylation has also been detected on human replication-dependent histone mRNAs. The turnover of these mRNAs is thought to be important for the prevention of potentially toxic histone accumulation following the completion or inhibition of chromosomal DNA replication. These mRNAs are distinguished by their lack of a 3′ poly(A) tail, the function of which is instead assumed by a stable stem-loop structure and its cognate stem-loop binding protein (SLBP); the latter carries out the same functions as those of PABP on polyadenylated mRNAs" (Norbury, "Cytoplasmic RNA: a case of the tail wagging the dog," Nature Reviews Molecular Cell Biology; AOP, published online 29 August 2013; doi:10.1038/nrm3645) the contents of which are incorporated herein by reference in its entirety.

[000617] Unique poly-A tail lengths provide certain advantages to the polynucleotides of the present invention.

[000618] Generally, the length of a poly-A tail, when present, is greater than 30 nucleotides in length. In another embodiment, the poly-A tail is greater than 35 nucleotides in length (e.g., at least or greater than about 35, 40, 45, 50, 55, 60, 70, 80, 90, 100, 120, 140, 160, 180, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1,000, 1,100, 1,200, 1,300, 1,400, 1,500, 1,600, 1,700, 1,800, 1,900, 2,000, 2,500, and 3,000 nucleotides). In some embodiments, the polynucleotide or region thereof includes from about 30 to about 3,000 nucleotides (e.g., from 30 to 50, from 30 to 100, from 30 to 250, from 30 to 500, from 30 to 750, from 30 to 1,000, from 30 to 1,500, from 30 to 2,000, from 30 to 2,500, from 50 to 100, from 50 to 250, from 50 to 500, from 50 to 750, from 50 to 1,000, from 50 to 1,500, from 50 to 2,000, from 50 to 2,500, from 50 to 3,000, from 100 to 500, from 100 to 750, from 100 to 1,000, from 100 to 1,500, from 100 to 2,000, from 100 to 2,500, from 100 to 3,000, from 500 to 750, from 500 to 1,000, from 500 to 1,500, from 500 to 2,000, from 500 to 2,500, from 500 to 3,000, from 1,000 to 1,500, from 1,000

to 2,000, from 1,000 to 2,500, from 1,000 to 3,000, from 1,500 to 2,000, from 1,500 to 2,500, from 1,500 to 3,000, from 2,000 to 3,000, from 2,000 to 2,500, and from 2,500 to 3,000).

[000619] In one embodiment, the poly-A tail is designed relative to the length of the overall polynucleotide or the length of a particular region of the polynucleotide. This design may be based on the length of a coding region, the length of a particular feature or region or based on the length of the ultimate product expressed from the polynucleotides.

[000620] In this context the poly-A tail may be 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100% greater in length than the polynucleotide or feature thereof. The poly-A tail may also be designed as a fraction of the polynucleotides to which it belongs. In this context, the poly-A tail may be 10, 20, 30, 40, 50, 60, 70, 80, or 90% or more of the total length of the construct, a construct region or the total length of the construct minus the poly-A tail. Further, engineered binding sites and conjugation of polynucleotides for Poly-A binding protein may enhance expression.

[000621] In one embodiment, engineered binding sites and/or the conjugation of polynucleotides for Poly-A binding protein may be used to enhance expression. The engineered binding sites may be sensor sequences which can operate as binding sites for ligands of the local microenvironment of the polynucleotides. As a non-limiting example, the polynucleotides may comprise at least one engineered binding site to alter the binding affinity of Poly-A binding protein (PABP) and analogs thereof. The incorporation of at least one engineered binding site may increase the binding affinity of the PABP and analogs thereof.

[000622] Additionally, multiple distinct polynucleotides may be linked together via the PABP (Poly-A binding protein) through the 3'-end using modified nucleotides at the 3'-terminus of the poly-A tail. Transfection experiments can be conducted in relevant cell lines at and protein production can be assayed by ELISA at 12hr, 24hr, 48hr, 72 hr and day 7 post-transfection. As a non-limiting example, the transfection experiments may be used to evaluate the effect on PABP or analogs thereof binding affinity as a result of the addition of at least one engineered binding site.

[000623] In one embodiment, a polyA tail may be used to modulate translation initiation. While not wishing to be bound by theory, the polyA til recruits PABP which in turn can interact with translation initiation complex and thus may be essential for protein synthesis.

[000624] In another embodiment, a polyA tail may also be used in the present invention to protect against 3'-5' exonuclease digestion.

Poly-A Tail and miR Sequences

[000625] In one embodiment, the polynucleotides of the present invention comprise a poly-A tail and at least one miR sequence. The miR sequence may be located in the 5'UTR, the 3'UTR and/or the polyA tailing region.

[000626] In one embodiment, the polynucleotides of the present invention comprise a poly-A tail and at least one miR sequence located in the poly-A tailing region. The miR sequence may be located anywhere in the poly-A tailing region such as, but not limited to, at the beginning of the poly-A tailing region, near the 5' end of the poly-A tailing region, in the middle of the poly-A tailing region, halfway between the 5' end and the 3'end of the poly-A tailing region, at the end of the poly-A tailing region and/or at the 3' end of the poly-A tailing region.

[000627] In one embodiment, the polynucleotides of the present invention comprise a poly-A tail and at least one miR-142-3p sequence or fragment thereof. As a non-limiting example, the polynucleotide may comprise a miR-142-3p sequence in the 3'UTR and a poly-A tail without a miR sequence. As another non-limiting example, the polynucleotide may comprise a miR-142-3p sequence at the beginning of the poly-A tail. As yet another non-limiting example, the polynucleotide may comprise a miR-142-3p sequence in the middle of the poly-A tail. As yet another non-limiting example, the polynucleotide may comprise a miR-142-3p sequence at the end of the poly-A tail.

[000628] In one embodiment, the polynucleotides of the present invention may comprise a poly-A tail of approximately 80 nucleotides where the poly-A tail also comprises at least one miR sequence or fragment thereof.

Poly A-G Quartet

[000629] In one embodiment, the polynucleotides of the present invention are designed to include a polyA-G Quartet region. The G-quartet is a cyclic hydrogen bonded array of four guanine nucleotides that can be formed by G-rich sequences in both DNA and RNA. In this embodiment, the G-quartet is incorporated at the end of the poly-A tail. The resultant polynucleotide is assayed for stability, protein production and other parameters including half-life at various time points. It has been discovered that the polyA-G quartet results in protein production from an mRNA equivalent to at least 75% of that seen using a poly-A tail of 120 nucleotides alone.

[000630] In another embodiment, the polynucleotides which comprise a polyA tail or a polyA-G Quartet may be stabilized by a modification to the 3'region of the nucleic acid that can prevent and/or inhibit the addition of oligio(U) (see e.g., International Patent Publication No. WO2013 103659, herein incorporated by reference in its entirety).

Poly-A tails and Chain Terminating Nucleosides

[000631] In one embodiment, the polynucleotides of the present invention may comprise a polyA tail and may be stabilized by the addition of a chain terminating nucleoside. The polynucleotides with a polyA tail may further comprise a 5'cap structure.

[000632] In another embodiment, the polynucleotides of the present invention may comprise a polyA-G Quartet and may be stabilized by the addition of a chain terminating nucleoside. The polynucleotides with a polyA-G Quartet may further comprise a 5'cap structure.

[000633] In one embodiment, the chain terminating nucleoside which may be used to stabilize the polynucleotides comprising a polyA tail or polyA-G Quartet may be, but is not limited to, those described in International Patent Publication No. WO2013 103659, herein incorporated by reference in its entirety. In another embodiment, the chain terminating nucleosides which may be used with the present invention includes, but is not limited to, 3'-deoxyadenosine (cordycepin), 3'-deoxyuridine, 3'-deoxycytosine, 3'-deoxyguanosine, 3'-deoxythymine, 2',3'-dideoxynucleosides, such as 2',3'- dideoxyadenosine, 2',3'-dideoxyuridine, 2',3'-dideoxycytosine, 2',3'- dideoxyguanosine, 2',3'-dideoxythymine, a 2'-deoxynucleoside, or a -O- methylnucleoside.

[000634] In yet another embodiment, the nucleic acid such as, but not limited to mRNA, which comprise a polyA tail or a polyA-G Quartet may be stabilized by the addition of an chain terminating nucleoside that terminates in a 3'-deoxynucleoside, 2',3'-dideoxynucleoside 3'-0-methylnucleosides, 3'-0-ethylnucleosides, 3'-arabinosides, and other modified nucleosides known in the art and/or described herein.

Start codon region

[000635] In some embodiments, the polynucleotides of the present invention may have regions that are analogous to or function like a start codon region.

[000636] In one embodiment, the translation of a polynucleotide may initiate on a codon which is not the start codon AUG. Translation of the polynucleotide may initiate on an alternative start codon such as, but not limited to, ACG, AGG, AAG, CTG/CUG, GTG/GUG, ATA/AUA, ATT/AUU, TTG/UUG (see Touriol et al. Biology of the Cell 95 (2003) 169-178 and Matsuda and Mauro PLoS ONE, 2010 5:11; the contents of each of which are herein incorporated by reference in its entirety). As a non-limiting example, the translation of a polynucleotide begins on the alternative start codon ACG. As another non-limiting example, polynucleotide translation begins on the alternative start codon CTG or CUG. As yet another non-limiting example, the translation of a polynucleotide begins on the alternative start codon GTG or GUG.

[000637] Nucleotides flanking a codon that initiates translation such as, but not limited to, a start codon or an alternative start codon, are known to affect the translation efficiency, the length and/or the structure of the polynucleotide. (See e.g., Matsuda and Mauro PLoS ONE, 2010 5:11;

the contents of which are herein incorporated by reference in its entirety). Masking any of the nucleotides flanking a codon that initiates translation may be used to alter the position of translation initiation, translation efficiency, length and/or structure of a polynucleotide.

[000638] In one embodiment, a masking agent may be used near the start codon or alternative start codon in order to mask or hide the codon to reduce the probability of translation initiation at the masked start codon or alternative start codon. Non-limiting examples of masking agents include antisense locked nucleic acids (LNA) polynucleotides and exon-junction complexes (EJCs) (See e.g., Matsuda and Mauro describing masking agents LNA polynucleotides and EJCs (PLoS ONE, 2010 5:11); the contents of which are herein incorporated by reference in its entirety).

[000639] In another embodiment, a masking agent may be used to mask a start codon of a polynucleotide in order to increase the likelihood that translation will initiate on an alternative start codon.

[000640] In one embodiment, a masking agent may be used to mask a first start codon or alternative start codon in order to increase the chance that translation will initiate on a start codon or alternative start codon downstream to the masked start codon or alternative start codon.

[000641] In one embodiment, a start codon or alternative start codon may be located within a perfect complement for a miR binding site. The perfect complement of a miR binding site may help control the translation, length and/or structure of the polynucleotide similar to a masking agent. As a non-limiting example, the start codon or alternative start codon may be located in the middle of a perfect complement for a miR-122 binding site. The start codon or alternative start codon may be located after the first nucleotide, second nucleotide, third nucleotide, fourth nucleotide, fifth nucleotide, sixth nucleotide, seventh nucleotide, eighth nucleotide, ninth nucleotide, tenth nucleotide, eleventh nucleotide, twelfth nucleotide, thirteenth nucleotide, fourteenth nucleotide, fifteenth nucleotide, sixteenth nucleotide, seventeenth nucleotide, eighteenth nucleotide, nineteenth nucleotide, twentieth nucleotide or twenty-first nucleotide.

[000642] In another embodiment, the start codon of a polynucleotide may be removed from the polynucleotide sequence in order to have the translation of the polynucleotide begin on a codon which is not the start codon. Translation of the polynucleotide may begin on the codon following the removed start codon or on a downstream start codon or an alternative start codon. In a non-limiting example, the start codon ATG or AUG is removed as the first 3 nucleotides of the polynucleotide sequence in order to have translation initiate on a downstream start codon or alternative start codon. The polynucleotide sequence where the start codon was removed may further comprise at least one masking agent for the downstream start codon and/or alternative

start codons in order to control or attempt to control the initiation of translation, the length of the polynucleotide and/or the structure of the polynucleotide.

Stop Codon Region

[000643] In one embodiment, the polynucleotides of the present invention may include at least one, at least two or more than two stop codons before the 3' untranslated region (UTR). The stop codon may be selected from TGA, TAA and TAG. In one embodiment, the polynucleotides of the present invention include the stop codon TGA and one additional stop codon. In a further embodiment the addition stop codon may be TAA. In another embodiment, the polynucleotides of the present invention include three stop codons.

Signal Sequences

[000644] The polynucleotides may also encode additional features which facilitate trafficking of the polypeptides to therapeutically relevant sites. One such feature which aids in protein trafficking is the signal sequence. As used herein, a "signal sequence" or "signal peptide" is a polynucleotide or polypeptide, respectively, which is from about 9 to 200 nucleotides (3-60 amino acids) in length which is incorporated at the 5' (or N-terminus) of the coding region or polypeptide encoded, respectively. Addition of these sequences result in trafficking of the encoded polypeptide to the endoplasmic reticulum through one or more secretory pathways. Some signal peptides are cleaved from the protein by signal peptidase after the proteins are transported.

[000645] Additional signal sequences which may be utilized in the present invention include those taught in, for example, databases such as those found at www.signalpeptide.de/ or http://proline.bic.nus.edu.sg/spdb/. Those described in US Patents 8,124,379; 7,413,875 and 7,385,034 are also within the scope of the invention and the contents of each are incorporated herein by reference in their entirety.

Target Selection

[000646] According to the present invention, the polynucleotides may comprise at least a first region of linked nucleosides encoding at least one polypeptide of interest. Non limiting examples of polypeptides of interest or "Targets" of the present invention are listed in Table 6 of International Publication Nos. WO2013151666, WO2013151668, WO2013151663, WO2013151669, WO2013151670, WO2013151664, WO2013151665, WO2013151736; Tables 6 and 7 International Publication No. WO2013151672; Tables 6, 178 and 179 of International Publication No. WO2013151671; Tables 6, 185 and 186 of International Publication No WO2013151667; the contents of each of which are herein incorporated by reference in their entireties.

Protein Cleavage Signals and Sites

[000647] In one embodiment, the polypeptides of the present invention may include at least one protein cleavage signal containing at least one protein cleavage site. Protein cleavage signals and sites are described in paragraphs [00339] - [00348] of copending International Publication No. WO2014081507, the contents of which are herein incorporated by reference in its entirety.

Insertions and Substitutions

[000648] In one embodiment, the UTR of the polynucleotide may be replaced by the insertion of at least one region and/or string of nucleosides of the same base. The region and/or string of nucleotides may include, but is not limited to, at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7 or at least 8 nucleotides and the nucleotides may be natural and/or unnatural. As a non-limiting example, the group of nucleotides may include 5-8 adenine, cytosine, thymine, a string of any of the other nucleotides disclosed herein and/or combinations thereof.

[000649] In one embodiment, the UTR of the polynucleotide may be replaced by the insertion of at least two regions and/or strings of nucleotides of two different bases such as, but not limited to, adenine, cytosine, thymine, any of the other nucleotides disclosed herein and/or combinations thereof. As a non-limiting example, the 5'UTR may be replaced by inserting 5-8 adenine bases followed by the insertion of 5-8 cytosine bases. In another example, the 5'UTR may be replaced by inserting 5-8 cytosine bases followed by the insertion of 5-8 adenine bases.

[000650] In one embodiment, the polynucleotide may include at least one substitution and/or insertion downstream of the transcription start site which may be recognized by an RNA polymerase. As a non-limiting example, at least one substitution and/or insertion may occur downstream the transcription start site by substituting at least one nucleic acid in the region just downstream of the transcription start site (such as, but not limited to, +1 to +6). Changes to region of nucleotides just downstream of the transcription start site may affect initiation rates, increase apparent nucleotide triphosphate (NTP) reaction constant values, and increase the dissociation of short transcripts from the transcription complex curing initial transcription (Brieba et al, Biochemistry (2002) 41: 5144-5149; herein incorporated by reference in its entirety). The modification, substitution and/or insertion of at least one nucleoside may cause a silent mutation of the sequence or may cause a mutation in the amino acid sequence.

[000651] In one embodiment, the polynucleotide may include the substitution of at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12 or at least 13 guanine bases downstream of the transcription start site.

[000652] In one embodiment, the polynucleotide may include the substitution of at least 1, at least 2, at least 3, at least 4, at least 5 or at least 6 guanine bases in the region just downstream of the transcription start site. As a non-limiting example, if the nucleotides in the region are GGGAGA the guanine bases may be substituted by at least 1, at least 2, at least 3 or at least 4 adenine nucleotides. In another non-limiting example, if the nucleotides in the region are GGGAGA the guanine bases may be substituted by at least 1, at least 2, at least 3 or at least 4 cytosine bases. In another non-limiting example, if the nucleotides in the region are GGGAGA the guanine bases may be substituted by at least 1, at least 2, at least 3 or at least 4 thymine, and/or any of the nucleotides described herein.

[000653] In one embodiment, the polynucleotide may include at least one substitution and/or insertion upstream of the start codon. For the purpose of clarity, one of skill in the art would appreciate that the start codon is the first codon of the protein coding region whereas the transcription start site is the site where transcription begins. The polynucleotide may include, but is not limited to, at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7 or at least 8 substitutions and/or insertions of nucleotide bases. The nucleotide bases may be inserted or substituted at 1, at least 1, at least 2, at least 3, at least 4 or at least 5 locations upstream of the start codon. The nucleotides inserted and/or substituted may be the same base (e.g., all A or all C or all T or all G), two different bases (e.g., A and C, A and T, or C and T), three different bases (e.g., A, C and T or A, C and T) or at least four different bases. As a non-limiting example, the guanine base upstream of the coding region in the polynucleotide may be substituted with adenine, cytosine, thymine, or any of the nucleotides described herein. In another non-limiting example the substitution of guanine bases in the polynucleotide may be designed so as to leave one guanine base in the region downstream of the transcription start site and before the start codon (see Esvelt *et al.* Nature (201 1) 472(7344):499-503; the contents of which is herein incorporated by reference in its entirety). As a non-limiting example, at least 5 nucleotides may be inserted at 1 location downstream of the transcription start site but upstream of the start codon and the at least 5 nucleotides may be the same base type.

Incorporating Post Transcriptional Control Modulators

[000654] In one embodiment, the polynucleotides of the present invention may include at least one post transcriptional control modulator. These post transcriptional control modulators may be, but are not limited to, small molecules, compounds and regulatory sequences. As a non-limiting example, post transcriptional control may be achieved using small molecules identified by PTC Therapeutics Inc. (South Plainfield, NJ) using their GEMS™ (Gene Expression Modulation by Small-Molecules) screening technology.

[000655] The post transcriptional control modulator may be a gene expression modulator which is screened by the method detailed in or a gene expression modulator described in International Publication No. WO2006022712, herein incorporated by reference in its entirety. Methods identifying RNA regulatory sequences involved in translational control are described in International Publication No. WO2004067728, herein incorporated by reference in its entirety; methods identifying compounds that modulate untranslated region dependent expression of a gene are described in International Publication No. WO2004065561, herein incorporated by reference in its entirety.

[000656] In one embodiment, the polynucleotides of the present invention may include at least one post transcriptional control modulator is located in the 5' and/or the 3' untranslated region of the polynucleotides of the present invention.

[000657] In another embodiment, the polynucleotides of the present invention may include at least one post transcription control modulator to modulate premature translation termination. The post transcription control modulators may be compounds described in or a compound found by methods outlined in International Publication Nos. WO2004010106, WO2006044456, WO2006044682, WO2006044503 and WO2006044505, each of which is herein incorporated by reference in its entirety. As a non-limiting example, the compound may bind to a region of the 28S ribosomal RNA in order to modulate premature translation termination (See e.g., WO2004010106, herein incorporated by reference in its entirety).

[000658] In one embodiment, polynucleotides of the present invention may include at least one post transcription control modulator to alter protein expression. As a non-limiting example, the expression of VEGF may be regulated using the compounds described in or a compound found by the methods described in International Publication Nos. WO2005118857, WO2006065480, WO2006065479 and WO2006058088, each of which is herein incorporated by reference in its entirety.

[000659] The polynucleotides of the present invention may include at least one post transcription control modulator to control translation. In one embodiment, the post transcription control modulator may be a RNA regulatory sequence. As a non-limiting example, the RNA regulatory sequence may be identified by the methods described in International Publication No. WO2006071903, herein incorporated by reference in its entirety.

II. Design, Synthesis and Quantitation of Polynucleotides

Design-Codon Optimization

[000660] The polynucleotides, their regions or parts or subregions may be codon optimized. Codon optimization methods are known in the art and may be useful in efforts to achieve one or

more of several goals. These goals include to match codon frequencies in target and host organisms to ensure proper folding, bias GC content to increase mRNA stability or reduce secondary structures, minimize tandem repeat codons or base runs that may impair gene construction or expression, customize transcriptional and translational control regions, insert or remove protein trafficking sequences, remove/add post translation modification sites in encoded protein (e.g. glycosylation sites), add, remove or shuffle protein domains, insert or delete restriction sites, modify ribosome binding sites and mRNA degradation sites, to adjust translational rates to allow the various domains of the protein to fold properly, or to reduce or eliminate problem secondary structures within the polynucleotide. Codon optimization tools, algorithms and services are known in the art, non-limiting examples include services from GeneArt (Life Technologies), DNA2.0 (Menlo Park CA) and/or proprietary methods. In one embodiment, the ORF sequence is optimized using optimization algorithms. Codon options for each amino acid are given in Table 1.

Table 1. Codon Options

Amino Acid	Single Letter Code	Codon Options
Isoleucine	I	ATT, ATC, ATA
Leucine	L	CTT, CTC, CTA, CTG, TTA, TTG
Valine	V	GTT, GTC, GTA, GTG
Phenylalanine	F	TTT, TTC
Methionine	M	ATG
Cysteine	C	TGT, TGC
Alanine	A	GCT, GCC, GCA, GCG
Glycine	G	GGT, GGC, GGA, GGG
Proline	P	CCT, CCC, CCA, CCG
Threonine	T	ACT, ACC, ACA, ACG
Serine	S	TCT, TCC, TCA, TCG, AGT, AGC
Tyrosine	Y	TAT, TAC
Tryptophan	W	TGG
Glutamine	Q	CAA, CAG
Asparagine	N	AAT, AAC
Histidine	H	CAT, CAC
Glutamic acid	E	GAA, GAG
Aspartic acid	D	GAT, GAC
Lysine	K	AAA, AAG
Arginine	R	CGT, CGC, CGA, CGG, AGA, AGG
Selenocysteine	Sec	UGA in mRNA in presence of Selenocysteine insertion element (SECIS)
Stop codons	Stop	TAA, TAG, TGA

[000661] Features, which may be considered beneficial in some embodiments of the present invention, may be encoded by regions of the polynucleotide and such regions may be upstream (5') or downstream (3') to a region which encodes a polypeptide. These regions may be incorporated into the polynucleotide before and/or after codon optimization of the protein

encoding region or open reading frame (ORF). It is not required that a polynucleotide contain both a 5' and 3' flanking region. Examples of such features include, but are not limited to, untranslated regions (UTRs), Kozak sequences, an oligo(dT) sequence, and detectable tags and may include multiple cloning sites which may have Xbal recognition.

[000662] In some embodiments, a 5' UTR and/or a 3' UTR region may be provided as flanking regions. Multiple 5′ or 3' UTRs may be included in the flanking regions and may be the same or of different sequences. Any portion of the flanking regions, including none, may be codon optimized and any may independently contain one or more different structural or chemical modifications, before and/or after codon optimization.

[000663] Tables 2 and 3 provide a listing of exemplary UTRs which may be utilized in the polynucleotides of the present invention. Shown in Table 2 is a listing of a 5'-untranslated region of the invention. Variants of 5' UTRs may be utilized wherein one or more nucleotides are added or removed to the termini, including A, T, C or G.

Table 2. 5'-Untranslated Regions

5' UTR Identifier	Name/ Description	SEQ ID NO.
5UTR-001	Upstream UTR	4
5UTR-002	Upstream UTR	5
5UTR-003	Upstream UTR	6
5UTR-004	Upstream UTR	7
5UTR-005	Upstream UTR	5
5UTR-006	Upstream UTR	6
5UTR-007	Upstream UTR	7
5UTR-008	Upstream UTR	8
5UTR-009	Upstream UTR	9
5UTR-010	Upstream UTR	10
5UTR-01 1	Upstream UTR	11
5UTR-012	Upstream UTR	12
5UTR-013	Upstream UTR	13
5UTR-014	Upstream UTR	14
5UTR-015	Upstream UTR	15
5UTR-016	Upstream UTR	16
5UTR-017	Upstream UTR	17
5UTR-018	Upstream UTR	5
5UTR-019	Upstream UTR	6
5UTR-020	Upstream UTR	7
5UTR-021	Upstream UTR	8
5UTR-022	Upstream UTR	9
5UTR-023	Upstream UTR	10
5UTR-024	Upstream UTR	18
5UTR-025	Upstream UTR	12
5UTR-026	Upstream UTR	13
5UTR-027	Upstream UTR	14
5UTR-028	Upstream UTR	15
5UTR-029	Upstream UTR	16
5UTR-030	Upstream UTR	17
5UTR-03 1	Synthetic UTR	19
5UTR-032	Synthetic UTR	20

5UTR-033	Synthetic UTR	21
5UTR-034	Synthetic UTR	4
5UTR-035	Synthetic UTR (no kozak)	22
5UTR-036	Synthetic UTR (3t5')	19
5UTR-037	Synthetic UTR (9t5')	20
5UTR-038	Synthetic UTR (9del5')	21
5UTR-039	Synthetic UTR (6t5')	23
5UTR-040	Synthetic UTR (21del5')	24
5UTR-041	Synthetic UTR (13nt5')	4
5UTR-042	Synthetic UTR (ggg-5') GGG	-

[000664] Shown in Table 3 is a listing of 3'-untranslated regions of the invention. Variants of 3' UTRs may be utilized wherein one or more nucleotides are added or removed to the termini, including A, T, C or G.

Table 3. 3'-Untranslated Regions

3′ UTR Identifier	Name/ Description	SEQ ID NO.
3UTR-001	Creatine Kinase	25
3UTR-002	Myoglobin	26
3UTR-003	α-actin	27
3UTR-004	Albumin	28
3UTR-005	α-globin	29
3UTR-006	G-CSF	30
3UTR-007	Col1a2; collagen, type I, alpha 2	31
3UTR-008	Col6a2; collagen, type VI, alpha 2	32
3UTR-009	RPN1; ribophorin I	33
3UTR-010	LRP1; low density lipoprotein receptor-related protein 1	34
3UTR-011	Nnt1; cardiotrophin-like cytokine factor 1	35
3UTR-012	Col6a1; collagen, type VI, alpha 1	36
3UTR-013	Calr; calreticulin	37
3UTR-014	Col1a1; collagen, type I, alpha 1	38
3UTR-015	Plod1; procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1	39
3UTR-016	Nucb1; nucleobindin 1	40
3UTR-017	α-globin	41

[000665] It should be understood that those listed in the previous tables are examples and that any UTR from any gene may be incorporated into the respective first or second flanking region of the primary construct. Furthermore, multiple wild-type UTRs of any known gene may be utilized. It is also within the scope of the present invention to provide artificial UTRs which are not variants of wild type genes. These UTRs or portions thereof may be placed in the same orientation as in the transcript from which they were selected or may be altered in orientation or location. Hence a 5' or 3' UTR may be inverted, shortened, lengthened, made chimeric with one or more other 5' UTRs or 3' UTRs. As used herein, the term "altered" as it relates to a UTR sequence, means that the UTR has been changed in some way in relation to a reference

sequence. For example, a 3' or 5' UTR may be altered relative to a wild type or native UTR by the change in orientation or location as taught above or may be altered by the inclusion of additional nucleotides, deletion of nucleotides, swapping or transposition of nucleotides. Any of these changes producing an "altered" UTR (whether 3' or 5') comprise a variant UTR.

[000666] In one embodiment, a double, triple or quadruple UTR such as a 5′ or 3' UTR may be used. As used herein, a "double" UTR is one in which two copies of the same UTR are encoded either in series or substantially in series. For example, a double beta-globin 3' UTR may be used as described in US Patent publication 20100129877, the contents of which are incorporated herein by reference in its entirety.

[000667] It is also within the scope of the present invention to have patterned UTRs. As used herein "patterned UTRs" are those UTRs which reflect a repeating or alternating pattern, such as ABABAB or AABBAABBAABB or ABCABCABC or variants thereof repeated once, twice, or more than 3 times. In these patterns, each letter, A, B, or C represent a different UTR at the nucleotide level.

[000668] In one embodiment, flanking regions are selected from a family of transcripts whose proteins share a common function, structure, feature of property. For example, polypeptides of interest may belong to a family of proteins which are expressed in a particular cell, tissue or at some time during development. The UTRs from any of these genes may be swapped for any other UTR of the same or different family of proteins to create a new primary transcript. As used herein, a "family of proteins" is used in the broadest sense to refer to a group of two or more polypeptides of interest which share at least one function, structure, feature, localization, origin, or expression pattern.

[000669] After optimization (if desired), the polynucleotides components are reconstituted and transformed into a vector such as, but not limited to, plasmids, viruses, cosmids, and artificial chromosomes. For example, the optimized polynucleotide may be reconstituted and transformed into chemically competent *E. coli*, yeast, neurospora, maize, drosophila, etc. where high copy plasmid-like or chromosome structures occur by methods described herein.

[000670] Synthetic polynucleotides and their nucleic acid analogs play an important role in the research and studies of biochemical processes. Various enzyme-assisted and chemical-based methods have been developed to synthesize polynucleotides and nucleic acids.

Synthesis Methods

[000671] Synthetic polynucleotides and their nucleic acid analogs play an important role in the research and studies of biochemical processes. Various enzyme-assisted and chemical-based methods have been developed to synthesize polynucleotides and nucleic acids.

[000672] Enzymatic methods include *in vitro* transcription which uses RNA polymerases to synthesize the polynucleotides of the present invention. Enzymatic methods and RNA polymerases for transcription are described in International Patent Application No. PCT/US2014/53907, the contents of which are herein incorporated by reference in its entirety, such as in paragraphs [000276]-[000297].

[000673] Solid-phase chemical synthesis may be used to manufacture the polynucleotides described herein or portions thereof. Solid-phase chemical synthesis manufacturing of the polynucleotides described herein are described in International Patent Application No. PCT/US2014/53907, the contents of which are herein incorporated by reference in its entirety, such as in paragraphs [000298]-[000307].

[000674] Liquid phase chemical synthesis may be used to manufacture the polynucleotides described herein or portions thereof. Liquid phase chemical synthesis manufacturing of the polynucleotides described herein are described in International Patent Application No. PCT/US2014/53907, the contents of which are herein incorporated by reference in its entirety, such as in paragraph [000308].

[000675] Combinations of different synthetic methods may be used to manufacture the polynucleotides described herein or portions thereof. These combinations are described in International Patent Application No. PCT/US2014/53907, the contents of which are herein incorporated by reference in its entirety, such as in paragraphs [000309] - [0003 12].

[000676] Small region synthesis which may be used for regions or subregions of the polynucleotides of the present invention. These synthesis methods are described in International Patent Application No. PCT/US2014/53907, the contents of which are herein incorporated by reference in its entirety, such as in paragraphs [0003 13] - [0003 14].

[000677] Ligation of polynucleotide regions or subregions may be used to prepare the polynucleotides described herein. These ligation methods are described in International Patent Application No. PCT/US2014/53907, the contents of which are herein incorporated by reference in its entirety, such as in paragraphs [0003 15] - [000322].

[000678] For example, polynucleotides of the invention having a sequence comprising Formula I:

$$[\mathbf{A}_n]\text{-}\mathbf{L}\text{^}\mathbf{B}_o],$$

Formula I

may be synthesized by reacting a compound having the structure of Formula XIV:

$$[A_n]\text{-}(R^1)_a\text{-}(R^2)_b\text{-}(R^3)_c\text{-}N_3$$

Formula XIV

with a compound having the structure of Formula XV:

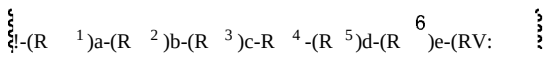
$$R^{27}\text{-}(R^5)_d\text{-}(R^6)_e\text{-}(R^7)_f\text{-}[B_0]$$

Formula XV

[000679] wherein each A and B is independently any nucleoside;

[000680] n and o are, independently 15 to 1000; and

[000681] L^1 has the structure of Formula III:



Formula III

[000682] wherein a, b, c, d, e, and f are each, independently, 0 or 1;

[000683] wherein each A and B is independently any nucleoside;

[000684] n and o are, independently 15 to 1000;

[000685] R^1, R^3, R^5, and R^7 each, independently, is selected from optionally substituted *Ci-Ce* alkylene, optionally substituted *Ci-Ce* heteroalkylene, O, S, and NR^8;

[000686] R^2 and R^6 are each, independently, selected from carbonyl, thiocarbonyl, sulfonyl, or phosphoryl;

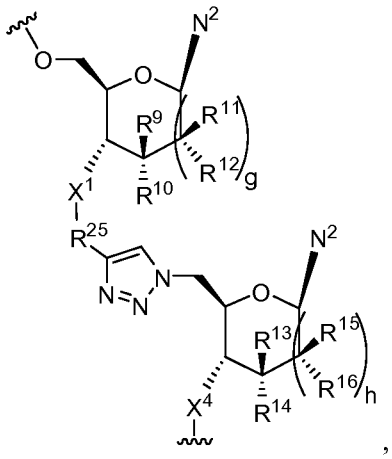
[000687] R^4 is an optionally substituted triazolene; and

[000688] R^8 is hydrogen, optionally substituted $_{C1\text{-}C4}$ alkyl, optionally substituted $_{C3\text{-}C4}$ alkenyl, optionally substituted $_{C2\text{-}C4}$ alkynyl, optionally substituted $_{C2\text{-}C6}$ heterocyclyl, optionally substituted $_{C6\text{-}C12}$ aryl, or optionally substituted $_{C1\text{-}C7}$ heteroalkyl; and

[000689] R^{27} is an optionally substituted $_{C2\text{-}C3}$ alkynyl or an optionally substituted $C_8\text{-}C_{12}$ cycloalkynyl,

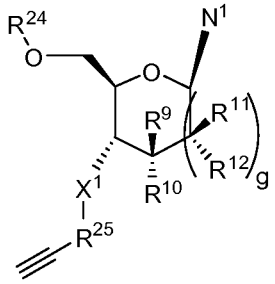
[000690] wherein L^1 is attached to $[A_n]$ and $[B_0]$ at the sugar of one of the nucleosides.

[000691] Polynucleotides of the invention including the structure of Formula XI:



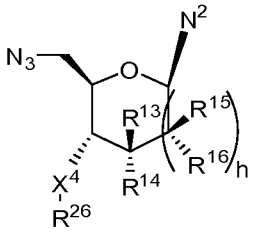
Formula XI

may be synthesized by reacting a compound having the structure of Formula XII:



Formula XII

with a compound having the structure of Formula XIII:



Formula XIII

[000692] wherein each of N^1 and N^2 is independently a nucleobase;

[000693] each of R^9, R^{10}, R^{11}, R^{12}, R^{13}, R^{14}, R^{15}, and R^{16} is, independently, H, halo, hydroxy, thiol, optionally substituted Ci-C_6 alkyl, optionally substituted Ci-C_6 heteroalkyl, optionally substituted C2-C6 heteroalkenyl, optionally substituted C2-C6 heteroalkynyl, optionally substituted amino, azido, or optionally substituted C_6-$C1_0$ aryl;

[000694] each of g and h is, independently, 0 or 1;

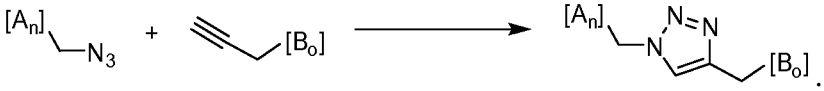
[000695] each X^4 is, independently, O, NH, or S; and

[000696] each X^2 and X^3 is independently O or S;

[000697] each of R^{24} and R^{26} is, independently, a region of linked nucleosides; and

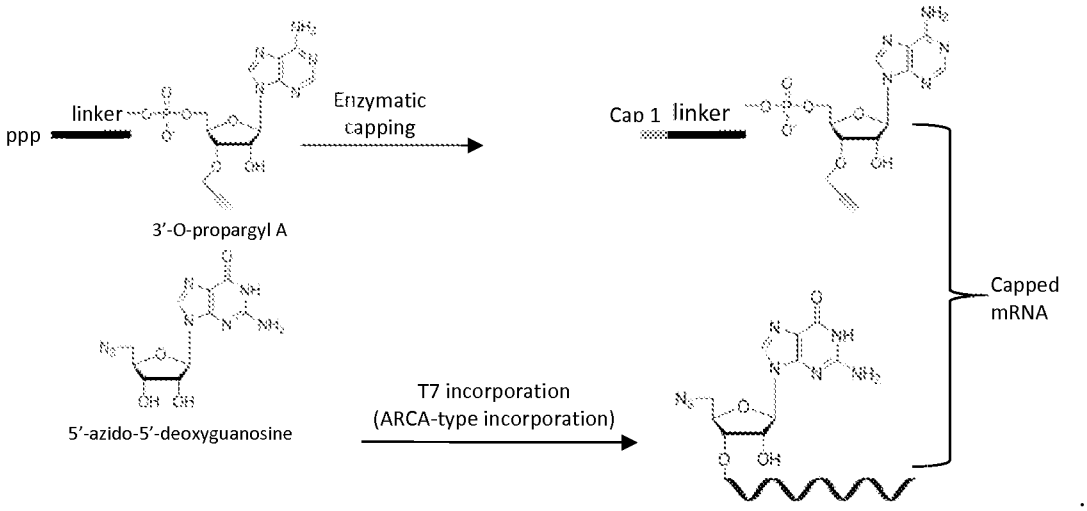
[000698] R^{25} is optionally substituted C1-C6 alkylene or optionally substituted C1-C6 heteroalkylene or R^{25} and the alkynyl group together form optionally substituted cycloalkynyl.

[000699] For example, the chimeric polynucleotides of the invention may be synthesized as shown below

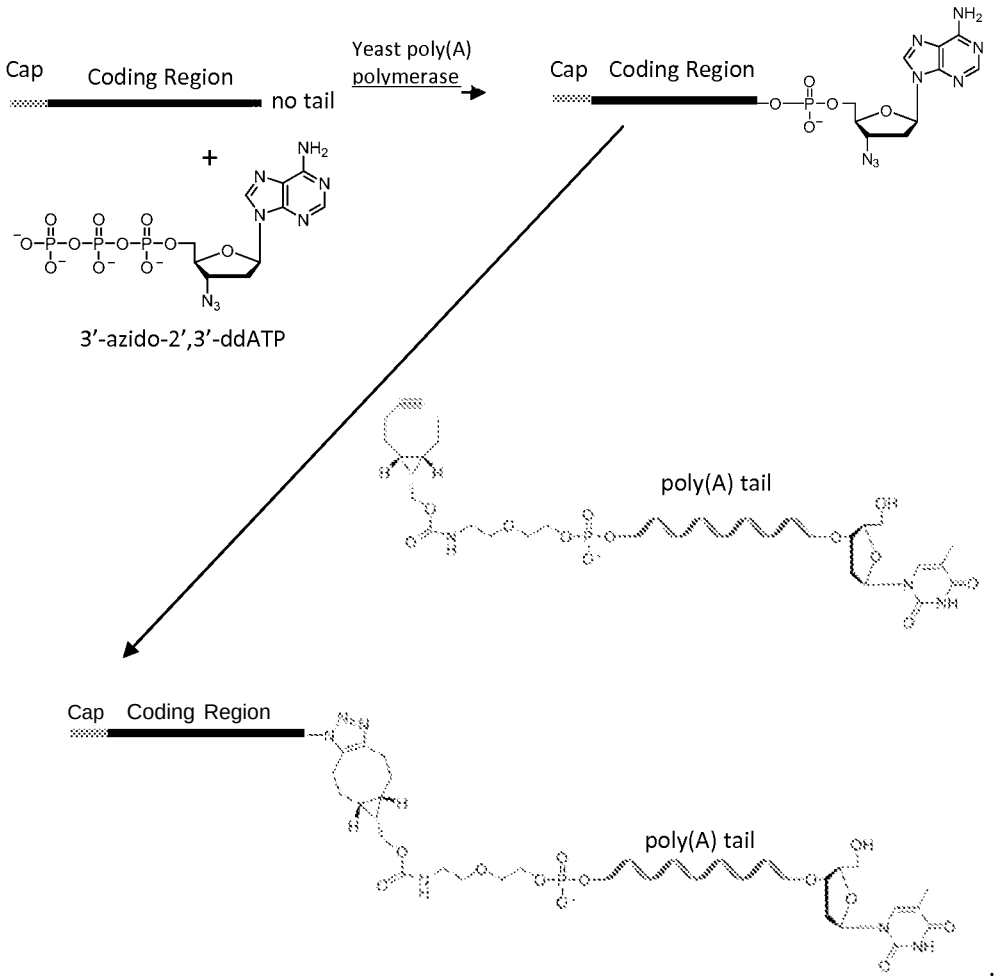


. In some embodiments, the 5' cap structure or poly-A tail may be attached to a chimeric polynucleotide of the invention with this method.

[000700] A 5' cap structure may be attached to a chimeric polynucleotide of the invention as shown below:



[000701] A poly-A tail may be attached to a chimeric polynucleotide of the invention as shown below:



[000702] Sequential ligation can be performed on a solid substrate. For example, initial linker DNA molecules modified with biotin at the end are attached to streptavidin-coated beads. The

3'-ends of the linker DNA molecules are complimentary with the 5'-ends of the incoming DNA fragments. The beads are washed and collected after each ligation step and the final linear constructs are released by a meganuclease. This method allows rapid and efficient assembly of genes in an optimized order and orientation. (Takita, *DNA Research,* vol. 20(4), 1-10 (2013), the contents of which are incorporated herein by reference in their entirety). Labeled polynucleotides synthesized on solid-supports are disclosed in US Pat. Pub. No. 2001/0014753 to Soloveichik et al. and US Pat. Pub. No. 2003/0191303 to Vinayak et al, the contents of which are incorporated herein by reference for their entirety.

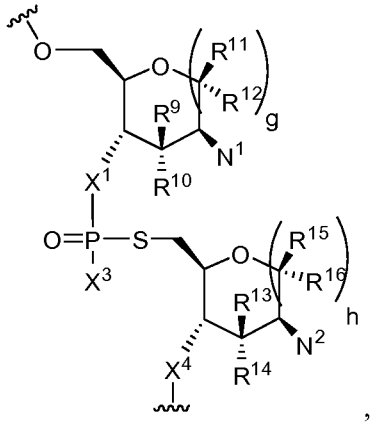
Modified and Conjugated Polynucleotides

[000703] Non-natural modified nucleotides may be introduced to polynucleotides or nucleic acids during synthesis or post-synthesis of the chains to achieve desired functions or properties. The modifications may be on internucleotide lineage, the purine or pyrimidine bases, or sugar. The modification may be introduced at the terminal of a chain or anywhere else in the chain; with chemical synthesis or with a polymerase enzyme. For example, hexitol nucleic acids (HNAs) are nuclease resistant and provide strong hybridization to RNA. Short messenger RNAs (mRNAs) with hexitol residues in two codons have been constructed (Lavrik et al, *Biochemistry,* 40, 11777-1 1784 (2001), the contents of which are incorporated herein by reference in their entirety). The antisense effects of a chimeric HNA gapmer oligonucleotide comprising a phosphorothioate central sequence flanked by 5' and 3' HNA sequences have also been studied (See e.g., Kang et al, *Nucleic Acids Research,* vol. 32(4), 441 1-4419 (2004), the contents of which are incorporated herein by reference in their entirety). The preparation and uses of modified nucleotides comprising 6-member rings in RNA interference, antisense therapy or other applications are disclosed in US Pat. Application No. 2008/0261905, US Pat. Application No. 2010/0009865, and International Publication No. WO97/30064 to Herdewijn et al; the contents of each of which are herein incorporated by reference in their entireties). Modified nucleic acids and their synthesis are disclosed in copending International publication No. WO20 13052523 (Attorney Docket Number M09), the contents of which are incorporated herein by reference for their entirety. The synthesis and strategy of modified polynucleotides is reviewed by Verma and Eckstein in *Annual Review of Biochemistry,* vol. 76, 99-134 (1998), the contents of which are incorporated herein by reference in their entirety.

[000704] Either enzymatic or chemical ligation methods can be used to conjugate polynucleotides or their regions with different functional blocks, such as fluorescent labels, liquids, nanoparticles, delivery agents, etc. The conjugates of polynucleotides and modified polynucleotides are reviewed by Goodchild in *Bioconjugate Chemistry,* vol. 1(3), 165-187

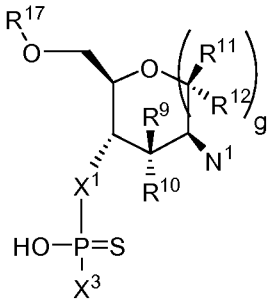
(1990), the contents of which are incorporated herein by reference in their entirety. US Pat. No. 6,835,827 and US Pat. No. 6,525,183 to Vinayak et al. (the contents of each of which are herein incorporated by reference in their entireties) teach synthesis of labeled oligonucleotides using a labeled solid support.

[000705] For example, chimeric polynucleotides of the invention including the structure of Formula V:



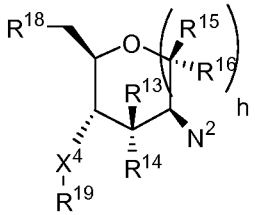
Formula V

[000706] This method includes reacting a compound having the structure of Formula VI:



Formula VI

with a compound having the structure of Formula VII:



Formula VII

[000707] wherein each of N^1 and N^2 is, independently, a nucleobase;

[000708] each of R^9, R^{10}, R^{11}, R^{12}, R^{13}, R^{14}, R^{15}, and R^{16} is, independently, H, halo, hydroxy, thiol, optionally substituted Ci-C_6 alkyl, optionally substituted Ci-C_6 heteroalkyl, optionally

substituted C_{2-C6} heteroalkenyl, optionally substituted C_{2-C6} heteroalkynyl, optionally substituted amino, azido, or optionally substituted C_{6}-C_{10} aryl;

[000709] each of g and h is, independently, 0 or 1;

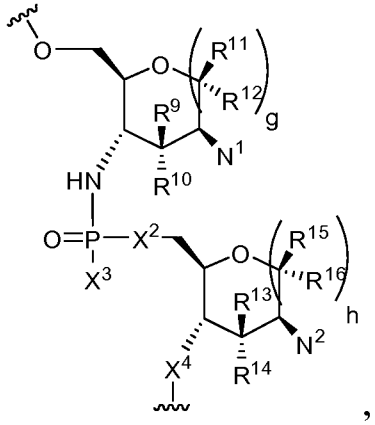
[000710] each X^1 and X^4 is, independently, O, NH, or S; and

[000711] each X^3 is independently OH or SH, or a salt thereof;

[000712] each of R^{17} and R^{19} is, independently, a region of linked nucleosides; and

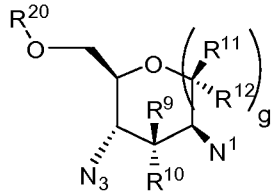
[000713] R^{18} is a halogen.

[000714] Chimeric polynucleotides of the invention including the structure of Formula VIII:



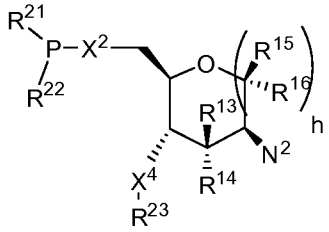
Formula VIII

[000715] This method includes reacting a compound having the structure of Formula IX:



Formula IX

with a compound having the structure of Formula X:



Formula X

[000716] wherein each of N^1 and N^2 is, independently, a nucleobase;

[000717] each of R^9, R^{10}, R^{11}, R^{12}, R^{13}, R^{14}, R^{15}, and R^{16} is, independently, H, halo, hydroxy, thiol, optionally substituted Ci-C_6 alkyl, optionally substituted Ci-C_6 heteroalkyl, optionally

substituted C2-C6 heteroalkenyl, optionally substituted C2-C6 heteroalkynyl, optionally substituted amino, azido, or optionally substituted C6-C10 aryl;

[000718] each of g and h is, independently, 0 or 1;

[000719] each X^4 is, independently, O, NH, or S; and

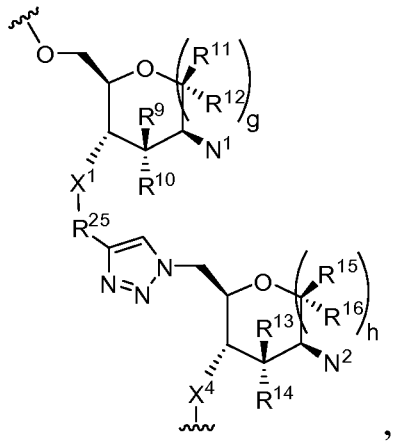
[000720] each X^2 is independently O or S;

[000721] each X^3 is independently OH, SH, or a salt thereof;

[000722] each of R^{20} and R^{23} is, independently, a region of linked nucleosides; and

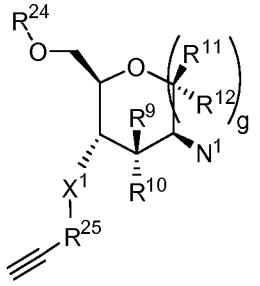
[000723] each of R^{21} and R^{22} is, independently, optionally substituted *Ci-Ce* alkoxy.

[000724] Chimeric polynucleotides of the invention including the structure of Formula XI:



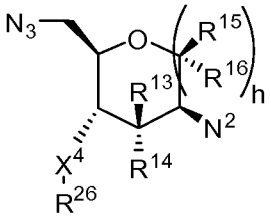
Formula XI

[000725] This method includes reacting a compound having the structure of Formula XII:



Formula XII

with a compound having the structure of Formula XIII:



Formula XIII

[000726] wherein each of N^1 and N^2 is, independently, a nucleobase;

[000727] each of R^9, R^{10}, R^{11}, R^{12}, R^{13}, R^{14}, R^{15}, and R^{16} is, independently, H, halo, hydroxy, thiol, optionally substituted Ci-C_6 alkyl, optionally substituted Ci-C_6 heteroalkyl, optionally substituted C2-C6 heteroalkenyl, optionally substituted C2-C6 heteroalkynyl, optionally substituted amino, azido, or optionally substituted C_6-$C1_0$ aryl;

[000728] each of g and h is, independently, 0 or 1;

[000729] each X^4 is, independently, O, NH, or S; and

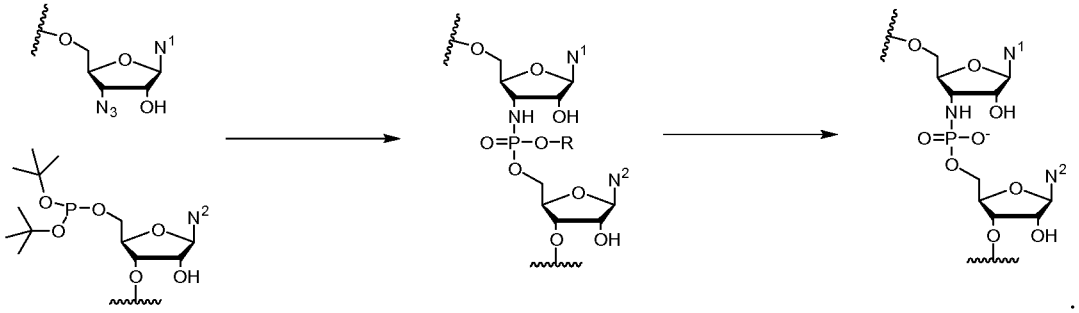
[000730] each X^2 is independently O or S;

[000731] each X^3 is independently OH, SH, or a salt thereof;

[000732] each of R^{24} and R^{26} is, independently, a region of linked nucleosides; and

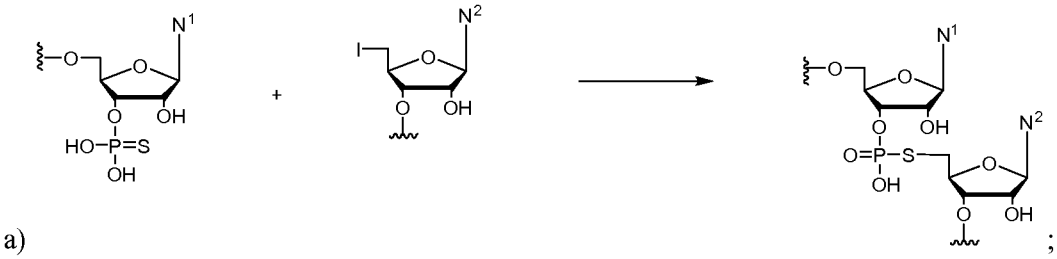
[000733] R^{25} is optionally substituted ***Ci-Ce*** alkylene or optionally substituted ***Ci-Ce*** heteroalkylene or R^{25} and the alkynyl group together form optionally substituted cycloalkynylene.

[000734] Chimeric polynucleotides of the invention may be synthesized as shown below:

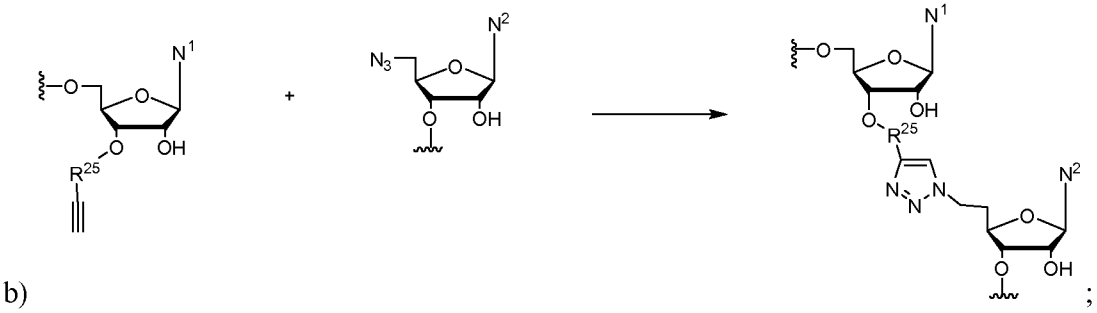


.

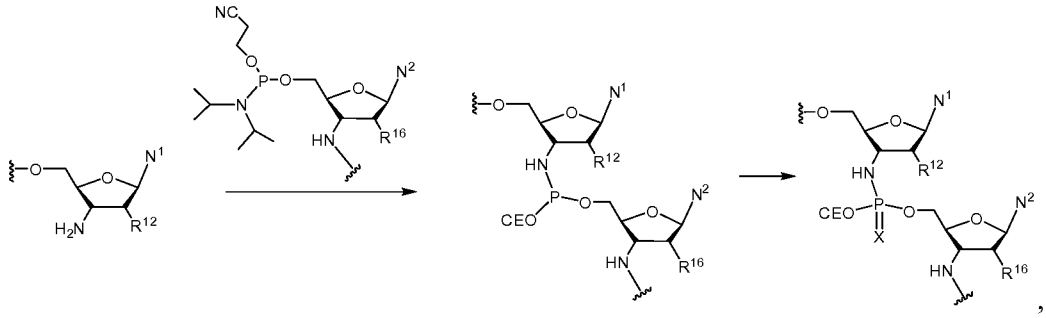
[000735] Other methods for the synthesis of the chimeric polynucleotides of the invention are shown below:



a) ;



b) ;

c)  , where CEO is 2-cyanoethoxy, and X is O or S.

[000736] It will be understood that the reactive group shown at the 3' (or 4' position, when g or h is 1) and at the 5' (or 6' position, when g or h is 1) can be reversed. For example, the halogen, azido, or alkynyl group may be attached to the 5' position (or 6' position, when g or h is 1), and the thiophosphate, (thio)phosphoryl, or azido group may be attached to the 3' position (or 4' position, when g or h is 1).

Quantification

[000737] In one embodiment, the polynucleotides of the present invention may be quantified in exosomes or when derived from one or more bodily fluid. As used herein "bodily fluids" include peripheral blood, serum, plasma, ascites, urine, cerebrospinal fluid (CSF), sputum, saliva, bone marrow, synovial fluid, aqueous humor, amniotic fluid, cerumen, breast milk, broncheoalveolar lavage fluid, semen, prostatic fluid, cowper's fluid or pre-ejaculatory fluid, sweat, fecal matter, hair, tears, cyst fluid, pleural and peritoneal fluid, pericardial fluid, lymph, chyme, chyle, bile, interstitial fluid, menses, pus, sebum, vomit, vaginal secretions, mucosal secretion, stool water, pancreatic juice, lavage fluids from sinus cavities, bronchopulmonary aspirates, blastocyl cavity fluid, and umbilical cord blood. Alternatively, exosomes may be retrieved from an organ selected from the group consisting of lung, heart, pancreas, stomach, intestine, bladder, kidney, ovary, testis, skin, colon, breast, prostate, brain, esophagus, liver, and placenta.

[000738] In the exosome quantification method, a sample of not more than 2mL is obtained from the subject and the exosomes isolated by size exclusion chromatography, density gradient centrifugation, differential centrifugation, nanomembrane ultrafiltration, immunoabsorbent capture, affinity purification, microfluidic separation, or combinations thereof. In the analysis, the level or concentration of a polynucleotide may be an expression level, presence, absence, truncation or alteration of the administered construct. It is advantageous to correlate the level with one or more clinical phenotypes or with an assay for a human disease biomarker. The assay may be performed using construct specific probes, cytometry, qRT-PCR, real-time PCR, PCR, flow cytometry, electrophoresis, mass spectrometry, or combinations thereof while the exosomes may be isolated using immunohistochemical methods such as enzyme linked immunosorbent

assay (ELISA) methods. Exosomes may also be isolated by size exclusion chromatography, density gradient centrifugation, differential centrifugation, nanomembrane ultrafiltration, immunoabsorbent capture, affinity purification, microfluidic separation, or combinations thereof.

[000739] These methods afford the investigator the ability to monitor, in real time, the level of polynucleotides remaining or delivered. This is possible because the polynucleotides of the present invention differ from the endogenous forms due to the structural or chemical modifications.

[000740] In one embodiment, the polynucleotide may be quantified using methods such as, but not limited to, ultraviolet visible spectroscopy (UV/Vis). A non-limiting example of a UV/Vis spectrometer is aNANODROP® spectrometer (ThermoFisher, Waltham, MA). The quantified polynucleotide may be analyzed in order to determine if the polynucleotide may be of proper size, check that no degradation of the polynucleotide has occurred. Degradation of the polynucleotide may be checked by methods such as, but not limited to, agarose gel electrophoresis, HPLC based purification methods such as, but not limited to, strong anion exchange HPLC, weak anion exchange HPLC, reverse phase HPLC (RP-HPLC), and hydrophobic interaction HPLC (HIC-HPLC), liquid chromatography-mass spectrometry (LCMS), capillary electrophoresis (CE) and capillary gel electrophoresis (CGE).

Purification

[000741] Purification of the polynucleotides described herein may include, but is not limited to, polynucleotide clean-up, quality assurance and quality control. Clean-up may be performed by methods known in the arts such as, but not limited to, AGENCOURT® beads (Beckman Coulter Genomics, Danvers, MA), poly-T beads, LNA™ oligo-T capture probes (EXIQON® Inc, Vedbaek, Denmark) or HPLC based purification methods such as, but not limited to, strong anion exchange HPLC, weak anion exchange HPLC, reverse phase HPLC (RP-HPLC), and hydrophobic interaction HPLC (HIC-HPLC). The term "purified" when used in relation to a polynucleotide such as a "purified polynucleotide" refers to one that is separated from at least one contaminant. As used herein, a "contaminant" is any substance which makes another unfit, impure or inferior. Thus, a purified polynucleotide (e.g., DNA and RNA) is present in a form or setting different from that in which it is found in nature, or a form or setting different from that which existed prior to subjecting it to a treatment or purification method.

[000742] A quality assurance and/or quality control check may be conducted using methods such as, but not limited to, gel electrophoresis, UV absorbance, or analytical HPLC.

[000743] In another embodiment, the polynucleotides may be sequenced by methods including, but not limited to reverse-transcriptase-PCR.

III. Modifications

[000744] As used herein in a polynucleotide (such as a chimeric polynucleotide, IVT polynucleotide or a circular polynucleotide), the terms "chemical modification" or, as appropriate, "chemically modified" refer to modification with respect to adenosine (A), guanosine (G), uridine (U), thymidine (T) or cytidine (C) ribo- or deoxyribnucleosides in one or more of their position, pattern, percent or population. Generally, herein, these terms are not intended to refer to the ribonucleotide modifications in naturally occurring 5'-terminal mRNA cap moieties.

[000745] In a polypeptide, the term "modification" refers to a modification as compared to the canonical set of 20 amino acids.

[000746] The modifications may be various distinct modifications. In some embodiments, the regions may contain one, two, or more (optionally different) nucleoside or nucleotide modifications. In some embodiments, a modified polynucleotide, introduced to a cell may exhibit reduced degradation in the cell, as compared to an unmodified polynucleotide.

[000747] Modifications which are useful in the present invention include, but are not limited to those in Table 4 of International Publication No. WO2015038892, the contents of which are herein incorporated by reference in its entirety. Noted in the table are the symbol of the modification, the nucleobase type and whether the modification is naturally occurring or not.

[000748] Non-limiting examples of modification which may be useful in the present invention include, 2-methylthio-N6-(cis-hydroxyisopentenyl)adenosine; 2-methylthio-N6-methyladenosine; 2-methylthio-N6-threonyl carbamoyladenosine; N6-glycinylcarbamoyladenosine; N6-isopentenyladenosine; N6-methyladenosine; N6-threonylcarbamoyladenosine; l,2'-0-dimethyladenosine; 1-methyladenosine; 2'-0-methyladenosine; 2'-0-ribosyladenosine (phosphate); 2-methyladenosine; 2-methylthio-N6 isopentenyladenosine; 2-methylthio-N6-hydroxynorvalyl carbamoyladenosine; 2'-0-methyladenosine; 2'-0-ribosyladenosine (phosphate); isopentenyladenosine; N6-(cis-hydroxyisopentenyl)adenosine; N6,2'-0-dimethyladenosine; N6,2'-0-dimethyladenosine; N6,N6,2'-0-trimethyladenosine; N6,N6-dimethyladenosine; N6-acetyladenosine; N6-hydroxynorvalylcarbamoyladenosine; N6-methyl-N6-threonylcarbamoyladenosine; 2-methyladenosine; 2-methylthio-N6-isopentenyladenosine; 7-deaza-adenosine; N 1-methyl-adenosine; N6, N6 (dimethyl) adenine; N6-cis-hydroxy-isopentenyl-adenosine; a-thio-adenosine; 2 (amino)adenine; 2 (aminopropyl)adenine; 2 (methylthio) N6 (isopentenyl)adenine; 2-(alkyl)adenine; 2-(aminoalkyl)adenine; 2-(aminopropyl)adenine; 2-(halo)adenine; 2-(halo)adenine; 2-(propyl)adenine; 2'-Amino-2'-deoxy-ATP; 2'-Azido-2'-deoxy-ATP; 2'-Deoxy-

2'-a-aminoadenosine TP; 2'-Deoxy-2'-a-azidoadenosine TP; 6 (alkyl)adenine; 6 (methyl)adenine; 6-(alkyl)adenine ; 6-(methyl)adenine; 7 (deaza)adenine; 8 (alkenyl)adenine; 8 (alkynyl)adenine; 8 (amino)adenine; 8 (thioalkyl)adenine; 8-(alkenyl)adenine; 8-(alkyl)adenine; 8-(alkynyl)adenine; 8-(amino)adenine; 8-(halo)adenine; 8-(hydroxyl)adenine; 8-(thioalkyl)adenine; 8-(thiol)adenine; 8-azido-adenosine; aza adenine; deaza adenine; N6 (methyl)adenine; N6-(isopentyl)adenine; 7-deaza-8-aza-adenosine; 7-methyladenine; 1-Deazaadenosine TP; 2'Fluoro-N6-Bz-deoxyadenosine TP; 2'-OMe-2-Amino-ATP; 2'0-methyl-N6-Bz-deoxyadenosine TP; 2'-a-Ethynyladenosine TP; 2-aminoadenine; 2-Aminoadenosine TP; 2-Amino-ATP; 2'-a-Trifluoromethyladenosine TP; 2-Azidoadenosine TP; 2'-b-Ethynyladenosine TP; 2-Bromoadenosine TP; 2'-b-Trifluoromethyladenosine TP; 2-Chloroadenosine TP; 2'-Deoxy-2',2'-difluoroadenosine TP; 2'-Deoxy-2'-a-mercaptoadenosine TP; 2'-Deoxy-2'-a-thiomethoxyadenosine TP; 2'-Deoxy-2'-b-aminoadenosine TP; 2'-Deoxy-2'-b-azidoadenosine TP; 2'-Deoxy-2'-b-bromoadenosine TP; 2'-Deoxy-2'-b-chloroadenosine TP; 2'-Deoxy-2'-b-fluoroadenosine TP; 2'-Deoxy-2'-b-iodoadenosine TP; 2'-Deoxy-2'-b-mercaptoadenosine TP; 2'-Deoxy-2'-b-thiomethoxyadenosine TP; 2-Fluoroadenosine TP; 2-Iodoadenosine TP; 2-Mercaptoadenosine TP; 2-methoxy-adenine; 2-methylthio-adenine; 2-Trifluoromethyladenosine TP; 3-Deaza-3-bromoadenosine TP; 3-Deaza-3-chloroadenosine TP; 3-Deaza-3-fluoroadenosine TP; 3-Deaza-3-iodoadenosine TP; 3-Deazaadenosine TP; 4'-Azidoadenosine TP; 4'-Carbocyclic adenosine TP; 4'-Ethynyladenosine TP; 5'-Homo-adenosine TP; 8-Aza-ATP; 8-bromo-adenosine TP; 8-Trifluoromethyladenosine TP; 9-Deazaadenosine TP; 2-aminopurine; 7-deaza-2,6-diaminopurine; 7-deaza-8-aza-2,6-diaminopurine; 7-deaza-8-aza-2-aminopurine; 2,6-diaminopurine; 7-deaza-8-aza-adenine, 7-deaza-2-aminopurine; 2-thiocytidine; 3-methylcytidine; 5-formylcytidine; 5-hydroxymethylcytidine; 5-methylcytidine; N4-acetylcytidine; 2'-0-methylcytidine; 2'-0-methylcytidine; 5,2'-0-dimethylcytidine; 5-formyl-2'-O-methylcytidine; lysidine; N4,2'-0-dimethylcytidine; N4-acetyl-2'-0-methylcytidine; N4-methylcytidine; N4,N4-Dimethyl-2'-OMe-Cytidine TP; 4-methylcytidine; 5-aza-cytidine; Pseudo-iso-cytidine; pyrrolo-cytidine; a-thio-cytidine; 2-(thio)cytosine; 2'-Amino-2'-deoxy-CTP; 2'-Azido-2'-deoxy-CTP; 2'-Deoxy-2'-a-aminocytidine TP; 2'-Deoxy-2'-a-azidocytidine TP; 3 (deaza) 5 (aza)cytosine; 3 (methyl)cytosine; 3-(alkyl)cytosine; 3-(deaza) 5 (aza)cytosine; 3-(methyl)cytidine; 4,2'-0-dimethylcytidine; 5 (halo)cytosine; 5 (methyl)cytosine; 5 (propynyl)cytosine; 5 (trifluoromethyl)cytosine; 5-(alkyl)cytosine; 5-(alkynyl)cytosine; 5-(halo)cytosine; 5-(propynyl)cytosine; 5-(trifluoromethyl)cytosine; 5-bromo-cytidine; 5-iodo-cytidine; 5-propynyl cytosine; 6-(azo)cytosine; 6-aza-cytidine; aza cytosine; deaza cytosine; N4 (acetyl)cytosine; 1-methyl-1-deaza-pseudoisocytidine; 1-methyl-pseudoisocytidine; 2-methoxy-

5-methyl-cytidine; 2-methoxy-cytidine; 2-thio-5-methyl-cytidine; 4-methoxy-l-methyl-pseudoisocytidine; 4-methoxy-pseudoisocytidine; 4-thio-l -methyl- 1-deaza-pseudoisocytidine; 4-thio-l-methyl-pseudoisocytidine; 4-thio-pseudoisocytidine; 5-aza-zebularine; 5-methyl-zebularine; pyrrolo-pseudoisocytidine; zebularine; (E)-5-(2-Bromo-vinyl)cytidine TP; 2,2'-anhydro-cytidine TP hydrochloride; 2'Fluor-N4-Bz-cytidine TP; 2'Fluoro-N4-Acetyl-cytidine TP; 2'-0-Methyl-N4-Acetyl-cytidine TP; 2'0-methyl-N4-Bz-cytidine TP; 2'-a-Ethynylcytidine TP; 2'-a-Trifluoromethylcytidine TP; 2'-b-Ethynylcytidine TP; 2'-b-Trifluoromethylcytidine TP; 2'-Deoxy-2',2'-difluorocytidine TP; 2'-Deoxy-2'-a-mercaptocytidine TP; 2'-Deoxy-2'-a-thiomethoxycytidine TP; 2'-Deoxy-2'-b-aminocytidine TP; 2'-Deoxy-2'-b-azidocytidine TP; 2'-Deoxy-2'-b-bromocytidine TP; 2'-Deoxy-2'-b-chlorocytidine TP; 2'-Deoxy-2'-b-fluorocytidine TP; 2'-Deoxy-2'-b-iodocytidine TP; 2'-Deoxy-2'-b-mercaptocytidine TP; 2'-Deoxy-2'-b-thiomethoxycytidine TP; 2'-0-Methyl-5-(l-propynyl)cytidine TP; 3'-Ethynylcytidine TP; 4'-Azidocytidine TP; 4'-Carbocyclic cytidine TP; 4'-Ethynylcytidine TP; 5-(l-Propynyl)ara-cytidine TP; 5-(2-Chloro-phenyl)-2-thiocytidine TP; 5-(4-Amino-phenyl)-2-thiocytidine TP; 5-Aminoallyl-CTP; 5-Cyanocytidine TP; 5-Ethynylara-cytidine TP; 5-Ethynylcytidine TP; 5'-Homo-cytidine TP; 5-Methoxycytidine TP; 5-Trifluoromethyl-Cytidine TP; N4-Amino-cytidine TP; N4-Benzoyl-cytidine TP; pseudoisocytidine; 7-methylguanosine; N2,2'-0-dimethylguanosine; N2-methylguanosine; wyosine; l,2'-0-dimethylguanosine; 1-methylguanosine; 2'-0-methylguanosine; 2'-0-ribosylguanosine (phosphate); 2'-0-methylguanosine; 2'-0-ribosylguanosine (phosphate); 7-aminomethyl-7-deazaguanosine; 7-cyano-7-deazaguanosine; archaeosine; methylwyosine; N2,7-dimethylguanosine; N2,N2,2'-0-trimethylguanosine; N2,N2,7-trimethylguanosine; N2,N2-dimethylguanosine; N2,7,2'-0-trimethylguanosine; 6-thio-guanosine; 7-deaza-guanosine; 8-oxo-guanosine; Nl-methyl-guanosine; a-thio-guanosine; 2 (propyl)guanine; 2-(alkyl)guanine; 2'-Amino-2'-deoxy-GTP; 2'-Azido-2'-deoxy-GTP; 2'-Deoxy-2'-a-aminoguanosine TP; 2'-Deoxy-2'-a-azidoguanosine TP; 6 (methyl)guanine; 6-(alkyl)guanine; 6-(methyl)guanine; 6-methyl-guanosine; 7 (alkyl)guanine; 7 (deaza)guanine; 7 (methyl)guanine; 7-(alkyl)guanine; 7-(deaza)guanine; 7-(methyl)guanine; 8 (alkyl)guanine; 8 (alkynyl)guanine; 8 (halo)guanine; 8 (thioalkyl)guanine; 8-(alkenyl)guanine; 8-(alkyl)guanine; 8-(alkynyl)guanine; 8-(amino)guanine; 8-(halo)guanine; 8-(hydroxyl)guanine; 8-(thioalkyl)guanine; 8-(thiol)guanine; aza guanine; deaza guanine; N (methyl)guanine; N-(methyl)guanine; l-methyl-6-thio-guanosine; 6-methoxy-guanosine; 6-thio-7-deaza-8-aza-guanosine; 6-thio-7-deaza-guanosine; 6-thio-7-methyl-guanosine; 7-deaza-8-aza-guanosine; 7-methyl-8-oxo-guanosine; N2,N2-dimethyl-6-thio-guanosine; N2-methyl-6-thio-guanosine; 1-Me-GTP; 2'Fluoro-N2-isobutyl-guanosine TP; 2'0-methyl-N2-isobutyl-guanosine TP; 2'-a-

Ethynylguanosine TP; 2'-a-Trifluoromethylguanosine TP; 2'-b-Ethynylguanosine TP; 2'-b-Trifluoromethylguanosine TP; 2'-Deoxy-2',2'-difluoroguanosine TP; 2'-Deoxy-2'-a-mercaptoguanosine TP; 2'-Deoxy-2'-a-thiomethoxyguanosine TP; 2'-Deoxy-2'-b-aminoguanosine TP; 2'-Deoxy-2'-b-azidoguanosine TP; 2'-Deoxy-2'-b-bromoguanosine TP; 2'-Deoxy-2'-b-chloroguanosine TP; 2'-Deoxy-2'-b-fluoroguanosine TP; 2'-Deoxy-2'-b-iodoguanosine TP; 2'-Deoxy-2'-b-mercaptoguanosine TP; 2'-Deoxy-2'-b-thiomethoxyguanosine TP; 4'-Azidoguanosine TP; 4'-Carbocyclic guanosine TP; 4'-Ethynylguanosine TP; 5'-Homo-guanosine TP; 8-bromo-guanosine TP; 9-Deazaguanosine TP; N2-isobutyl-guanosine TP; 1-methylinosine; inosine; l,2'-0-dimethylinosine; 2'-0-methylinosine; 7-methylinosine; 2'-0-methylinosine; epoxyqueuosine; galactosyl-queuosine; mannosylqueuosine; queuosine; allyamino-thymidine; aza thymidine; deaza thymidine; deoxy-thymidine; 2'-O-methyluridine; 2-thiouridine; 3-methyluridine; 5-carboxymethyluridine; 5-hydroxyuridine; 5-methyluridine; 5-taurinomethyl-2-thiouridine; 5-taurinomethyluridine; dihydrouridine; pseudouridine; (3-(3-amino-3-carboxypropyl)uridine; 1-methyl-3-(3-amino-5-carboxypropyl)pseudouridine; 1-methylpseduouridine; 1-methyl-pseudouridine; 2'-O-methyluridine; 2'-0-methylpseudouridine; 2'-0-methyluridine; 2-thio-2'-0-methyluridine; 3-(3-amino-3-carboxypropyl)uridine; 3,2'-0-dimethyluridine; 3-Methyl-pseudo-Uridine TP; 4-thiouridine; 5-(carboxyhydroxymethyl)uridine; 5-(carboxyhydroxymethyl)uridine methyl ester; 5,2'-0-dimethyluridine; 5,6-dihydro-uridine; 5-aminomethyl-2-thiouridine; 5-carbamoylmethyl-2'-O-methyluridine; 5-carbamoylmethyluridine; 5-carboxyhydroxymethyluridine; 5-carboxyhydroxymethyluridine methyl ester; 5-carboxymethylaminomethyl-2'-0-methyluridine; 5-carboxymethylaminomethyl-2-thiouridine; 5-carboxymethylaminomethyl-2-thiouridine; 5-carboxymethylaminomethyluridine; 5-carboxymethylaminomethyluridine; 5-Carbamoylmethyluridine TP; 5-methoxycarbonylmethyl-2'-O-methyluridine; 5-methoxycarbonylmethyl-2-thiouridine; 5-methoxycarbonylmethyluridine; 5-methoxyuridine; 5-methyl-2-thiouridine; 5-methylaminomethyl-2-selenouridine; 5-methylaminomethyl-2-thiouridine; 5-methylaminomethyluridine; 5-Methyldihydrouridine; 5-Oxyacetic acid- Uridine TP; 5-Oxyacetic acid-methyl ester-Uridine TP; N1-methyl-pseudo-uridine; uridine 5-oxyacetic acid; uridine 5-oxyacetic acid methyl ester; 3-(3-Amino-3-carboxypropyl)-Uridine TP; 5-(iso-Pentenylaminomethyl)- 2-thiouridine TP; 5-(iso-Pentenylaminomethyl)-2'-0-methyluridine TP; 5-(iso-Pentenylaminomethyl)uridine TP; 5-propynyl uracil; a-thio-uridine; 1 (aminoalkylamino-carbonylethylenyl)-2(thio)-pseudouracil; 1 (aminoalkylaminocarbonylethylenyl)-2,4-(dithio)pseudouracil; 1 (aminoalkylaminocarbonylethylenyl)-4 (thio)pseudouracil; 1 (aminoalkylaminocarbonylethylenyl)-pseudouracil; 1 (aminocarbonylethylenyl)-2(thio)-

pseudouracil; 1 (aminocarbonylethylenyl)-2,4-(dithio)pseudouracil; 1 (aminocarbonylethylenyl)-4 (thio)pseudouracil; 1 (aminocarbonylethylenyl)-pseudouracil; 1 substituted 2(thio)-pseudouracil; 1 substituted 2,4-(dithio)pseudouracil; 1 substituted 4 (thio)pseudouracil; 1 substituted pseudouracil; l-(aminoalkylamino-carbonylethylenyl)-2-(thio)-pseudouracil; 1-Methyl-3-(3-amino-3-carboxypropyl) pseudouridine TP; l-Methyl-3-(3-amino-3-carboxypropyl)pseudo-UTP ; 1-Methyl-pseudo-UTP ; 2 (thio)pseudouracil; 2' deoxy uridine; 2' fluorouridine; 2-(thio)uracil; 2,4-(dithio)psuedouracil; 2' methyl, 2'amino, 2'azido, 2'fluro-guanosine; 2'-Amino-2'-deoxy-UTP; 2'-Azido-2'-deoxy-UTP; 2'-Azido-deoxyuridine TP; 2'-0-methylpseudouridine; 2' deoxy uridine; 2' fluorouridine; 2'-Deoxy-2'-a-aminouridine TP; 2'-Deoxy-2'-a-azidouridine TP; 2-methylpseudouridine; 3 (3 amino-3 carboxypropyl)uracil; 4 (thio)pseudouracil; 4-(thio)pseudouracil; 4-(thio)uracil; 4-thiouracil; 5 (1,3-diazole-l-alkyl)uracil; 5 (2-aminopropyl)uracil; 5 (aminoalkyl)uracil; 5 (dimethylaminoalkyl)uracil; 5 (guanidiniumalkyl)uracil; 5 (methoxycarbonylmethyl)-2-(thio)uracil; 5 (methoxycarbonyl-methyl)uracil; 5 (methyl) 2 (thio)uracil; 5 (methyl) 2,4 (dithio)uracil; 5 (methyl) 4 (thio)uracil; 5 (methylaminomethyl)-2 (thio)uracil; 5 (methylaminomethyl)-2,4 (dithio)uracil; 5 (methylaminomethyl)-4 (thio)uracil; 5 (propynyl)uracil; 5 (trifluoromethyl)uracil; 5-(2-aminopropyl)uracil; 5-(alkyl)-2-(thio)pseudouracil; 5-(alkyl)-2,4 (dithio)pseudouracil; 5-(alkyl)-4 (thio)pseudouracil; 5-(alkyl)pseudouracil; 5-(alkyl)uracil; 5-(alkynyl)uracil; 5-(allylamino)uracil; 5-(cyanoalkyl)uracil; 5-(dialkylaminoalkyl)uracil; 5-(dimethylaminoalkyl)uracil; 5-(guanidiniumalkyl)uracil; 5-(halo)uracil; 5-(l,3-diazole-l-alkyl)uracil; 5-(methoxy)uracil; 5-(methoxycarbonylmethyl)-2-(thio)uracil; 5-(methoxycarbonyl-methyl)uracil; 5-(methyl) 2(thio)uracil; 5-(methyl) 2,4 (dithio)uracil; 5-(methyl) 4 (thio)uracil; 5-(methyl)-2-(thio)pseudouracil; 5-(methyl)-2,4 (dithio)pseudouracil; 5-(methyl)-4 (thio)pseudouracil; 5-(methyl)pseudouracil; 5-(methylaminomethyl)-2 (thio)uracil; 5-(methylaminomethyl)-2,4(dithio)uracil; 5-(methylaminomethyl)-4-(thio)uracil; 5-(propynyl)uracil; 5-(trifluoromethyl)uracil; 5-aminoallyl-uridine; 5-bromo-uridine; 5-iodo-uridine; 5-uracil; 6 (azo)uracil; 6-(azo)uracil; 6-aza-uridine; allyamino-uracil; aza uracil; deaza uracil; N3 (methyl)uracil; P seudo-UTP-l-2-ethanoic acid ; pseudouracil; 4-Thio-pseudo-UTP ; 1-carboxymethyl-pseudouridine; 1-methyl- 1-deaza-pseudouridine; 1-propynyl-uridine; 1-taurinomethyl- 1-methyl-uridine; 1-taurinomethyl-4-thio-uridine; 1-taurinomethyl-pseudouridine; 2-methoxy-4-thio-pseudouridine; 2-thio- 1-methyl- 1-deaza-pseudouridine; 2-thio- 1-methyl-pseudouridine; 2-thio-5-aza-uridine; 2-thio-dihydropseudouridine; 2-thio-dihydrouridine; 2-thio-pseudouridine; 4-methoxy-2-thio-pseudouridine; 4-methoxy-pseudouridine; 4-thio- 1-methyl-pseudouridine; 4-thio-pseudouridine; 5-aza-uridine; dihydropseudouridine; (±)l-(2-

Hydroxypropyl)pseudouridine TP; (2R)-l-(2-Hydroxypropyl)pseudouridine TP; (2S)-l-(2-Hydroxypropyl)pseudouridine TP; (E)-5-(2-Bromo-vinyl)ara-uridine TP; (E)-5-(2-Bromo-vinyl)uridine TP; (Z)-5-(2-Bromo-vinyl)ara-uridine TP; (Z)-5-(2-Bromo-vinyl)uridine TP; 1-(2,2,2-Trifluoroethyl)-pseudo-UTP ; l-(2,2,3,3,3-Pentafluoropropyl)pseudouridine TP; l-(2,2-Diethoxyethyl)pseudouridine TP; l-(2,4,6-Trimethylbenzyl)pseudouridine TP; 1-(2,4,6-Trimethyl-benzyl)pseudo-UTP ; l-(2,4,6-Trimethyl-phenyl)pseudo-UTP ; l-(2-Amino-2-carboxyethyl)pseudo-UTP; 1-(2-Amino-ethyl)pseudo-UTP; 1-(2-Hydroxyethyl)pseudouridine TP; l-(2-Methoxyethyl)pseudouridine TP; l-(3,4-Bis-trifluoromethoxybenzyl)pseudouridine TP; l-(3,4-Dimethoxybenzyl)pseudouridine TP; l-(3-Amino-3-carboxypropyl)pseudo-UTP ; l-(3-Amino-propyl)pseudo-UTP; l-(3-Cyclopropyl-prop-2-ynyl)pseudouridine TP; l-(4-Amino-4-carboxybutyl)pseudo-UTP; l-(4-Amino-benzyl)pseudo-UTP; l-(4-Amino-butyl)pseudo-UTP; 1-(4-Amino-phenyl)pseudo-UTP ; l-(4-Azidobenzyl)pseudouridine TP; l-(4-Bromobenzyl)pseudouridine TP; l-(4-Chlorobenzyl)pseudouridine TP; l-(4-Fluorobenzyl)pseudouridine TP; l-(4-Iodobenzyl)pseudouridine TP; l-(4-Methanesulfonylbenzyl)pseudouridine TP; l-(4-Methoxybenzyl)pseudouridine TP; l-(4-Methoxy-benzyl)pseudo-UTP; l-(4-Methoxy-phenyl)pseudo-UTP ; l-(4-Methylbenzyl)pseudouridine TP; l-(4-Methyl-benzyl)pseudo-UTP ; l-(4-Nitrobenzyl)pseudouridine TP; l-(4-Nitro-benzyl)pseudo-UTP; 1(4-Nitro-phenyl)pseudo-UTP ; l-(4-Thiomethoxybenzyl)pseudouridine TP; l-(4-Trifluoromethoxybenzyl)pseudouridine TP; 1-(4-Trifluoromethylbenzyl)pseudouridine TP; l-(5-Amino-pentyl)pseudo-UTP; l-(6-Amino-hexyl)pseudo-UTP; 1,6-Dimethyl-pseudo-UTP; l-[3-(2- {2-[2-(2-Aminoethoxy)-ethoxy]-ethoxy}-ethoxy)-propionyl]pseudouridine TP; l-{3-[2-(2-Aminoethoxy)-ethoxy]-propionyl } pseudouridine TP; 1-Acetylpseudouridine TP; l-Alkyl-6-(l-propynyl)-pseudo-UTP; l-Alkyl-6-(2-propynyl)-pseudo-UTP; l-Alkyl-6-allyl-pseudo-UTP; l-Alkyl-6-ethynyl-pseudo-UTP; 1-Alkyl-6-homoallyl-pseudo-UTP; l-Alkyl-6-vinyl-pseudo-UTP; 1-Allylpseudouridine TP; 1-Aminomethyl-pseudo-UTP ; 1-Benzoylpseudouridine TP; 1-Benzyloxymethylpseudouridine TP; 1-Benzyl-pseudo-UTP ; l-Biotinyl-PEG2 -pseudouridine TP; 1-Biotinylpseudouridine TP; 1-Butyl-pseudo-UTP ; 1-Cyanomethylpseudouridine TP; 1-Cyclobutylmethyl-pseudo-UTP ; 1-Cyclobutyl-pseudo-UTP ; 1-Cycloheptylmethyl-pseudo-UTP ; 1-Cycloheptyl-pseudo-UTP ; 1-Cyclohexylmethyl-pseudo-UTP ; 1-Cyclohexyl-pseudo-UTP ; l-Cyclooctylmethyl-pseudo-UTP ; 1-Cyclooctyl-pseudo-UTP ; 1-Cyclopentylmethyl-pseudo-UTP ; 1-Cyclopentyl-pseudo-UTP ; 1-Cyclopropylmethyl-pseudo-UTP ; 1-Cyclopropyl-pseudo-UTP ; 1-Ethyl-pseudo-UTP ; 1-Hexyl-pseudo-UTP ; 1-Homoallylpseudouridine TP; 1-Hydroxymethylpseudouridine TP; 1-iso-propyl-pseudo-UTP ; l-Me-2-thio-pseudo-UTP; l-Me-4-thio-pseudo-UTP; 1-Me-alpha-thio-

pseudo-UTP ; 1-Methanesulfonylmethylpseudouridine TP; 1-Methoxymethylpseudouridine TP; l-Methyl-6-(2,2,2-Trifluoroethyl)pseudo-UTP; l-Methyl-6-(4-morpholino)-pseudo-UTP; 1-Methyl-6-(4-thiomorpholino)-pseudo-UTP; l-Methyl-6-(substituted phenyl)pseudo-UTP; 1-Methyl-6-amino-pseudo-UTP; l-Methyl-6-azido-pseudo-UTP; l-Methyl-6-bromo-pseudo-UTP; l-Methyl-6-butyl-pseudo-UTP; l-Methyl-6-chloro-pseudo-UTP; l-Methyl-6-cyano-pseudo-UTP; l-Methyl-6-dimethylamino-pseudo-UTP; l-Methyl-6-ethoxy-pseudo-UTP; l-Methyl-6-ethylcarboxylate-pseudo-UTP; l-Methyl-6-ethyl-pseudo-UTP; l-Methyl-6-fluoro-pseudo-UTP; l-Methyl-6-formyl-pseudo-UTP; l-Methyl-6-hydroxyamino-pseudo-UTP; 1-Methyl-6-hydroxy-pseudo-UTP; l-Methyl-6-iodo-pseudo-UTP; l-Methyl-6-iso-propyl-pseudo-UTP; 1-Methyl-6-methoxy-pseudo-UTP; l-Methyl-6-methylamino-pseudo-UTP; l-Methyl-6-phenyl-pseudo-UTP; 1-Methyl-6-propyl-pseudo-UTP; 1-Methyl-6-tert-butyl-pseudo-UTP; 1-Methyl-6-trifluoromethoxy-pseudo-UTP; l-Methyl-6-trifluoromethyl-pseudo-UTP; 1-Morpholinomethylpseudouridine TP; 1-Pentyl-pseudo-UTP ; 1-Phenyl-pseudo-UTP ; 1-Pivaloylpseudouridine TP; 1-Propargylpseudouridine TP; 1-Propyl-pseudo-UTP ; 1-propynyl-pseudouridine; 1-p-tolyl-pseudo-UTP ; 1-tert-Butyl-pseudo-UTP ; 1-Thiomethoxymethylpseudouridine TP; 1-Thiomorpholinomethylpseudouridine TP; 1-Trifluoroacetylpseudouridine TP; 1-Trifluoromethyl-pseudo-UTP ; 1-Vinylpseudouridine TP; 2,2'-anhydro-uridine TP; 2'-bromo-deoxyuridine TP; 2'-F-5-Methyl-2'-deoxy-UTP; 2'-OMe-5-Me-UTP; 2'-OMe-pseudo-UTP; 2'-a-Ethynyluridine TP; 2'-a-Trifluoromethyluridine TP; 2'-b-Ethynyluridine TP; 2'-b-Trifluoromethyluridine TP; 2'-Deoxy-2',2'-difluorouridine TP; 2'-Deoxy-2'-a-mercaptouridine TP; 2'-Deoxy-2'-a-thiomethoxyuridine TP; 2'-Deoxy-2'-b-aminouridine TP; 2'-Deoxy-2'-b-azidouridine TP; 2'-Deoxy-2'-b-bromouridine TP; 2'-Deoxy-2'-b-chlorouridine TP; 2'-Deoxy-2'-b-fluorouridine TP; 2'-Deoxy-2'-b-iodouridine TP; 2'-Deoxy-2'-b-mercaptouridine TP; 2'-Deoxy-2'-b-thiomethoxyuridine TP; 2-methoxy-4-thio-uridine; 2-methoxyuridine; 2'-0-Methyl-5-(l-propynyl)uridine TP; 3-Alkyl-pseudo-UTP; 4'-Azidouridine TP; 4'-Carbocyclic uridine TP; 4'-Ethynyluridine TP; 5-(l-Propynyl)ara-uridine TP; 5-(2-Furanyl)uridine TP; 5-Cyanouridine TP; 5-Dimethylaminouridine TP; 5'-Homo-uridine TP; 5-iodo-2'-fluoro-deoxyuridine TP; 5-Phenylethynyluridine TP; 5-Trideuteromethyl-6-deuterouridine TP; 5-Trifluoromethyl-Uridine TP; 5-Vinylarauridine TP; 6-(2,2,2-Trifluoroethyl)-pseudo-UTP; 6-(4-Morpholino)-pseudo-UTP; 6-(4-Thiomorpholino)-pseudo-UTP; 6-(Substituted-Phenyl)-pseudo-UTP; 6-Amino-pseudo-UTP; 6-Azido-pseudo-UTP; 6-Bromo-pseudo-UTP ; 6-Butyl-pseudo-UTP; 6-Chloro-pseudo-UTP ; 6-Cyano-pseudo-UTP; 6-Dimethylamino-pseudo-UTP; 6-Ethoxy-pseudo-UTP; 6-Ethylcarboxylate-pseudo-UTP; 6-Ethyl-pseudo-UTP; 6-Fluoro-pseudo-UTP ; 6-Formyl-pseudo-UTP; 6-Hydroxyamino-pseudo-UTP; 6-

Hydroxy-pseudo-UTP; 6-Iodo-pseudo-UTP ; 6-iso-Propyl-pseudo-UTP; 6-Methoxy-pseudo-UTP ; 6-Methylamino-pseudo-UTP; 6-Methyl-pseudo-UTP ; 6-Phenyl-pseudo-UTP; 6-Phenyl-pseudo-UTP ; 6-Propyl-pseudo-UTP; 6-tert-Butyl-pseudo-UTP; 6-Trifluoromethoxy-pseudo-UTP; 6-Trifluoromethyl-pseudo-UTP ; Alpha-thio-pseudo-UTP ; Pseudouridine l-(4-methylbenzenesulfonic acid) TP; Pseudouridine 1-(4-methylbenzoic acid) TP; Pseudouridine TP l-[3-(2-ethoxy)]propionic acid; Pseudouridine TP l-[3-{2-(2-[2-(2-ethoxy)-ethoxy]-ethoxy)-ethoxy}]propionic acid; Pseudouridine TP l-[3-{2-(2-[2-[2(2-ethoxy)-ethoxy}-ethoxy]-ethoxy)-ethoxy}]propionic acid; Pseudouridine TP l-[3-{2-(2-[2-ethoxy]-ethoxy)-ethoxy}]propionic acid; Pseudouridine TP l-[3-{2-(2-ethoxy)-ethoxy}] propionic acid; Pseudouridine TP 1-methylphosphonic acid; Pseudouridine TP 1-methylphosphonic acid diethyl ester; Pseudo-UTP-N1-3-propionic acid; Pseudo-UTP-Nl-4-butanoic acid; Pseudo-UTP-Nl-5-pentanoic acid; Pseudo-UTP-Nl-6-hexanoic acid; Pseudo-UTP-Nl-7-heptanoic acid; Pseudo-UTP-Nl-methyl-p-benzoic acid; Pseudo-UTP-Nl -p-benzoic acid ; wybutosine; hydroxywybutosine; isowyosine; peroxywybutosine; undermodified hydroxywybutosine; and 4-demethylwyosine.

[000749] Other modifications which may be useful in the polynucleotides of the present invention are listed in Table 5 of International Publication No. WO2015038892, the contents of which are herein incorporated by reference in its entirety.

[000750] The polynucleotides can include any useful linker between the nucleosides. Such linkers, including backbone modifications are given in Table 6 of International Publication No. WO2015038892, the contents of which are herein incorporated by reference in its entirety. Non limiting examples of linkers which may be included in the polynucleotides described herein include 3'-alkylene phosphonates; 3'-amino phosphoramidate; alkene containing backbones; aminoalkylphosphoramidates; aminoalkylphosphotriesters; boranophosphates; -CH2-0-N(CH3)-CH2-; -CH2-N(CH3)-N(CH3)-CH2-; -CH2-NH-CH2-; chiral phosphonates; chiral phosphorothioates; formacetyl and thioformacetyl backbones; methylene (methylimino); methylene formacetyl and thioformacetyl backbones; methyleneimino and methylenehydrazino backbones; morpholino linkages; -N(CH3)-CH2-CH2-; oligonucleosides with heteroatom internucleoside linkage; phosphinates; phosphoramidates ; phosphorodithioates; phosphorothioate internucleoside linkages; phosphorothioates; phosphotriesters; PNA; siloxane backbones; sulfamate backbones; sulfide sulfoxide and sulfone backbones; sulfonate and sulfonamide backbones; thionoalkylphosphonates; thionoalkylphosphotriesters; and thionophosphoramidates .

[000751] The polynucleotides can include any useful modification, such as to the sugar, the nucleobase, or the internucleoside linkage *(e.g.* to a linking phosphate / to a phosphodiester

linkage / to the phosphodiester backbone). One or more atoms of a pyrimidine nucleobase may be replaced or substituted with optionally substituted amino, optionally substituted thiol, optionally substituted alkyl (e.g., methyl or ethyl), or halo (e.g., chloro or fluoro). In certain embodiments, modifications (e.g., one or more modifications) are present in each of the sugar and the internucleoside linkage. Modifications according to the present invention may be modifications of ribonucleic acids (RNAs) to deoxyribonucleic acids (DNAs), threose nucleic acids (TNAs), glycol nucleic acids (GNAs), peptide nucleic acids (PNAs), locked nucleic acids (LNAs) or hybrids thereof). Additional modifications are described herein.

[000752] In some embodiments, the polynucleotides of the invention do not substantially induce an innate immune response of a cell into which the mRNA is introduced. Features of an induced innate immune response include 1) increased expression of pro-inflammatory cytokines, 2) activation of intracellular PRRs (RIG-I, MDA5, etc, and/or 3) termination or reduction in protein translation.

[000753] In certain embodiments, it may desirable to intracellularly degrade a polynucleotide introduced into the cell. For example, degradation of a polynucleotide may be preferable if precise timing of protein production is desired. Thus, in some embodiments, the invention provides a polynucleotide containing a degradation domain, which is capable of being acted on in a directed manner within a cell.

[000754] Traditionally, the basic components of an mRNA molecule include at least a coding region, a 5'UTR, a 3'UTR, a 5' cap and a poly-A tail. Building on this wild type modular structure, the present invention expands the scope of functionality of traditional mRNA molecules by providing polynucleotides which maintain a modular organization, but which comprise one or more structural and/or chemical modifications or alterations which impart useful properties to the polynucleotide including, in some embodiments, the lack of a substantial induction of the innate immune response of a cell into which the polynucleotides are introduced. As used herein, a "structural" feature or modification is one in which two or more linked nucleotides are inserted, deleted, duplicated, inverted or randomized in a polynucleotide without significant chemical modification to the nucleotides themselves. Because chemical bonds will necessarily be broken and reformed to effect a structural modification, structural modifications are of a chemical nature and hence are chemical modifications. However, structural modifications will result in a different sequence of nucleotides. For example, the polynucleotide "ATCG" may be chemically modified to "AT-5meC-G". The same polynucleotide may be structurally modified from "ATCG" to "ATCCCG". Here, the dinucleotide "CC" has been inserted, resulting in a structural modification to the polynucleotide.

[000755] Any of the regions of the polynucleotides may be chemically modified as taught herein or as taught in International Publication Number WO2013052523 filed October 3, 2012 (Attorney Docket Number M9) and International Publication No. WO2014093924, filed December 13, 2013 (Attorney Docket Number M36) the contents of each of which are incorporated herein by reference in its entirety.

Modified Polynucleotide Molecules

[000756] The present invention also includes building blocks, e.g., modified ribonucleosides, and modified ribonucleotides, of polynucleotide molecules. For example, these building blocks can be useful for preparing the polynucleotides of the invention. Such building blocks are taught in International Publication Number WO2013052523 filed October 3, 2012 (Attorney Docket Number M9) and International Publication No. WO2014093924, filed December 13, 2013 (Attorney Docket Number M36) the contents of each of which are incorporated herein by reference in its entirety.

Modifications on the Sugar

[000757] The modified nucleosides and nucleotides (e.g., building block molecules), which may be incorporated into a polynucleotide (e.g., RNA or mRNA, as described herein), can be modified on the sugar of the ribonucleic acid. For example, the 2' hydroxyl group (OH) can be modified or replaced with a number of different substituents. Exemplary substitutions at the 2'-position include, but are not limited to, H, halo, optionally substituted C_{i_6} alkyl; optionally substituted C_{i_6} alkoxy; optionally substituted $_{C6-10}$ aryloxy; optionally substituted $_{C3-8}$ cycloalkyl; optionally substituted $_{C3-8}$ cycloalkoxy; optionally substituted $_{C6-10}$ aryloxy; optionally substituted $_{C6-10}$ aryl-Ci-6 alkoxy, optionally substituted $Ci-_{12}$ (heterocyclyl)oxy; a sugar (e.g., ribose, pentose, or any described herein); a polyethyleneglycol (PEG), $-0(CH_2CH_20)_nCH_2CH_2OR$, where R is H or optionally substituted alkyl, and n is an integer from 0 to 20 (e.g., from 0 to 4, from 0 to 8, from 0 to 10, from 0 to 16, from 1 to 4, from 1 to 8, from 1 to 10, from 1 to 16, from 1 to 20, from 2 to 4, from 2 to 8, from 2 to 10, from 2 to 16, from 2 to 20, from 4 to 8, from 4 to 10, from 4 to 16, and from 4 to 20); "locked" nucleic acids (LNA) in which the 2'-hydroxyl is connected by a C_{i_6} alkylene or C_{i_6} heteroalkylene bridge to the 4'-carbon of the same ribose sugar, where exemplary bridges included methylene, propylene, ether, or amino bridges; aminoalkyl, as defined herein; aminoalkoxy, as defined herein; amino as defined herein; and amino acid, as defined herein

[000758] Generally, RNA includes the sugar group ribose, which is a 5-membered ring having an oxygen. Exemplary, non-limiting modified nucleotides include replacement of the oxygen in ribose (e.g., with S, Se, or alkylene, such as methylene or ethylene); addition of a double bond

(e.g., to replace ribose with cyclopentenyl or cyclohexenyl); ring contraction of ribose (e.g., to form a 4-membered ring of cyclobutane or oxetane); ring expansion of ribose (e.g., to form a 6- or 7-membered ring having an additional carbon or heteroatom, such as for anhydrohexitol, altritol, mannitol, cyclohexanyl, cyclohexenyl, and morpholino that also has a phosphoramidate backbone); multicyclic forms (e.g., tricyclo; and "unlocked" forms, such as glycol nucleic acid (GNA) (e.g., R-GNA or S-GNA, where ribose is replaced by glycol units attached to phosphodiester bonds), threose nucleic acid (TNA, where ribose is replace with a-L-threofuranosyl-(3'→2')) , and peptide nucleic acid (PNA, where 2-amino-ethyl-glycine linkages replace the ribose and phosphodiester backbone). The sugar group can also contain one or more carbons that possess the opposite stereochemical configuration than that of the corresponding carbon in ribose. Thus, a polynucleotide molecule can include nucleotides containing, e.g., arabinose, as the sugar. Such sugar modifications are taught in International Publication Number WO2013052523 filed October 3, 2012 (Attorney Docket Number M9) and International Application No. WO2014093924, filed December 13, 2013 (Attorney Docket Number M36) the contents of each of which are incorporated herein by reference in its entirety.

Modifications on the Nucieobase

[000759] The present disclosure provides for modified nucleosides and nucleotides. As described herein "nucleoside" is defined as a compound containing a sugar molecule (e.g., a pentose or ribose) or a derivative thereof in combination with an organic base (e.g., a purine or pyrimidine) or a derivative thereof (also referred to herein as "nucieobase"). As described herein, "nucleotide" is defined as a nucleoside including a phosphate group. The modified nucleotides may by synthesized by any useful method, as described herein (e.g., chemically, enzymatically, or recombinantly to include one or more modified or non-natural nucleosides). The polynucleotides may comprise a region or regions of linked nucleosides. Such regions may have variable backbone linkages. The linkages may be standard phosphoester linkages, in which case the polynucleotides would comprise regions of nucleotides.

[000760] The modified nucleotide base pairing encompasses not only the standard adenosine-thymine, adenosine-uracil, or guanosine-cytosine base pairs, but also base pairs formed between nucleotides and/or modified nucleotides comprising non-standard or modified bases, wherein the arrangement of hydrogen bond donors and hydrogen bond acceptors permits hydrogen bonding between a non-standard base and a standard base or between two complementary non-standard base structures. One example of such non-standard base pairing is the base pairing between the modified nucleotide inosine and adenine, cytosine or uracil.

[000761] The modified nucleosides and nucleotides can include a modified nucleobase. Examples of nucleobases found in RNA include, but are not limited to, adenine, guanine, cytosine, and uracil. Examples of nucleobase found in DNA include, but are not limited to, adenine, guanine, cytosine, and thymine. Such modified nucleobases (including the distinctions between naturally occurring and non-naturally occurring) are taught in International Publication Number WO2013052523 filed October 3, 2012 (Attorney Docket Number M9) and International Publicatoin No. WO20 14093924, filed December 13, 2013 (Attorney Docket Number M36) the contents of each of which are incorporated herein by reference in its entirety.

Combinations of Modified Sugars, Nucleobases, and Internucleoside Linkages

[000762] The polynucleotides of the invention can include a combination of modifications to the sugar, the nucleobase, and/or the internucleoside linkage. These combinations can include any one or more modifications described herein.

[000763] Examples of modified nucleotides and modified nucleotide combinations are provided below in Tables 4 and 5. These combinations of modified nucleotides can be used to form the polynucleotides of the invention. Unless otherwise noted, the modified nucleotides may be completely substituted for the natural nucleotides of the polynucleotides of the invention. As a non-limiting example, the natural nucleotide uridine may be substituted with a modified nucleoside described herein. In another non-limiting example, the natural nucleotide uridine may be partially substituted (e.g., about 0.1%, 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 99.9%) with at least one of the modified nucleoside disclosed herein. Any combination of base/sugar or linker may be incorporated into the polynucleotides of the invention and such modifications are taught in International Publication Number WO20 13052523 filed October 3, 2012 (Attorney Docket Number M9), International Publication No. WO20 14093924, filed December 13, 2013 (Attorney Docket Number M36), International Publication No. WO201505 1173 filed October 2, 2014 (Attorney Docket Number M71) and International Publication No. WO2015051 169, filed October 2, 2014 (Attorney Docket Number M72), the contents of each of which are incorporated herein by reference in its entirety.

Table 4. Combinations

Modified Nucleotide	Modified Nucleotide Combination
a-thio-cytidine	a-thio-cytidine/5-iodo-uridine
	a-thio-cytidine/Nl-methyl-pseudouridine
	a-thio-cytidine/a-thio-uridine
	a-thio-cytidine/5-methyl-uridine
	a-thio-cytidine/pseudo-uridine
	about 50% of the cytosines are a-thio-cytidine
pseudoisocytidine	pseudoisocytidine/5-iodo-uridine

	pseudoisocytidine/N 1-methyl-pseudouridine
	pseudoisocytidine/a-thio-uridine
	pseudoisocytidine/5-methyl-uridine
	pseudoisocytidine/pseudouridine
	about 25% of cytosines are pseudoisocytidine
	pseudoisocytidine/about *50%* of uridines are N1-methyl-pseudouridine and about *50%* of uridines are pseudouridine
	pseudoisocytidine/about *25%* of uridines are N1-methyl-pseudouridine and about *25%* of uridines are pseudouridine
pyrrolo-cytidine	pyrrolo-cytidine/5-iodo-uridine
	pyrrolo-cytidine/N 1-methyl-pseudouridine
	pyrrolo-cytidine/a-thio-uridine
	pyrrolo-cytidine/ 5-methyl-uridine
	pyrrolo-cytidine/pseudouridine
	about 50%, of the cytosines are pyrrolo-cytidine
5-methyl-cytidine	5-methyl-cytidine/5-iodo-uridine
	5-methyl-cytidine/Nl -methyl-pseudouridine
	5-methyl-cytidine/a-thio-uridine
	5-methyl-cytidine/ 5-methyl-uridine
	5-methyl-cytidine/pseudouridine
	about 25%o of cytosines are 5-methyl-cytidine
	about 50%, of cytosines are 5-methyl-cytidine
	5-methyl-cytidine/ 5-methoxy-uridine
	5-methyl-cytidine/ 5-bromo -uridine
	5-methyl-cytidine/2-thio-uridine
	5-methyl-cytidine/ about 50%o of uridines are 2-thio -uridine
	about 50%o of uridines are 5-methyl-cytidine/ about 50%o of uridines are 2-thio-uridine
N4-acetyl-cytidine	N4-acetyl-cytidine /5-iodo-uridine
	N4-acetyl-cytidine /Nl -methyl-pseudouridine
	N4-acetyl-cytidine /a-thio-uridine
	N4-acetyl-cytidine /5-methyl-uridine
	N4-acetyl-cytidine /pseudouridine
	about 50%o of cytosines are N4-acetyl-cytidine
	about 25%o of cytosines are N4-acetyl-cytidine
	N4-acetyl-cytidine /5-methoxy-uridine
	N4-acetyl-cytidine /5-bromo-uridine
	N4-acetyl-cytidine /2-thio-uridine
	about 50%o of cytosines are N4-acetyl-cytidine/ about 50%o of uridines are 2-thio-uridine

Table 5. Combinations

1-(2,2,2 -Trifluoroethyl)pseudo-UTP
1-Ethyl-pseudo-UTP
1-Methyl-pseudo-U-alpha-thio-TP
1-methyl-pseudouridine TP, ATP, GTP, CTP
l-methyl-pseudo-UTP/5-methyl-CTP/ATP/GTP
1-methyl-pseudo-UTP/CTP/ATP/GTP
1-Propyl -pseudo-UTP
25 % 5-Aminoallyl-CTP + 75 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
25 % 5-Aminoallyl-CTP + 75 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
25 % 5-Bromo-CTP + 75 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
25 % 5-Bromo-CTP + 75 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
25 % 5-Bromo-CTP + 75 % CTP/l-Methyl-pseudo-UTP
25 % 5-Carboxy-CTP + 75 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
25 % 5-Carboxy-CTP + 75 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
25 % 5-Ethyl-CTP + 75 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP

25 % 5-Ethyl-CTP + 75 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
25 % 5-Ethynyl-CTP + 75 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
25 % 5-Ethynyl-CTP + 75 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
25 % 5-Fluoro-CTP + 75 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
25 % 5-Fluoro-CTP + 75 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
25 % 5-Formyl-CTP + 75 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
25 % 5-Formyl-CTP + 75 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
25 % 5-Hydroxymethyl-CTP + 75 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
25 % 5-Hydroxymethyl-CTP + 75 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
25 % 5-Iodo-CTP + 75 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
25 % 5-Iodo-CTP + 75 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
25 % 5-Methoxy-CTP + 75 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
25 % 5-Methoxy-CTP + 75 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
25 % 5-Methyl-CTP + 75 % CTP/25 % 5-Methoxy-UTP + 75 % 1-Methyl-pseudo-UTP
25 % 5-Methyl-CTP + 75 % CTP/25 % 5-Methoxy-UTP + 75 % UTP
25 % 5-Methyl-CTP + 75 % CTP/50 % 5-Methoxy-UTP + 50 % 1-Methyl-pseudo-UTP
25 % 5-Methyl-CTP + 75 % CTP/50 % 5-Methoxy-UTP + 50 % UTP
25 % 5-Methyl-CTP + 75 % CTP/5-Methoxy-UTP
25 % 5-Methyl-CTP + 75 % CTP/75 % 5-Methoxy-UTP + 25 % 1-Methyl-pseudo-UTP
25 % 5-Methyl-CTP + 75 % CTP/75 % 5-Methoxy-UTP + 25 % UTP
25 % 5-Phenyl-CTP + 75 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
25 % 5-Phenyl-CTP + 75 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
25 % 5-Trifluoromethyl-CTP + 75 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
25 % 5-Trifluoromethyl-CTP + 75 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
25 % 5-Trifluoromethyl-CTP + 75 % CTP/l-Methyl-pseudo-UTP
25 % N4-Ac-CTP + 75 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
25 % N4-Ac-CTP + 75 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
25 % N4-Bz-CTP + 75 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
25 % N4-Bz-CTP + 75 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
25 % N4-Methyl-CTP + 75 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
25 % N4-Methyl-CTP + 75 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
25 % Pseudo-iso-CTP + 75 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
25 % Pseudo-iso-CTP + 75 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
25% 5-Bromo-CTP/75% CTP/ Pseudo-UTP
25% 5-methoxy-UTP/25% 5-methyl-CTP/ATP/GTP
25% 5-methoxy-UTP/5-methyl-CTP/ATP/GTP
25% 5-methoxy-UTP/75% 5-methyl-CTP/ATP/GTP
25% 5-methoxy-UTP/CTP/ATP/GTP
25% 5-metoxy-UTP/50% 5-methyl-CTP/ATP/GTP
2-Amino-ATP
2-Thio-CTP
2-thio-pseudouridine TP, ATP, GTP, CTP
2-Thio-pseudo-UTP
2-Thio-UTP
3-Methyl-CTP
3-Methyl-pseudo-UTP
4-Thio-UTP
50 % 5-Bromo-CTP + 50 % CTP/l-Methyl-pseudo-UTP
50 % 5-Hydroxymethyl-CTP + 50 % CTP/l-Methyl-pseudo-UTP
50 % 5-methoxy-UTP/5-methyl-CTP/ATP/GTP
50 % 5-Methyl-CTP + 50 % CTP/25 % 5-Methoxy-UTP + 75 % 1-Methyl-pseudo-UTP
50 % 5-Methyl-CTP + 50 % CTP/25 % 5-Methoxy-UTP + 75 % UTP
50 % 5-Methyl-CTP + 50 % CTP/50 % 5-Methoxy-UTP + 50 % 1-Methyl-pseudo-UTP
50 % 5-Methyl-CTP + 50 % CTP/50 % 5-Methoxy-UTP + 50 % UTP
50 % 5-Methyl-CTP + 50 % CTP/5-Methoxy-UTP
50 % 5-Methyl-CTP + 50 % CTP/75 % 5-Methoxy-UTP + 25 % 1-Methyl-pseudo-UTP
50 % 5-Methyl-CTP + 50 % CTP/75 % 5-Methoxy-UTP + 25 % UTP
50 % 5-Trifluoromethyl-CTP + 50 % CTP/l-Methyl-pseudo-UTP

50% 5-Bromo-CTP/ 50% CTP/Pseudo-UTP
50% 5-methoxy-UTP/25% 5-methyl-CTP/ATP/GTP
50% 5-methoxy-UTP/50% 5-methyl-CTP/ATP/GTP
50% 5-methoxy-UTP/75% 5-methyl-CTP/ATP/GTP
50% 5-methoxy-UTP/CTP/ATP/GTP
5-Aminoallyl-CTP
5-Aminoallyl-CTP/ 5-Methoxy-UTP
5-Aminoallyl-UTP
5-Bromo-CTP
5-Bromo-CTP/ 5-Methoxy-UTP
5-Bromo-CTP/ 1-Methyl-pseudo-UTP
5-Bromo-CTP/Pseudo-UTP
5-bromocytidine TP, ATP, GTP, UTP
5-Bromo-UTP
5-Carboxy-CTP/ 5-Methoxy-UTP
5-Ethyl-CTP/5-Methoxy-UTP
5-Ethynyl-CTP/5-Methoxy-UTP
5-Fluoro-CTP/ 5-Methoxy-UTP
5-Formyl-CTP/ 5-Methoxy-UTP
5-Hydroxy- methyl-CTP/ 5-Methoxy-UTP
5-Hydroxymethyl-CTP
5-Hydroxymethyl-CTP/l-Methyl-pseudo-UTP
5-Hydroxymethyl-CTP/5-Methoxy-UTP
5-hydroxymethyl-cytidine TP, ATP, GTP, UTP
5-Iodo-CTP/ 5-Methoxy-UTP
5-Me-CTP/5-Methoxy-UTP
5-Methoxy carbonyl methyl-UTP
5-Methoxy-CTP/5-Methoxy-UTP
5-methoxy-uridine TP, ATP, GTP, UTP
5-methoxy-UTP
5-Methoxy-UTP
5-Methoxy-UTP/ N6-Isopentenyl-ATP
5-methoxy-UTP/25% 5-methyl-CTP/ATP/GTP
5-methoxy-UTP/5-methyl-CTP/ATP/GTP
5-methoxy-UTP/75% 5-methyl-CTP/ATP/GTP
5-methoxy-UTP/CTP/ATP/GTP
5-Methyl-2-thio-UTP
5-Methylaminomethyl-UTP
5-Methyl-CTP/ 5-Methoxy-UTP
5-Methyl-CTP/ 5-Methoxy-UTP(cap 0)
5-Methyl-CTP/ 5-Methoxy-UTP(No cap)
5-Methyl-CTP/25 % 5-Methoxy-UTP + 75 % 1-Methyl-pseudo-UTP
5-Methyl-CTP/25 % 5-Methoxy-UTP + 75 % UTP
5-Methyl-CTP/50 % 5-Methoxy-UTP + 50 % 1-Methyl-pseudo-UTP
5-Methyl-CTP/50 % 5-Methoxy-UTP + 50 % UTP
5-Methyl-CTP/5-Methoxy-UTP/N6-Me-ATP
5-Methyl-CTP/75 % 5-Methoxy-UTP + 25 % 1-Methyl-pseudo-UTP
5-Methyl-CTP/75 % 5-Methoxy-UTP + 25 % UTP
5-Phenyl-CTP/ 5-Methoxy-UTP
5-Trifluoro- methyl-CTP/ 5-Methoxy-UTP
5-Trifluoromethyl-CTP
5-Trifluoromethyl-CTP/ 5-Methoxy-UTP
5-Trifluoromethyl-CTP/l-Methyl-pseudo-UTP
5-Trifluoromethyl-CTP/Pseudo-UTP
5-Trifluoromethyl-UTP
5-trifluromethylcytidine TP, ATP, GTP, UTP
75 % 5-Aminoallyl-CTP + 25 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
75 % 5-Aminoallyl-CTP + 25 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP

75 % 5-Bromo-CTP + 25 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
75 % 5-Bromo-CTP + 25 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
75 % 5-Carboxy-CTP + 25 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
75 % 5-Carboxy-CTP + 25 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
75 % 5-Ethyl-CTP + 25 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
75 % 5-Ethyl-CTP + 25 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
75 % 5-Ethynyl-CTP + 25 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
75 % 5-Ethynyl-CTP + 25 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
75 % 5-Fluoro-CTP + 25 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
75 % 5-Fluoro-CTP + 25 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
75 % 5-Formyl-CTP + 25 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
75 % 5-Formyl-CTP + 25 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
75 % 5-Hydroxymethyl-CTP + 25 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
75 % 5-Hydroxymethyl-CTP + 25 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
75 % 5-Iodo-CTP + 25 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
75 % 5-Iodo-CTP + 25 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
75 % 5-Methoxy-CTP + 25 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
75 % 5-Methoxy-CTP + 25 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
75 % 5-methoxy-UTP/5-methyl-CTP/ATP/GTP
75 % 5-Methyl-CTP + 25 % CTP/25 % 5-Methoxy-UTP + 75 % 1-Methyl-pseudo-UTP
75 % 5-Methyl-CTP + 25 % CTP/25 % 5-Methoxy-UTP + 75 % UTP
75 % 5-Methyl-CTP + 25 % CTP/50 % 5-Methoxy-UTP + 50 % 1-Methyl-pseudo-UTP
75 % 5-Methyl-CTP + 25 % CTP/50 % 5-Methoxy-UTP + 50 % UTP
75 % 5-Methyl-CTP + 25 % CTP/5-Methoxy-UTP
75 % 5-Methyl-CTP + 25 % CTP/75 % 5-Methoxy-UTP + 25 % 1-Methyl-pseudo-UTP
75 % 5-Methyl-CTP + 25 % CTP/75 % 5-Methoxy-UTP + 25 % UTP
75 % 5-Phenyl-CTP + 25 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
75 % 5-Phenyl-CTP + 25 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
75 % 5-Trifluoromethyl-CTP + 25 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
75 % 5-Trifluoromethyl-CTP + 25 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
75 % 5-Trifluoromethyl-CTP + 25 % CTP/1-Methyl-pseudo-UTP
75 % N4-Ac-CTP + 25 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
75 % N4-Ac-CTP + 25 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
75 % N4-Bz-CTP + 25 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
75 % N4-Bz-CTP + 25 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
75 % N4-Methyl-CTP + 25 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
75 % N4-Methyl-CTP + 25 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
75 % Pseudo-iso-CTP + 25 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
75 % Pseudo-iso-CTP + 25 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
75% 5-Bromo-CTP/25% CTP/ 1-Methyl-pseudo-UTP
75% 5-Bromo-CTP/25% CTP/ Pseudo-UTP
75% 5-methoxy-UTP/25% 5-methyl-CTP/ATP/GTP
75% 5-methoxy-UTP/50% 5-methyl-CTP/ATP/GTP
75% 5-methoxy-UTP/75% 5-methyl-CTP/ATP/GTP
75% 5-methoxy-UTP/CTP/ATP/GTP
8-Aza-ATP
Alpha-thio-CTP
CTP/25 % 5-Methoxy-UTP + 75 % 1-Methyl-pseudo-UTP
CTP/25 % 5-Methoxy-UTP + 75 % UTP
CTP/50 % 5-Methoxy-UTP + 50 % 1-Methyl-pseudo-UTP
CTP/50 % 5-Methoxy-UTP + 50 % UTP
CTP/5-Methoxy-UTP
CTP/5-Methoxy-UTP (cap 0)
CTP/5-Methoxy-UTP(No cap)
CTP/75 % 5-Methoxy-UTP + 25 % 1-Methyl-pseudo-UTP
CTP/75 % 5-Methoxy-UTP + 25 % UTP
CTP/UTP(No cap)
N1-Me-GTP

N4-Ac-CTP
N4Ac-CTP/l-Methyl-pseudo-UTP
N4Ac-CTP/5-Methoxy-UTP
N4-acetyl-cytidine TP, ATP, GTP, UTP
N4-$_{BZ}$ -CTP/ 5-Methoxy-UTP
N4-methyl CTP
N4-Methyl-CTP/ 5-Methoxy-UTP
Pseudo-iso-CTP/ 5-Methoxy-UTP
PseudoU-alpha-thio-TP
pseudouridine TP, ATP, GTP, CTP
pseudo-UTP/5-methyl-CTP/ATP/GTP
UTP-5-oxyacetic acid Me ester
Xanthosine

[000764] According to the invention, polynucleotides of the invention may be synthesized to comprise the combinations or single modifications of Table 5.

[000765] Where a single modification is listed, the listed nucleoside or nucleotide represents 100 percent of that A, U, G or C nucleotide or nucleoside having been modified. Where percentages are listed, these represent the percentage of that particular A, U, G or C nucleobase triphosphate of the total amount of A, U, G, or C triphosphate present. For example, the combination: 25 % 5-Aminoallyl-CTP + 75 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP refers to a polynucleotide where 25% of the cytosine triphosphates are 5-Aminoallyl-CTP while 75% of the cytosines are CTP; whereas 25% of the uracils are 5-methoxy UTP while 75% of the uracils are UTP. Where no modified UTP is listed then the naturally occurring ATP, UTP, GTP and/or CTP is used at 100% of the sites of those nucleotides found in the polynucleotide. In this example all of the GTP and ATP nucleotides are left unmodified.

IV. Pharmaceutical Compositions

Formulation, Administration, Delivery and Dosing

[000766] The present invention provides polynucleotides compositions and complexes in combination with one or more pharmaceutically acceptable excipients. Pharmaceutical compositions may optionally comprise one or more additional active substances, e.g. therapeutically and/or prophylactically active substances. Pharmaceutical compositions of the present invention may be sterile and/or pyrogen-free. General considerations in the formulation and/or manufacture of pharmaceutical agents may be found, for example, in *Remington: The Science and Practice of Pharmacy* 21st ed., Lippincott Williams & Wilkins, 2005 (the contents of which is incorporated herein by reference in its entirety).

[000767] In some embodiments, compositions are administered to humans, human patients or subjects. For the purposes of the present disclosure, the phrase "active ingredient" generally refers to polynucleotides to be delivered as described herein.

[000768] Although the descriptions of pharmaceutical compositions provided herein are principally directed to pharmaceutical compositions which are suitable for administration to humans, it will be understood by the skilled artisan that such compositions are generally suitable for administration to any other animal, *e.g.,* to non-human animals, e.g. non-human mammals. Modification of pharmaceutical compositions suitable for administration to humans in order to render the compositions suitable for administration to various animals is well understood, and the ordinarily skilled veterinary pharmacologist can design and/or perform such modification with merely ordinary, if any, experimentation. Subjects to which administration of the pharmaceutical compositions is contemplated include, but are not limited to, humans and/or other primates; mammals, including commercially relevant mammals such as cattle, pigs, horses, sheep, cats, dogs, mice, and/or rats; and/or birds, including commercially relevant birds such as poultry, chickens, ducks, geese, and/or turkeys.

[000769] Formulations of the pharmaceutical compositions described herein may be prepared by any method known or hereafter developed in the art of pharmacology. In general, such preparatory methods include the step of bringing the active ingredient into association with an excipient and/or one or more other accessory ingredients, and then, if necessary and/or desirable, dividing, shaping and/or packaging the product into a desired single- or multi-dose unit.

[000770] Relative amounts of the active ingredient, the pharmaceutically acceptable excipient, and/or any additional ingredients in a pharmaceutical composition in accordance with the invention will vary, depending upon the identity, size, and/or condition of the subject treated and further depending upon the route by which the composition is to be administered. By way of example, the composition may comprise between 0.1% and 100%, e.g., between .5 and 50%, between 1-30%, between 5-80%, at least 80% (w/w) active ingredient.

Formulations

[000771] The polynucleotides of the invention can be formulated using one or more excipients to: (1) increase stability; (2) increase cell transfection; (3) permit the sustained or delayed release (e.g., from a depot formulation of the polynucleotide); (4) alter the biodistribution (e.g., target the polynucleotide to specific tissues or cell types); (5) increase the translation of encoded protein *in vivo;* and/or (6) alter the release profile of encoded protein in vivo. In addition to traditional excipients such as any and all solvents, dispersion media, diluents, or other liquid vehicles, dispersion or suspension aids, surface active agents, isotonic agents, thickening or emulsifying agents, preservatives, excipients of the present invention can include, without limitation, lipidoids, liposomes, lipid nanoparticles, polymers, lipoplexes, core-shell nanoparticles, peptides, proteins, cells transfected with polynucleotides (e.g., for transplantation

into a subject), hyaluronidase, nanoparticle mimics and combinations thereof. Accordingly, the formulations of the invention can include one or more excipients, each in an amount that together increases the stability of the polynucleotide, increases cell transfection by the polynucleotide, increases the expression of polynucleotides encoded protein, and/or alters the release profile of polynucleotide encoded proteins. Further, the polynucleotides of the present invention may be formulated using self-assembled nucleic acid nanoparticles.

[000772] Formulations of the pharmaceutical compositions described herein may be prepared by any method known or hereafter developed in the art of pharmacology. In general, such preparatory methods include the step of associating the active ingredient with an excipient and/or one or more other accessory ingredients.

[000773] A pharmaceutical composition in accordance with the present disclosure may be prepared, packaged, and/or sold in bulk, as a single unit dose, and/or as a plurality of single unit doses. As used herein, a "unit dose" refers to a discrete amount of the pharmaceutical composition comprising a predetermined amount of the active ingredient. The amount of the active ingredient is generally equal to the dosage of the active ingredient which would be administered to a subject and/or a convenient fraction of such a dosage such as, for example, one-half or one-third of such a dosage.

[000774] Relative amounts of the active ingredient, the pharmaceutically acceptable excipient, and/or any additional ingredients in a pharmaceutical composition in accordance with the present disclosure may vary, depending upon the identity, size, and/or condition of the subject being treated and further depending upon the route by which the composition is to be administered. For example, the composition may comprise between 0.1% and 99% (w/w) of the active ingredient. By way of example, the composition may comprise between 0.1% and 100%, e.g., between .5 and 50%, between 1-30%, between 5-80%, at least 80% (w/w) active ingredient.

[000775] In some embodiments, the formulations described herein may contain at least one polynucleotide. As a non-limiting example, the formulations may contain 1, 2, 3, **4** or 5 polynucleotides.

[000776] In one embodiment, the formulations described herein may comprise more than one type of polynucleotide. In one embodiment, the formulation may comprise a chimeric polynucleotide in linear and circular form. In another embodiment, the formulation may comprise a circular polynucleotide and an IVT polynucleotide. In yet another embodiment, the formulation may comprise an IVT polynucleotide, a chimeric polynucleotide and a circular polynucleotide.

[000777] In one embodiment the formulation may contain polynucleotide encoding proteins selected from categories such as, but not limited to, human proteins, veterinary proteins, bacterial proteins, biological proteins, antibodies, immunogenic proteins, therapeutic peptides and proteins, secreted proteins, plasma membrane proteins, cytoplasmic and cytoskeletal proteins, intracellular membrane bound proteins, nuclear proteins, proteins associated with human disease and/or proteins associated with non-human diseases. In one embodiment, the formulation contains at least three polynucleotides encoding proteins. In one embodiment, the formulation contains at least five polynucleotide encoding proteins.

[000778] Pharmaceutical formulations may additionally comprise a pharmaceutically acceptable excipient, which, as used herein, includes, but is not limited to, any and all solvents, dispersion media, diluents, or other liquid vehicles, dispersion or suspension aids, surface active agents, isotonic agents, thickening or emulsifying agents, preservatives, and the like, as suited to the particular dosage form desired. Various excipients for formulating pharmaceutical compositions and techniques for preparing the composition are known in the art (see Remington: The Science and Practice of Pharmacy, 21st Edition, A. R. Gennaro, Lippincott, Williams & Wilkins, Baltimore, MD, 2006; incorporated herein by reference in its entirety). The use of a conventional excipient medium may be contemplated within the scope of the present disclosure, except insofar as any conventional excipient medium may be incompatible with a substance or its derivatives, such as by producing any undesirable biological effect or otherwise interacting in a deleterious manner with any other component(s) of the pharmaceutical composition.

[000779] In one embodiment, the formulations of the polynucleotides described herein may also comprise a component such as, but not limited to, DLin-MC3-DMA lipid, cholesterol, PEG-DMG, DOPE, DSPC, Methoxy PEG-DSPC, Hydrogenated soy phospatidyl glycerol, sphingomyelin, DOPC, DPPC, dierucoylphophadtidylcholine (DEPC), tricaprylin (C8:0), triolein (C18:l), soybean oil, methoxy-PEG-40-carbonyl-distearoylphosphatidylethanolamine, L-dimyristoylphosphatidylcholine, L-dimyristoylphosphatidylglycerol, egg phosphatidylglycerol, MPEG5000 DPPE, DPPA (dipalmitoyl phosphatide), phosphatidylcholine, DPPG, LECIVA-S90 Ipurified PC from soy), LECIVA-S70 (pure phospholipid from soy lecithin), LIPOVA-E120 (purified egg lecithin USP), Egg lecithin, propylene glycol, glycerol, polysorbate 80, glutathione (reduced), butylated hydroxytoluene (BHA), ascorbyl palmitate, alpha-tocopherol, sodium carbonate, TRIS, histidine, calcium chloride, sodium phosphate, sodium citrate, ammonium sulfate, mannitol, sucrose, lactose, trehalose, disodium succinate hexahydrate and nitrogen.

[000780] In some embodiments, the particle size of the lipid nanoparticle may be increased and/or decreased. The change in particle size may be able to help counter biological reaction such as, but not limited to, inflammation or may increase the biological effect of the polynucleotides, such as modified mRNA, delivered to mammals.

[000781] Pharmaceutically acceptable excipients used in the manufacture of pharmaceutical compositions include, but are not limited to, inert diluents, surface active agents and/or emulsifiers, preservatives, buffering agents, lubricating agents, and/or oils. Such excipients may optionally be included in the pharmaceutical formulations of the invention.

[000782] Non-limiting examples of formulations and methods of delivery of polynucleotides are taught in International Pub. No. WO2013090648 (Attorney Docket No. M011.20), and International Pub. No. WO2014152211 (Attorney Docket No. M30.20), the contents of each of which are herein incorporated by reference in its entirety.

Lipidoids

[000783] The synthesis of lipidoids has been extensively described and formulations containing these compounds are particularly suited for delivery of polynucleotides (see Mahon et al, Bioconjug Chem. 2010 21:1448-1454; Schroeder et al., J Intern Med. 2010 267:9-21; Akinc et al, Nat Biotechnol. 2008 26:561-569; Love et al, Proc Natl Acad Sci U S A. 2010 107:1864-1869; Siegwart et al, Proc Natl Acad Sci U S A. 2011 108:12996-3001; all of which are incorporated herein in their entireties).

[000784] While these lipidoids have been used to effectively deliver double stranded small interfering RNA molecules in rodents and non-human primates (see Akinc et al, Nat Biotechnol. 2008 26:561-569; Frank-Kamenetsky et al, Proc Natl Acad Sci U S A. 2008 105:11915-11920; Akinc et al, Mol Ther. 2009 17:872-879; Love et al, Proc Natl Acad Sci U S A. 2010 107:1864-1869; Leuschner et al, Nat Biotechnol. 2011 29:1005-1010; all of which is incorporated herein in their entirety), the present disclosure describes their formulation and use in delivering polynucleotides.

[000785] Complexes, micelles, liposomes or particles can be prepared containing these lipidoids and therefore, can result in an effective delivery of the polynucleotide, as judged by the production of an encoded protein, following the injection of a lipidoid formulation via localized and/or systemic routes of administration. Lipidoid complexes of polynucleotides can be administered by various means including, but not limited to, intravenous, intramuscular, or subcutaneous routes.

[000786] *In vivo* delivery of nucleic acids may be affected by many parameters, including, but not limited to, the formulation composition, nature of particle PEGylation, degree of loading,

polynucleotide to lipid ratio, and biophysical parameters such as, but not limited to, particle size (Akinc et al, Mol Ther. 2009 17:872-879; herein incorporated by reference in its entirety). As an example, small changes in the anchor chain length of poly(ethylene glycol) (PEG) lipids may result in significant effects on in vivo efficacy. Formulations with the different lipidoids, including, but not limited to penta[3-(l-laurylaminopropionyl)]-triethylenetetramine hydrochloride (TETA-5LAP; aka 98N12-5, see Murugaiah et al, Analytical Biochemistry, 401:61 (2010); herein incorporated by reference in its entirety), C12-200 (including derivatives and variants), and MD1, can be tested for *in vivo* activity.

[000787] The lipidoid referred to herein as "98N12-5" is disclosed by Akinc et al., Mol Ther. 2009 17:872-879 and is incorporated by reference in its entirety.

[000788] The lipidoid referred to herein as "C12-200" is disclosed by Love et al, Proc Natl Acad Sci U S A. 2010 107:1864-1869 and Liu and Huang, Molecular Therapy. 2010 669-670; both of which are herein incorporated by reference in their entirety. The lipidoid formulations can include particles comprising either 3 or 4 or more components in addition to polynucleotides.

[000789] Lipidoids and polynucleotide formulations comprising lipidoids are described in International Patent Publication No. WO201415221 1 (Attorney Docket No. M030.20), the contents of which are herein incorporated by reference in its entirety, such as in paragraphs [000415] - [000422].

Liposomes, Lipoplexes, and Lipid Nanoparticies

[000790] The polynucleotides of the invention can be formulated using one or more liposomes, lipoplexes, or lipid nanoparticies. In one embodiment, pharmaceutical compositions of polynucleotides include liposomes. Liposomes are artificially-prepared vesicles which may primarily be composed of a lipid bilayer and may be used as a delivery vehicle for the administration of nutrients and pharmaceutical formulations. Liposomes can be of different sizes such as, but not limited to, a multilamellar vesicle (MLV) which may be hundreds of nanometers in diameter and may contain a series of concentric bilayers separated by narrow aqueous compartments, a small unicellular vesicle (SUV) which may be smaller than 50 nm in diameter, and a large unilamellar vesicle (LUV) which may be between 50 and 500 nm in diameter. Liposome design may include, but is not limited to, opsonins or ligands in order to improve the attachment of liposomes to unhealthy tissue or to activate events such as, but not limited to, endocytosis. Liposomes may contain a low or a high pH in order to improve the delivery of the pharmaceutical formulations.

[000791] The formation of liposomes may depend on the physicochemical characteristics such as, but not limited to, the pharmaceutical formulation entrapped and the liposomal ingredients ,

the nature of the medium in which the lipid vesicles are dispersed, the effective concentration of the entrapped substance and its potential toxicity, any additional processes involved during the application and/or delivery of the vesicles, the optimization size, polydispersity and the shelf-life of the vesicles for the intended application, and the batch-to-batch reproducibility and possibility of large-scale production of safe and efficient liposomal products.

[000792] As a non-limiting example, liposomes such as synthetic membrane vesicles may be prepared by the methods, apparatus and devices described in US Patent Publication No. US20130177638, US20130177637, US20130177636, US20130177635, US20130177634, US20130177633, US20130183375, US20130183373 and US20 1301 83372 and International Patent Publication No WO2008042973, the contents of each of which are herein incorporated by reference in its entirety.

[000793] In one embodiment, pharmaceutical compositions described herein may include, without limitation, liposomes such as those formed from l,2-dioleyloxy -*N*,*N*-dimethylaminopropane (DODMA) liposomes, DiLa2 liposomes from Marina Biotech (Bothell, WA), l,2-dilinoleyloxy-3-dimethylaminopropane (DLin-DMA), 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[l,3]-dioxolane (DLin-KC2-DMA), and MC3 (US20100324120; herein incorporated by reference in its entirety) and liposomes which may deliver small molecule drugs such as, but not limited to, DOXIL® from Janssen Biotech, Inc. (Horsham, PA).

[000794] In one embodiment, the polynucleotides of the invention may be formulated with liposomes comprising a lipid bilayer and a polymer-conjugated lipid, wherein said polymer-conjugated lipid, including but not limited to a glycosaminoglycan (GAG)-conjugated lipid, is incorporated into said lipid bilayer as described in and/or made by the methods of International Patent Publication No. WO2012153338, the contents of which are herein incorporated by reference in its entirety.

[000795] In one embodiment, pharmaceutical compositions described herein may include, without limitation, liposomes such as those formed from the synthesis of stabilized plasmid-lipid particles (SPLP) or stabilized nucleic acid lipid particle (SNALP) that have been previously described and shown to be suitable for oligonucleotide delivery *in vitro* and *in vivo* (see Wheeler et al. Gene Therapy. 1999 6:271-281; Zhang et al. Gene Therapy. 1999 6:1438-1447; Jeffs et al. Pharm Res. 2005 22:362-372; Morrissey et al, Nat Biotechnol. 2005 2:1002-1007; Zimmermann et al, Nature. 2006 441:1 11-114; Heyes et al. J Contr Rel. 2005 107:276-287; Semple et al. Nature Biotech. 2010 28:172-176; Judge et al. J Clin Invest. 2009 119:661-673; deFougerolles *Hum Gene Ther*. 2008 19:125-132; U.S. Patent Publication No US20130122104 and US20130303587; the contents of each of which are incorporated herein in their entireties). The

original manufacture method by Wheeler et al. was a detergent dialysis method, which was later improved by Jeffs et al. and is referred to as the spontaneous vesicle formation method. The liposome formulations are composed of 3 to 4 lipid components in addition to the polynucleotide. As an example a liposome can contain, but is not limited to, 55% cholesterol, 20% disteroylphosphatidyl choline (DSPC), 10% PEG-S-DSG, and 15% 1,2-dioleyloxy-*NN*-dimethylaminopropane (DODMA), as described by Jeffs et al. As another example, certain liposome formulations may contain, but are not limited to, 48% cholesterol, 20% DSPC, 2% PEG-c-DMA, and 30% cationic lipid, where the cationic lipid can be l,2-distearloxy -*N,N*-dimethylaminopropane (DSDMA), DODMA, DLin-DMA, or l,2-dilinolenyloxy-3-dimethylaminopropane (DLenDMA), as described by Heyes et al. the contents of which are herein incorporated by reference in its entirety.

[000796] In some embodiments, the polynucleotides of the invention may be formulated as components of SNALP particles described in and made by the methods of US Patent Publication No. 20140065228, the contents of which is herein incorporated by reference in its entirety, further comprising: a cationic lipid comprising from 50 mol % to 65 mol % of the total lipid present in the particle; a non-cationic lipid comprising up to 49.5 mol % of the total lipid present in the particle and comprising a mixture of a phospholipid and cholesterol or a derivative thereof, wherein the cholesterol or derivative thereof comprises from 30 mol % to 40 mol % of the total lipid present in the particle; and a conjugated lipid that inhibits aggregation of particles comprising from 0.5 mol % to 2 mol % of the total lipid present in the particle.

[000797] In some embodiments, liposome formulations may comprise from about about 25.0% cholesterol to about 40.0% cholesterol, from about 30.0% cholesterol to about 45.0% cholesterol, from about 35.0% cholesterol to about 50.0% cholesterol and/or from about 48.5% cholesterol to about 60% cholesterol. In a preferred embodiment, formulations may comprise a percentage of cholesterol selected from the group consisting of 28.5%, 31.5%, 33.5%, 36.5%, 37.0%, 38.5%, 39.0% and 43.5%. In some embodiments, formulations may comprise from about 5.0% to about 10.0% DSPC and/or from about 7.0% to about 15.0% DSPC.

[000798] In one embodiment, pharmaceutical compositions may include liposomes which may be formed to deliver polynucleotides which may encode at least one immunogen or any other polypeptide of interest. The polynucleotide may be encapsulated by the liposome and/or it may be contained in an aqueous core which may then be encapsulated by the liposome (see International Pub. Nos. WO2012031046, WO2012031043, WO2012030901 and WO2012006378 and US Patent Publication No. US20130189351, US20130195969 and

US20 13 0202684; the contents of each of which are herein incorporated by reference in their entirety).

[000799] In one embodiment, the pharmaceutical compositions may include liposomes comprising liposomal shells consisting of distearoyl phosphocholine (DSPC) and distearoyl phosphatidyl ethanolamine-m-poly ethylene glycol (DSPE-m-PEG) as described in International Patent Publication No. WO20 14054026, the contents of which are incorporated herein by reference in its entirety.

[000800] In another embodiment, liposomes may be formulated for targeted delivery. As a non-limiting example, the liposome may be formulated for targeted delivery to the liver. The liposome used for targeted delivery may include, but is not limited to, the liposomes described in and methods of making liposomes described in US Patent Publication No. US20130195967, the contents of which are herein incorporated by reference in its entirety. In a non-limiting example, according to US Patent Publication No. US20130195967, the polynucleotides may be formulated in a liposome that further comprises a polyamine; and a lipid component; wherein the lipid component comprises a neutral phospholipid and essentially no cationic lipid, and wherein the polynucleotide and the lipid component are present at certain ratios as described in US Patent Publication No. US20 1301 95967, the contents of which are herein incorporated by reference in its entirety, and wherein the liposome is from 30 to 500 nanometers in diameter.

[000801] In one embodiment, the polynucleotides of the invention may be formulated in liposomes described in and made by the methods of US Patent Application No. 20140065204, the contents of which is herein incorporated by reference in its entirety.

[000802] In one embodiment, the polynucleotides of the invention may be formulated in liposomal vaccine compositions, for example to stimulate an immune response, according to the compositions and methods of International Patent Publication No. WO20 12 149045, the contents of which are herein incorporated by reference in its entirety.

[000803] In another embodiment, the polynucleotide which may encode an immunogen may be formulated in a cationic oil-in-water emulsion where the emulsion particle comprises an oil core and a cationic lipid which can interact with the polynucleotide anchoring the molecule to the emulsion particle (see International Pub. No. WO2012006380; herein incorporated by reference in its entirety).

[000804] In one embodiment, the polynucleotides may be formulated in a water-in-oil emulsion comprising a continuous hydrophobic phase in which the hydrophilic phase is dispersed. As a non-limiting example, the emulsion may be made by the methods described in International Publication No. WO20 1087791, herein incorporated by reference in its entirety.

[000805] In another embodiment, the lipid formulation may include at least cationic lipid, a lipid which may enhance transfection and a least one lipid which contains a hydrophilic head group linked to a lipid moiety (International Pub. No. WO201 1076807 and U.S. Pub. No. 201 10200582; the contents of each of which is herein incorporated by reference in their entirety). In another embodiment, the polynucleotides encoding an immunogen may be formulated in a lipid vesicle which may have crosslinks between functionalized lipid bilayers (see U.S. Pub. No. 20120177724, the contents of which is herein incorporated by reference in its entirety).

[000806] In one embodiment, the polynucleotides may be formulated in a liposome as described in International Patent Publication No. WO20 13086526, herein incorporated by reference in its entirety. The polynucleotides may be encapsulated in a liposome using reverse pH gradients and/or optimized internal buffer compositions as described in International Patent Publication No. WO20 13 086526, herein incorporated by reference in its entirety.

[000807] In one embodiment, the polynucleotides may be delivered in a liposome comprising an ionizable lipid. As a non-limiting example, the ionizable lipid may be any of the formulas of ionizable lipids described in International Patent Publication No. WO2013149140 and US Patent Publication No. US20130330401, the contents of each of which are herein incorporated by reference in their entirety.

[000808] In one embodiment, the polynucleotides may be administered using the nucleic acid based therapy methods described in International Publication No. WO2008042973 and US Patent No. US8642076, the contents of each of which are herein incorporated by reference in its entirety. As a non-limiting example, the polynucleotides may be administered by association complexes such as liposomes and lipoplexes as described in International Publication No. WO2008042973, the contents of which are herein incorporated by reference in its entirety. As another non-limiting example, the liposomes or lipoplexes may include a polyamine compound or a lipid moiety described in International Publication No. WO2008042973 and US Patent No. US8642076, the contents of each of which are herein incorporated by reference in its entirety. As yet another non-limiting example, the liposomes or lipoplexes may include a polyamine compound or a lipid moiety described by formula (XV) in US Patent No. US8642076, the contents of which are herein incorporated by reference in its entirety.

[000809] In one embodiment, the pharmaceutical compositions may be formulated in liposomes such as, but not limited to, DiLa2 liposomes (Marina Biotech, Bothell, WA), SMARTICLES® (Marina Biotech, Bothell, WA), neutral DOPC (l,2-dioleoyl-sn-glycero-3-phosphocholine) based liposomes (e.g., siRNA delivery for ovarian cancer (Landen et al. Cancer

Biology & Therapy 2006 5(12)1708-1713); herein incorporated by reference in its entirety) and hyaluronan-coated liposomes (Quiet Therapeutics, Israel).

[000810] In one embodiment, the polynucleotides may be formulated in a liposome which can target the $\alpha_v\beta_3$ integrin receptor such as, but not limited to, the liposomes described in European Patent No. EP 1404860, the contents of which are herein incorporated by reference in its entirety.

[000811] In one embodiment, the polynucleotides may be formulated in amphoteric liposomes such as, but not limited to, the liposomes comprising amphoteric lipids described in US Patent No. US8580297, the contents of which are herein incorporated by reference in its entirety. Non-limiting examples of amphoteric liposomes and methods to make amphoteric liposomes are also described in US Patent Publication No. 20140056970, the contents of which is herein incorporated by reference in its entirety.

[000812] In one embodiment, the liposomes for formulation and/or delivery of the polynucleotides may be made using the apparatus and/or methods described in US Patent Publication No. US20 140044772, the contents of which are herein incorporated by reference in its entirety. As a non-limiting example, the method may include providing a buffer solution in a first reservoir and a lipid solution in a second reservoir and continuously diluting the lipid solution with the buffer solution in a mixing chamber until a liposome is produced. The lipid solution may also comprise an organic solvent such as, but not limited to, a lower alkanol (see e.g., the method described by Maclachlan et al. in US Patent Publication No. US20 140044772, the contents of which are herein incorporated by reference in its entirety).

[000813] In one embodiment, the liposomes for formulation and/or delivery of the polynucleotides may be internal structured self assembled liposomes (ISSALs). As a non-limiting example, the ISSAL may be any of the ISSALs described in International Patent Publication No. WO2014026284, the contents of which are herein incorporated by reference in its entirety. In one embodiment, the ISSAL comprises a nuclear core molecule or complex comprising a first affinity enhance molecule and a liposome encompassing the nuclear core molecule or complex (see e.g., International Patent Publication No. WO2014026284, the contents of which are herein incorporated by reference in its entirety).

[000814] In one embodiment, the polynucleotides of the invention may be in a formulation comprising delivery system complexes that include a nano-precipitaed bioactive compound encapsulated by a liposome, as described in International Patent Publication WO2014052634, the contents of which are incorporated by reference in its entirety.

[000815] In one embodiment, the cationic lipid may be a low molecular weight cationic lipid such as those described in US Patent Application Nos. 20130090372, 20130274504 and 20130274523, the contents of each of which are herein incorporated by reference in its entirety.

[000816] In one embodiment, the polynucleotides may be formulated in a lipid vesicle which may have crosslinks between functionalized lipid bilayers.

[000817] In one embodiment, the polynucleotides may be formulated in a liposome comprising a cationic lipid. The liposome may have a molar ratio of nitrogen atoms in the cationic lipid to the phophates in the RNA (N:P ratio) of between 1:1 and 20:1 as described in International Publication No. WO20 13 006825, herein incorporated by reference in its entirety. In another embodiment, the liposome may have a N:P ratio of greater than 20: 1 or less than 1:1.

[000818] In one embodiment, the polynucleotides may be formulated in a lipid-polycation complex. The formation of the lipid-polycation complex may be accomplished by methods known in the art and/or as described in U.S. Pub. No. 20120178702, herein incorporated by reference in its entirety. As a non-limiting example, the polycation may include a cationic peptide or a polypeptide such as, but not limited to, polylysine, polyornithine and/or polyarginine and the cationic peptides described in International Pub. No. WO2012013326 or US Patent Pub. No. US20130142818; each of which is herein incorporated by reference in its entirety. In another embodiment, the polynucleotides may be formulated in a lipid-polycation complex which may further include a neutral lipid such as, but not limited to, cholesterol or dioleoyl phosphatidylethanolamine (DOPE).

[000819] In one embodiment, the polynucleotide may be formulated in an aminoalcohol lipidoid. Aminoalcohol lipidoids which may be used in the present invention may be prepared by the methods described in U.S. Patent No. 8,450,298, herein incorporated by reference in its entirety.

[000820] The liposome formulation may be influenced by, but not limited to, the selection of the cationic lipid component, the degree of cationic lipid saturation, the nature of the PEGylation, ratio of all components and biophysical parameters such as size. In one example by Semple et al. (Semple et al. Nature Biotech. 2010 28:172-176; herein incorporated by reference in its entirety), the liposome formulation was composed of 57.1 % cationic lipid, 7.1% dipalmitoylphosphatidylcholine, 34.3 % cholesterol, and 1.4% PEG-c-DMA. As another example, changing the composition of the cationic lipid could more effectively deliver siRNA to various antigen presenting cells (Basha et al. Mol Ther. 201 1 19:2186-2200; herein incorporated by reference in its entirety). In some embodiments, liposome formulations may comprise from about 35 to about 45% cationic lipid, from about 40% to about 50% cationic lipid, from about

50% to about 60% cationic lipid and/or from about 55% to about 65% cationic lipid. In some embodiments, the ratio of lipid to mRNA in liposomes may be from about about 5:1 to about 20:1, from about 10:1 to about 25:1, from about 15:1 to about 30:1 and/or at least 30:1.

[000821] In some embodiments, the ratio of PEG in the lipid nanoparticle (LNP) formulations may be increased or decreased and/or the carbon chain length of the PEG lipid may be modified from C14 to C18 to alter the pharmacokinetics and/or biodistribution of the LNP formulations. As a non-limiting example, LNP formulations may contain from about 0.5% to about 3.0%, from about 1.0% to about 3.5%, from about 1.5% to about 4.0%, from about 2.0% to about 4.5%, from about 2.5% to about 5.0% and/or from about 3.0% to about 6.0% of the lipid molar ratio of PEG-c-DOMG as compared to the cationic lipid, DSPC and cholesterol. In another embodiment the PEG-c-DOMG may be replaced with a PEG lipid such as, but not limited to, PEG- DSG (1,2-Distearoyl-sn-glycerol, methoxypolyethylene glycol), PEG-DMG (1,2-Dimyristoyl-sn-glycerol) and/or PEG-DPG (1,2-Dipalmitoyl-sn-glycerol, methoxypolyethylene glycol). The cationic lipid may be selected from any lipid known in the art such as, but not limited to, DLin-MC3-DMA, DLin-DMA, C12-200 and DLin-KC2-DMA.

[000822] In one embodiment, the polynucleotides may be formulated in a lipid nanoparticle that comprises at least one lipid, and at least one albumin-polymer conjugate (APC), wherein the polymer comprises at least one positively -charged polymer, as described in International Publication No. WO2012 170930, the contents of which are incorporated herein by reference in its entirety.

[000823] In one embodiment, the polynucleotides may be formulated in a lipid nanoparticle such as those described in or made by the method of International Patent Publication No. WO2013 177421, the contents of which are incorporated herein by reference in its entirety.

[000824] In one embodiment, the lipid nanoparticles described herein may comprise a cationic lipid, a non-cationic lipid, cholesterol and a PEG lipid. The components of the lipid nanoparticle may be tailored for optimal delivery of the polynucleotides based on the delivery route and the desired outcome. As a non-limiting example, the lipid nanoparticle may comprise 40-60% cationic lipid, 8-16% non-cationic lipid, 30-45% cholesterol and 1-5% PEG lipid. As another non limiting example, the lipid nanoparticle may comprise 50% cationic lipid, 10% non-cationic lipid, 38.5% cholesterol and 1.5% PEG lipid. As yet another non-limiting example, the 40-60%, cationic lipid may be DODMA, DLin-KC2-DMA or DLin-MC3 -DMA, the 8-15% non-cationic lipid may be DSPC or DOPE and the 1-5% PEG lipid may be PEG 2000-DMG or anionic mPEG-DSPC and the lipid nanoparticle may comprise 30-45% cholesterol. The lipid nanoparticle may further comprise a buffer such as, but not limited to, citrate or phosphate at a

pH of 7, salt and/or sugar. Salt and/or sugar may be included in the formulations described herein for isotonicity.

[000825] In one embodiment, the lipid nanoparticles described herein may comprise polynucleotides (e.g., mRNA) in a lipid:mRNA weight ratio of 5:1, 10:1, 15:1, 20:1, 25:1, 30:1, 35: 1, 40: 1, 45: 1 or 50: 1. As a non-limiting example, the lipid nanoparticle described herein may comprise mRNA in a lipid:mRNA weight ratio of 20:1. As another non-limiting example, the lipid nanoparticle comprises 40-60% cationic lipid (e.g., DODMA, DLin-KC2-DMA or DLin-MC3-DMA), 8-15% non-cationic lipid (e.g., DSPC or DOPE), 30-45% cholesterol and 1-5% PEG lipid (e.g., PEG 2000-DMG or anionic mPEG-DSPC). As yet another non-limiting example, the lipid nanoparticle comprises 50% cationic lipid (e.g., DODMA, DLin-KC2-DMA or DLin-MC3-DMA), 10% non-cationic lipid (e.g., DSPC or DOPE), 38.5% cholesterol and 1.5% PEG lipid (e.g., PEG 2000-DMG).

[000826] In one embodiment, formulations comprising the polynucleotides and lipid nanoparticles described herein may comprise 0.15 mg/ml to 2 mg/ml of the polynucleotide described herein (e.g., mRNA), 50% cationic lipid (e.g., DLin-MC3 -DMA), 38.5% Cholesterol, 10% non-cationic lipid (e.g., DSPC), 1.5% PEG lipid (e.g., PEG-2K-DMG), 10 mM of citrate buffer and the formulation may additionally comprise 9% w/w of sucrose.

[000827] In one embodiment, the lipid nanoparticles described herein may comprise a PEG lipid which is a non-diffusible PEG. Non-limiting examples of non-diffusible PEGs include PEG-DSG and PEG-DSPE. As a non-limiting example, the lipid nanoparticle comprising the PEG lipid comprises 40-60% cationic lipid (e.g., DODMA, DLin-KC2-DMA or DLin-MC3-DMA), 8-15% non-cationic lipid (e.g., DSPC or DOPE), 30-45% cholesterol and 0.5-10% PEG lipid (e.g., PEG-DSG or PEG-DSPE). As another non-limiting example, the lipid nanoparticle comprising the PEG lipid comprises 50% cationic lipid (e.g., DODMA, DLin-KC2-DMA or DLin-MC3 -DMA), 10% non-cationic lipid (e.g., DSPC or DOPE), 39.5%, 38.5%, 35% or 30% cholesterol and 0.5%, 1.5%, 5% or 10% PEG lipid (e.g., PEG-DSG or PEG-DSPE).

[000828] In one embodiment, the lipid nanoparticles described herein may comprise 50% DLin-KC2-DMA, 10% DSPC, 39.5% cholesterol and 0.5% PEG-DSG. In one embodiment, the lipid nanoparticles described herein may comprise 50%> DLin-KC2-DMA, 10%> DSPC, 39.5% cholesterol and 0.5% PEG-DSPE.

[000829] In one embodiment, the lipid nanoparticles described herein may comprise 50% DLin-KC2-DMA, 10% DSPC, 38.5% cholesterol and 1.5% PEG-DSG. In one embodiment, the lipid nanoparticles described herein may comprise 50%> DLin-KC2-DMA, 10%> DSPC, 38.5% cholesterol and 1.5% PEG-DSPE.

[000830] In one embodiment, the lipid nanoparticles described herein may comprise 50% DLin-KC2-DMA, 10% DSPC, 35% cholesterol and 5% PEG-DSG. In one embodiment, the lipid nanoparticles described herein may comprise 50% DLin-KC2-DMA, 10% DSPC, 35% cholesterol and 5% PEG-DSPE.

[000831] In one embodiment, the lipid nanoparticles described herein may comprise 50% DLin-KC2-DMA, 10% DSPC, 39.5% cholesterol and 0.5% PEG-DSG. In one embodiment, the lipid nanoparticles described herein may comprise 50% DLin-KC2-DMA, 10% DSPC, 30% cholesterol and 10% PEG-DSPE.

[000832] In one embodiment, the lipid nanoparticles described herein may comprise the polynucleotides described herein in a concentration from approximately 0.1 mg/ml to 2 mg/ml such as, but not limited to, 0.1 mg/ml, 0.2 mg/ml, 0.3 mg/ml, 0.4 mg/ml, 0.5 mg/ml, 0.6 mg/ml, 0.7 mg/ml, 0.8 mg/ml, 0.9 mg/ml, 1.0 mg/ml, 1.1 mg/ml, 1.2 mg/ml, 1.3 mg/ml, 1.4 mg/ml, 1.5 mg/ml, 1.6 mg/ml, 1.7 mg/ml, 1.8 mg/ml, 1.9 mg/ml, 2.0 mg/ml or greater than 2.0 mg/ml.

[000833] In one embodiment, the lipid nanoparticles described herein may be lyophilized in order to improve storage stability of the formulation and/or polynucleotides.

[000834] In one embodiment, the polynucleotides may be formulated in a lipid nanoparticle such as those described in International Publication No. WO2012170930, herein incorporated by reference in its entirety.

[000835] In one embodiment, the formulation comprising the polynucleotide is a nanoparticle which may comprise at least one lipid. The lipid may be selected from, but is not limited to, DLin-DMA, DLin-K-DMA, 98N12-5, C12-200, DLin-MC3-DMA, DLin-KC2-DMA, DODMA, PLGA, PEG, PEG-DMG, PEGylated lipids and amino alcohol lipids. In another aspect, the lipid may be a cationic lipid such as, but not limited to, DLin-DMA, DLin-D-DMA, DLin-MC3-DMA, DLin-KC2-DMA, DODMA and amino alcohol lipids. The amino alcohol cationic lipid may be the lipids described in and/or made by the methods described in US Patent Publication No. US20130150625, herein incorporated by reference in its entirety. As a non-limiting example, the cationic lipid may be 2-amino-3-[(9Z,12Z)-octadeca-9,12-dien-l-yloxy]-2-{[(9Z,2Z)-octadeca-9,12-dien-l-yloxy]methyl}propan-l-ol (Compound 1 in US20130150625); 2-amino-3-[(9Z)-octadec-9-en-l-yloxy]-2-{[(9Z)-octadec-9-en-l-yloxy]methyl}propan-l-ol (Compound 2 in US20130150625); 2-amino-3-[(9Z,12Z)-octadeca-9,12-dien-l-yloxy]-2-[(octyloxy)methyl]propan-l-ol (Compound 3 in US20130150625); and 2-(dimethylamino)-3-[(9Z, 12Z)-octadeca-9, 12-dien-l -yloxy]-2- {[(9Z, 12Z)-octadeca-9, 12-dien- 1-yloxy]methyl}propan-l-ol (Compound 4 in US20130150625); or any pharmaceutically acceptable salt or stereoisomer thereof.

[000836] In one embodiment, the cationic lipid may be selected from, but not limited to, a cationic lipid described paragraph [000444] in International Publication No. WO2015038892, the contents of which are herein incorporated by reference in its entirety.

[000837] In one embodiment, the lipid may be a cleavable lipid such as those described in International Publication No. WO2012 170889, herein incorporated by reference in its entirety.

[000838] In another embodiment, the lipid may be a cationic lipid such as, but not limited to, Formula (I) of U.S. Patent Application No. US20130064894, the contents of which are herein incorporated by reference in its entirety.

[000839] In one embodiment, the cationic lipid may be synthesized by methods known in the art and/or as described in International Publication Nos. WO2012040184, WO201 1153 120, WO201 1149733, WO201 1090965, WO201 1043913, WO201 1022460, WO2012061259, WO2012054365, WO2012044638, WO2010080724, WO201021865, WO2013086373 and WO2013086354; the contents of each of which are herein incorporated by reference in their entirety.

[000840] In another embodiment, the cationic lipid may be a trialkyl cationic lipid. Non-limiting examples of trialkyl cationic lipids and methods of making and using the trialkyl cationic lipids are described in International Patent Publication No. WO2013 126803, the contents of which are herein incorporated by reference in its entirety.

[000841] In one embodiment, the cationic lipid may have a positively charged hydrophilic head and a hydrophobic tail that are connected via a linker structure. As a non-limiting example, the hydrophilic head group may be primary, secondary, tertiary amines or quaternary ammonium salts. As another non-limiting example, the lipids may have guanidino, imidazole, pyridinium, phosphorus, and arsenic groups.

[000842] In one embodiment, the lipid or lipids which may be used in the formulation and/or delivery of polynucleotides described herein may be, but is not limited to, 1,2-Dioleoyl-sn-glycero-3 -phosphatidylcholine (DOPC), l,2-Dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE), cholesterol, *N*-[l-(2,3-Dioleyloxy)propyl]*N*,*N*,*N*-trimethylammonium chloride (DOTMA), l,2-Dioleoyloxy-3-trimethylammonium-propane (DOTAP), Dioctadecylamidoglycylspermine (DOGS), *N*-(3-Aminopropyl)-*N*,*N*-dimethyl-2,3-bis(dodecyloxy)- 1-propanaminium bromide (GAP-DLRIE), cetyltrimethylammonium bromide (CTAB), 6-lauroxyhexyl ornithinate (LHON), l-)2,3-Dioleoloxypropyl)2,4,6-trimethylpyridinium (20c), 2,3-Dioleyloxy -*N*-[2(sperminecarboxamido)-ehtyl]-*N*,*N*-dimethyl- 1-propanaminium trifluoroacetate (DOSPA), l,2-Dioleyl-3-trimethylammonium-propane (DOPA), *N*-(2-Hydroxyethyl)-*N*,*N*-dimethyl-2,3 -bis(tetradecyloxy)- 1-propanaminium bromide (MDRIE),

Dimyristooxypropyl dimethyl hydroxyethyl ammonium bromide (DMRI), 3β-*[N-(N',N'*-Dimethylaminoethane)-carbamoyl]cholesterol (DC-Choi), Bis-guanidium-tren-cholesterol (BGTC), l,3-Dioleoxy-2-(6-carboxy-spermyl)-propylamide (DOSPER), Dimethyloctadecylammonium bromide (DDAB), Dioctadecylamidoglicylspermidin (DSL), rac-[(2,3-Dioctadecyloxypropyl)(2-hydroxyethyl)]-dimethylammonium chloride (CLIP-1), rac-[2(2,3-Dihexadecyloxypropyl-oxymethyloxy)ehtyl]trimethylammonium chloride (CLIP-6), Ethyldimyrisotylphosphatidylcholine (EDMPC), l,2-Distearyloxy -*N*,*N*-dimethyl-3-aminopropane (DSDMA), 1,2-Dimyristoyl-trimethylammoniumpropane (DMTAP), O,O'-Dimyristyl-*N*-lysyl asparate (DMKE), l,2-Distearoyl-sn-glycero-3-ethylphosphocholine (DSEPC), *N*-Palmitoyl-D-erythro-spingosyl carbamoyl-spermine (CCS), *N*-i-Butyl-*No*-tetradecyl-3-tetradecylaminopropionamidine (diC 14-amidine), Octadecenolyoxy[ethyl-2-heptadecenyl-3 hydroxyethyl] imidazolinium chloride (DOTIM), *N*1-Cholesteryloxycarbonyl-3,7-diazanonane-l,9-diamine (CDAN) and 2-(3-[Bis-(3-amino-propyl)-amino]propylamino)-N-ditetradecylcarbamoylme-ethyl-acetamide (RPR209 1290).

[000843] In one embodiment, the cationic lipid which may be used in the formulations and delivery agents described herein may be represented by formula (I) in US Patent Publication No. US20 140039032, the contents of which are herein incorporated by reference in its entirety. As a non-limiting example, the cationic lipid having formula (I) in US Patent Publication No. US20 140039032 may be used in a lipid nanoparticle to deliver nucleic acid molecules (e.g., polynucleotides described herein).

[000844] In one embodiment, the lipids which may be used in the formulations and/or delivery of the polynucleotides described herein may be a cleavable lipid. As a non-limiting example, the cleavable lipid and/or pharmaceutical compositions comprising cleavable lipids may be those described in International Patent Publication No. WO2012 170889, the contents of which are herein incorporated by reference in its entirety. As another non-limiting example, the cleavable lipid may be HGT4001, HGT4002, HGT4003, HGT4004 and/or HGT4005 as described in International Patent Publication No. WO2012170889, the contents of which are herein incorporated by reference in its entirety.

[000845] In one embodiment, the polymers which may be used in the formulation and/or delivery of polynucleotides described herein may be, but is not limited to, poly(ethylene)glycol (PEG), polyethylenimine (PEI), dithiobis(succinimidylpropionate) (DSP), Dimethyl-3,3'-dithiobispropionimidate (DTBP), poly(ethylene imine) biscarbamate (PEIC), poly(L-lysine) (PLL), histidine modified PLL, poly(*N*-vinylpyrrolidone) (PVP), poly(propylenimine (PPI), poly(amidoamine) (PAMAM), poly(amido ethylenimine) (SS-PAEI), triehtylenetetramine

(TETA), poly(β-aminoester), poly(4-hydroxy-L-proine ester) (PHP), poly(allylamine), poly(a-[4-aminobutyl]-L-glycolic acid (PAGA), Poly(D,L-lactic-co-glycolid acid (PLGA), Poly(*N*-ethyl-4-vinylpyridinium bromide), poly(phosphazene)s (PPZ), poly(phosphoester)s (PPE), poly(phosphoramidate)s (PPA), poly(*N*-2-hydroxypropylmethacrylamide) (pHPMA), poly(2-(dimethylamino) ethyl methacrylate) (pDMAEMA), poly(2-aminoethyl propylene phosphate) PPE_EA), Chitsoan, galactosylated chitosan, *N*-dodecylated chitosan, histone, collagen and dextran-spermine. In one embodiment, the polymer may be an inert polymer such as, but not limited to, PEG. In one embodiment, the polymer may be a cationic polymer such as, but not limited to, PEI, PLL, TETA, poly(allylamine), Poly(*N*-ethyl-4-vinylpyridinium bromide), pHPMA and pDMAEMA. In one embodiment, the polymer may be a biodegradable PEI such as, but not limited to, DSP, DTBP and PEIC. In one embodiment, the polymer may be biodegradable such as, but not limited to, histine modified PLL, SS-PAEI, poly(β-aminoester), PHP, PAGA, PLGA, PPZ, PPE, PPA and PPE-EA.

[000846] In one embodiment, the LNP formulations of the polynucleotides may contain PEG-c-DOMG at 3% lipid molar ratio. In another embodiment, the LNP formulations polynucleotides may contain PEG-c-DOMG at 1.5% lipid molar ratio.

[000847] In one embodiment, the pharmaceutical compositions of the polynucleotides may include at least one of the PEGylated lipids described in International Publication No. WO20 12099755, herein incorporated by reference.

[000848] In one embodiment, the LNP formulation may contain PEG-DMG 2000 (1,2-dimyristoyl-sn-glycero-3-phophoethanolamine-N-[methoxy(polyethylene glycol)-2000). In one embodiment, the LNP formulation may contain PEG-DMG 2000, a cationic lipid known in the art and at least one other component. In another embodiment, the LNP formulation may contain PEG-DMG 2000, a cationic lipid known in the art, DSPC and cholesterol. As a non-limiting example, the LNP formulation may contain PEG-DMG 2000, DLin-DMA, DSPC and cholesterol. As another non-limiting example the LNP formulation may contain PEG-DMG 2000, DLin-DMA, DSPC and cholesterol in a molar ratio of 2:40:10:48 (see e.g., Geall et al, Nonviral delivery of self-amplifying RNA vaccines, PNAS 2012; PMID: 22908294; herein incorporated by reference in its entirety).

[000849] In one embodiment, the LNP formulation may be formulated by the methods described in International Publication Nos. WO201 1127255 or WO2008 103276, the contents of each of which is herein incorporated by reference in their entirety. As a non-limiting example, the polynucleotides described herein may be encapsulated in LNP formulations as described in WO201 1127255 and/or WO2008103276; each of which is herein incorporated by reference in

their entirety. As another non-limiting example, polynucleotides described herein may be formulated in a nanoparticle to be delivered by a parenteral route as described in U.S. Pub. No. 20120207845 and International Publication No. WO2014008334; the contents of each of which are herein incorporated by reference in its entirety.

[000850] In one embodiment, the polynucleotides described herein may be formulated in a nanoparticle to be delivered by a parenteral route as described in U.S. Pub. No. US20 120207845; the contents of which are herein incorporated by reference in its entirety.

[000851] In one embodiment, the polynucleotides may be formulated in a lipid nanoparticle made by the methods described in US Patent Publication No US20130156845 or International Publication No WO2013093648 or WO2012024526, each of which is herein incorporated by reference in its entirety.

[000852] The lipid nanoparticles described herein may be made in a sterile environment by the system and/or methods described in US Patent Publication No. US20 1301 64400, herein incorporated by reference in its entirety.

[000853] In one embodiment, the lipid nanoparticles which may be used to deliver the polynucleotides described herein may be Particle Replication in Non-wetting Templates (PRINT) nanoparticles as described by Morton et al. (Scalable Manufacture of Built-to-Order Nanomedicine: Spray Assisted Layer-by-layer Functionalization of PRINT nanoparticles, *Adv. Mat.* 2013, 25, 4707-4713; the contents of which is herein incorporated by reference in its entirety). The PRINT nanoparticles may be manufactured by the methods outlined by Morton et al. in order to generate uniform nanoparticles which may have a desired composition, size, shape and surface functionality. As a non-limiting example, the polynucleotides described herein may be formulated in PRINT nanoparticles. As another non-limiting example, the polynucleotides may be formulated in PRINT nanoparticles for targeted interaction with cancer cells.

[000854] In one embodiment, the LNP formulation may be formulated in a nanoparticle such as a nucleic acid-lipid particle described in US Patent No. 8,492,359, the contents of which are herein incorporated by reference in its entirety. As a non-limiting example, the lipid particle may comprise one or more active agents or therapeutic agents; one or more cationic lipids comprising from about 50 mol % to about 85 mol % of the total lipid present in the particle; one or more non-cationic lipids comprising from about 13 mol % to about 49.5 mol % of the total lipid present in the particle; and one or more conjugated lipids that inhibit aggregation of particles comprising from about 0.5 mol % to about 2 mol % of the total lipid present in the particle. The nucleic acid in the nanoparticle may be the polynucleotides described herein and/or are known in the art.

[000855] In one embodiment, the lipid nanoparticle may comprise a lipidoid prepare by conjugate addition of alklamines to acrylates as described in International Patent Publication No. WO20 14028487, the contents of which are herein incorporated by reference in its entirety.

[000856] In one embodiment, the LNP formulation may be formulated by the methods described in International Publication Nos. WO201 1127255 or WO2008 103276, the contents of each of which are herein incorporated by reference in their entirety. As a non-limiting example, modified RNA described herein may be encapsulated in LNP formulations as described in WO201 1127255 and/or WO2008103276; the contents of each of which are herein incorporated by reference in their entirety.

[000857] In one embodiment, LNP formulations described herein may comprise a polycationic composition. As a non-limiting example, the polycationic composition may be selected from formula 1-60 of US Patent Publication No. US20050222064; the content of which is herein incorporated by reference in its entirety. In another embodiment, the LNP formulations comprising a polycationic composition may be used for the delivery of the modified RNA described herein *in vivo* and/or *in vitro.*

[000858] In one embodiment, the LNP formulations described herein may additionally comprise a permeability enhancer molecule. Non-limiting permeability enhancer molecules are described in US Patent Publication No. US20050222064; the content of which is herein incorporated by reference in its entirety.

[000859] In one embodiment, the pharmaceutical compositions may be formulated in liposomes such as, but not limited to, DiLa2 liposomes (Marina Biotech, Bothell, WA), SMARTICLES® (Marina Biotech, Bothell, WA), neutral DOPC (l,2-dioleoyl-sn-glycero-3-phosphocholine) based liposomes (e.g., siRNA delivery for ovarian cancer (Landen et al. Cancer Biology & Therapy 2006 5(12)1708-1713); herein incorporated by reference in its entirety) and hyaluronan-coated liposomes (Quiet Therapeutics, Israel).

[000860] In one embodiment, the polynucleotides may be formulated in a lyophilized gel-phase liposomal composition as described in US Publication No. US2012060293, herein incorporated by reference in its entirety.

[000861] The nanoparticle formulations may comprise a phosphate conjugate. The phosphate conjugate may increase *in vivo* circulation times and/or increase the targeted delivery of the nanoparticle. Phosphate conjugates for use with the present invention may be made by the methods described in International Application No. WO2013033438 or US Patent Publication No. US20130196948, the contents of each of which are herein incorporated by reference in its entirety. As a non-limiting example, the phosphate conjugates may include a compound of any

one of the formulas described in International Application No. WO2013033438, herein incorporated by reference in its entirety.

[000862] The nanoparticle formulation may comprise a polymer conjugate. The polymer conjugate may be a water soluble conjugate. The polymer conjugate may have a structure as described in U.S. Patent Application No. 20130059360, the contents of which are herein incorporated by reference in its entirety. In one aspect, polymer conjugates with the polynucleotides of the present invention may be made using the methods and/or segmented polymeric reagents described in U.S. Patent Application No. 20130072709, herein incorporated by reference in its entirety. In another aspect, the polymer conjugate may have pendant side groups comprising ring moieties such as, but not limited to, the polymer conjugates described in US Patent Publication No. US20 1301 96948, the contents of which is herein incorporated by reference in its entirety.

[000863] In one embodiment, the polynucleotides of the invention may be part of a nucleic acid conjugate comprising a hydrophobic polymer covalently bound to the polynucleotide through a first linker wherein said conjugate forms nanoparticulate micelles having a hydrophobic core and a hydrophilic shell, for example, to to render nucleic acids resistant to nuclease digestion, as described in International Patent Publication No. WO20 14047649, the contents of which is herein incorporated by reference in its entirety.

[000864] The nanoparticle formulations may comprise a conjugate to enhance the delivery of nanoparticles of the present invention in a subject. Further, the conjugate may inhibit phagocytic clearance of the nanoparticles in a subject. In one aspect, the conjugate may be a "self peptide designed from the human membrane protein CD47 (e.g., the "self particles described by Rodriguez et al (Science 2013 339, 971-975), herein incorporated by reference in its entirety). "Self peptides are described in paragraphs [000471] - [000473] of copending International Publication No. WO2015038892, the contents of which are herein incorporated by reference in its entirety.

[000865] In one embodiment, the conjugate may be for conjugated delivery of the polynucleotides to the liver. As a non-limiting example, the conjugate delivery system described in US Patent Publication No. US20130245091, the contents of which are herein incorporated by reference in its entirety, may be used to deliver the polynucleotides described herein.

[000866] In one embodiment, a non-linear multi-block copolymer-drug conjugate may be used to deliver active agents such as the polymer-drug conjugates and the formulas described in International Publication No. WO2013 138346, incorporated by reference in its entirety. As a non-limiting example, a non-linear multi-block copolymer may be conjugated to a nucleic acid

such as the polynucleotides described herein. As another non-limiting example, a non-linear multi-block copolymer may be conjugated to a nucleic acid such as the polynucleotides described herein to treat intraocular neovascular diseases.

[000867] In one embodiment, the polynucleotides of the invention may be formulated with monodisperse polymer particles as described in and made by the method described in US Patent No. 8,658,733, the contents of which is herein incorporated by refererence in its entirety.

[000868] In one embodiment, the polynucleotides of the invention may be formulated in polymer particles as described in amd made by the methods of US Patent Publication No. 20140057109, the contents of which is incorporated by refererence in its entirety.

[000869] In another embodiment, HIF-1 inhibitors may be conjugated to or dispersed in controlled release formulations such as a polymer-conjugate as described in International Publication No. WO2013 138343, the contents of which are herein incorporated by reference in its entirety. The polynucleotides described herein may encode HIF-1 inhibitors and may be delivered using the controlled release formulations of polymer-conjugates. The polymer-conjugates comprising HIF-1 inhibitors may be used to treat a disease and/or disorder that is associated with vascularization such as, but not limited to, cancer, obesity, and ocular diseases such as wet AMD.

[000870] In one embodiment, albumin-binding lipids may be conjugated to cargo (e.g., the polynucleotides and formulations thereof) for targeted delivery to the lymph nodes. Non-limiting examples of albumin-binding lipids and conjugates thereof are described in International Patent Publication No. WO20 13151771, the contents of which are herein incorporated by reference in its entirety.

[000871] In another embodiment, pharmaceutical compositions comprising the polynucleotides of the present invention and a conjugate which may have a degradable linkage. Non-limiting examples of conjugates include an aromatic moiety comprising an ionizable hydrogen atom, a spacer moiety, and a water-soluble polymer. As a non-limiting example, pharmaceutical compositions comprising a conjugate with a degradable linkage and methods for delivering such pharmaceutical compositions are described in US Patent Publication No. US20130184443, the contents of which are herein incorporated by reference in its entirety.

[000872] In one embodiment, pharmaceutical compositions comprising the polynucleotides of the present invention contain nanoparticles, liposomes, polymers, agents and proteins with reversible disulfide linkers. In a non-limiting example, the polynucleotides may be reversibly linked to delivery vehicles via the linkages described in US Patent Publication No. 20140081012, the contents of which is herein incorporated by reference in its entirety.

[000873] The nanoparticle formulations may be a carbohydrate nanoparticle comprising a carbohydrate carrier and a polynucleotide. As a non-limiting example, the carbohydrate carrier may include, but is not limited to, an anhydride-modified phytoglycogen or glycogen-type material, phtoglycogen octenyl succinate, phytoglycogen beta-dextrin, anhydride-modified phytoglycogen beta-dextrin. (See e.g., International Publication No. WO2012109121 and US Patent Publication No. 20140066363, the contents of each of which are herein incorporated by reference in their entirety). Nanoparticle formulations of the present invention may be coated with a surfactant or polymer in order to improve the delivery of the particle. In one embodiment, the nanoparticle may be coated with a hydrophilic coating such as, but not limited to, PEG coatings and/or coatings that have a neutral surface charge. The hydrophilic coatings may help to deliver nanoparticles with larger payloads such as, but not limited to, polynucleotides within the central nervous system. As a non-limiting example nanoparticles comprising a hydrophilic coating and methods of making such nanoparticles are described in US Patent Publication No. US20 1301 83244, the contents of which are herein incorporated by reference in its entirety.

[000874] In one embodiment, the lipid nanoparticles of the present invention may be hydrophilic polymer particles. Non-limiting examples of hydrophilic polymer particles and methods of making hydrophilic polymer particles are described in US Patent Publication No. US20130210991 and in US Patent Publication No. 20140073738 and 20140073715, the contents of each of which are herein incorporated by reference in their entirety.

[000875] In another embodiment, the lipid nanoparticles of the present invention may be hydrophobic polymer particles.

[000876] Lipid nanoparticle formulations may be improved by replacing the cationic lipid with a biodegradable cationic lipid which is known as a rapidly eliminated lipid nanoparticle (reLNP). Ionizable cationic lipids, such as, but not limited to, DLinDMA, DLin-KC2-DMA, and DLin-MC3-DMA, have been shown to accumulate in plasma and tissues over time and may be a potential source of toxicity. The rapid metabolism of the rapidly eliminated lipids can improve the tolerability and therapeutic index of the lipid nanoparticles by an order of magnitude from a 1 mg/kg dose to a 10 mg/kg dose in rat. Inclusion of an enzymatically degraded ester linkage can improve the degradation and metabolism profile of the cationic component, while still maintaining the activity of the reLNP formulation. The ester linkage can be internally located within the lipid chain or it may be terminally located at the terminal end of the lipid chain. The internal ester linkage may replace any carbon in the lipid chain.

[000877] In one embodiment, the internal ester linkage may be located on either side of the saturated carbon.

[000878] In one embodiment, an immune response may be elicited by delivering a lipid nanoparticle which may include a nanospecies, a polymer and an immunogen. (U.S. Publication No. 20120189700 and International Publication No. WO2012099805; each of which is herein incorporated by reference in their entirety). The polymer may encapsulate the nanospecies or partially encapsulate the nanospecies. The immunogen may be a recombinant protein, a modified RNA and/or a polynucleotide described herein. In one embodiment, the lipid nanoparticle may be formulated for use in a vaccine such as, but not limited to, against a pathogen.

[000879] Lipid nanoparticles may be engineered to alter the surface properties of particles so the lipid nanoparticles may penetrate the mucosal barrier. Lipid nanoparticles to penetrate the mucosal barrier and areas where mucus is located is described in International Patent Application No. PCT/US2014/027077 (Attorney Docket No. M030.20), the contents of which are herein incorporated by reference in its entirety, for example in paragraphs [000491] - [000501].

[000880] In one embodiment, the polynucleotide is formulated as a lipoplex, such as, without limitation, the ATUPLEX™ system, the DACC system, the DBTC system and other siRNA-lipoplex technology from Silence Therapeutics (London, United Kingdom), STEMFECT™ from STEMGENT® (Cambridge, MA), and polyethylenimine (PEI) or protamine-based targeted and non-targeted delivery of nucleic acids acids (Aleku et al. Cancer Res. 2008 68:9788-9798; Strumberg et al. Int J Clin Pharmacol Ther 2012 50:76-78; Santel et al, Gene Ther 2006 13:1222-1234; Santel et al, Gene Ther 2006 13:1360-1370; Gutbier et al, Pulm Pharmacol. Ther. 2010 23:334-344; Kaufmann et al. Microvasc Res 2010 80:286-293Weide et al. J Immunother. 2009 32:498-507; Weide et al. J Immunother. 2008 31:180-188; Pascolo Expert Opin. Biol. Ther. 4:1285-1294; Fotin-Mleczek et al, 2011 J. Immunother. 34:1-15; Song et al, Nature Biotechnol. 2005, 23:709-717; Peer et al., Proc Natl Acad Sci U S A. 2007 6;104:4095-4100; deFougerolles *Hum Gene Ther.* 2008 19:125-132; all of which are incorporated herein by reference in its entirety).

[000881] In one embodiment such formulations may also be constructed or compositions altered such that they passively or actively are directed to different cell types *in vivo,* including but not limited to hepatocytes, immune cells, tumor cells, endothelial cells, antigen presenting cells, and leukocytes (Akinc et al. Mol Ther. 2010 18:1357-1364; Song et al, Nat Biotechnol. 2005 23:709-717; Judge et al, J Clin Invest. 2009 119:661-673; Kaufmann et al, Microvasc Res 2010 80:286-293; Santel et al, Gene Ther 2006 13:1222-1234; Santel et al, Gene Ther 2006 13:1360-1370; Gutbier et al, Pulm Pharmacol. Ther. 2010 23:334-344; Basha et al., Mol. Ther. 201 1 19:2186-2200; Fenske and Cullis, Expert Opin Drug Deliv. 2008 5:25-44; Peer et al,

Science. 2008 319:627-630; Peer and Lieberman, Gene Ther. 201 1 18:1127-1133; all of which are incorporated herein by reference in its entirety). One example of passive targeting of formulations to liver cells includes the DLin-DMA, DLin-KC2-DMA and DLin-MC3 -DMA-based lipid nanoparticle formulations which have been shown to bind to apolipoprotein E and promote binding and uptake of these formulations into hepatocytes in vivo (Akinc et al. Mol Ther. 2010 18:1357-1364; herein incorporated by reference in its entirety). Formulations can also be selectively targeted through expression of different ligands on their surface as exemplified by, but not limited by, folate, transferrin, N-acetylgalactosamine (GalNAc), and antibody targeted approaches (Kolhatkar et al, Curr Drug Discov Technol. 201 1 8:197-206; Musacchio and Torchilin, *Fro«t 5 iosci*. 2011 16:1388-1412; Yu et al, Mol Membr Biol. 2010 27:286-298; Patil et al, Crit Rev Ther Drug Carrier Syst. 2008 25:1-61; Benoit et al, Biomacromolecules. 201 1 12:2708-2714; Zhao et al, Expert Opin Drug Deliv. 2008 5:309-319; Akinc et al, Mol Ther. 2010 18:1357-1364; Srinivasan et al, Methods Mol Biol. 2012 820:105-116; Ben-Arie et al, Methods Mol Biol. 2012 757:497-507; Peer 2010 J Control Release. 20:63-68; Peer et al, Proc Natl Acad Sci U S A. 2007 104:4095-4100; Kim et al, Methods Mol Biol. 201 1 721:339-353; Subramanya et al, Mol Ther. 2010 18:2028-2037; Song et al, Nat Biotechnol. 2005 23:709-717; Peer et al., Science. 2008 319:627-630; Peer and Lieberman, Gene Ther. 201 1 18:1 127-1 133; all of which are incorporated herein by reference in its entirety).

[000882] In one embodiment, the polynucleotide is formulated as a solid lipid nanoparticle. A solid lipid nanoparticle (SLN) may be spherical with an average diameter between 10 to 1000 nm. SLN possess a solid lipid core matrix that can solubilize lipophilic molecules and may be stabilized with surfactants and/or emulsifiers. In a further embodiment, the lipid nanoparticle may be a self-assembly lipid-polymer nanoparticle (see Zhang et al., ACS Nano, 2008, 2 (8), pp 1696-1702; the contents of which are herein incorporated by reference in its entirety). As a non-limiting example, the SLN may be the SLN described in International Patent Publication No. WO2013 105101, the contents of which are herein incorporated by reference in its entirety. As another non-limiting example, the SLN may be made by the methods or processes described in International Patent Publication No. WO20 13105 10 1, the contents of which are herein incorporated by reference in its entirety.

[000883] Liposomes, lipoplexes, or lipid nanoparticles may be used to improve the efficacy of polynucleotides directed protein production as these formulations may be able to increase cell transfection by the polynucleotide; and/or increase the translation of encoded protein. One such example involves the use of lipid encapsulation to enable the effective systemic delivery of polyplex plasmid DNA (Heyes et al, Mol Ther. 2007 15:713-720; herein incorporated by

reference in its entirety). The liposomes, lipoplexes, or lipid nanoparticles may also be used to increase the stability of the polynucleotide.

[000884] In one embodiment, the polynucleotides of the present invention can be formulated for controlled release and/or targeted delivery. As used herein, "controlled release" refers to a pharmaceutical composition or compound release profile that conforms to a particular pattern of release to effect a therapeutic outcome. In one embodiment, the polynucleotides may be encapsulated into a delivery agent described herein and/or known in the art for controlled release and/or targeted delivery. As used herein, the term "encapsulate" means to enclose, surround or encase. As it relates to the formulation of the compounds of the invention, encapsulation may be substantial, complete or partial. The term "substantially encapsulated" means that at least greater than 50, 60, 70, 80, 85, 90, 95, 96, 97, 98, 99, 99.9, 99.9 or greater than 99.999% of the pharmaceutical composition or compound of the invention may be enclosed, surrounded or encased within the delivery agent. "Partially encapsulation" means that less than 10, 10, 20, 30, 40 50 or less of the pharmaceutical composition or compound of the invention may be enclosed, surrounded or encased within the delivery agent. Advantageously, encapsulation may be determined by measuring the escape or the activity of the pharmaceutical composition or compound of the invention using fluorescence and/or electron micrograph. For example, at least 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 85, 90, 95, 96, 97, 98, 99, 99.9, 99.99 or greater than 99.99% of the pharmaceutical composition or compound of the invention are encapsulated in the delivery agent.

[000885] In one embodiment, the controlled release formulation may include, but is not limited to, tri-block co-polymers. As a non-limiting example, the formulation may include two different types of tri-block co-polymers (International Pub. No. WO201213 1104 and WO201213 1106; each of which is herein incorporated by reference in its entirety).

[000886] In another embodiment, the polynucleotides may be encapsulated into a lipid nanoparticle or a rapidly eliminated lipid nanoparticle and the lipid nanoparticles or a rapidly eliminated lipid nanoparticle may then be encapsulated into a polymer, hydrogel and/or surgical sealant described herein and/or known in the art. As a non-limiting example, the polymer, hydrogel or surgical sealant may be PLGA, ethylene vinyl acetate (EVAc), poloxamer, GELSITE® (Nanotherapeutics, Inc. Alachua, FL), HYLENEX® (Halozyme Therapeutics, San Diego CA), surgical sealants such as fibrinogen polymers (Ethicon Inc. Cornelia, GA), TISSELL® (Baxter International, Inc Deerfield, IL), PEG-based sealants, and COSEAL® (Baxter International, Inc Deerfield, IL).

[000887] In another embodiment, the lipid nanoparticle may be encapsulated into any polymer known in the art which may form a gel when injected into a subject. As another non-limiting example, the lipid nanoparticle may be encapsulated into a polymer matrix which may be biodegradable.

[000888] In one embodiment, the polynucleotide formulation for controlled release and/or targeted delivery may also include at least one controlled release coating. Controlled release coatings include, but are not limited to, OPADRY®, polyvinylpyrrolidone/vinyl acetate copolymer, polyvinylpyrrolidone, hydroxypropyl methylcellulose, hydroxypropyl cellulose, hydroxyethyl cellulose, EUDRAGIT RL®, EUDRAGIT RS® and cellulose derivatives such as ethylcellulose aqueous dispersions (AQUACOAT® and SURELEASE®). Controlled release and/or targeted delivery formulations are described in International Patent Application No. PCT/US2014/027077, the contents of which are herein incorporated by reference in its entirety, and non-limiting examples of the formulations are in paragraphs [000515] - [000519].

[000889] In one embodiment, the polynucleotides of the invention may be formulated in an aquaeous dispersion of polymer encapsulated particulate material described in or made by the method described in International Patent Publication No. WO2012 162742, the contents of which is herein incorporated by reference in its entirety.

[000890] In one embodiment, the polynucleotides of the present invention may be encapsulated in a therapeutic nanoparticle including ACCURINS™. Therapeutic nanoparticles may be formulated by methods described herein and known in the art such as, but not limited to, in Patent Publication No. WO201415221 1 (Attorney Docket No. M030.20), the contents of which are herein incorporated by reference in its entirety, such as in paragraphs [000519] - [000551]. As a non-limiting example, the therapeutic nanoparticle may be a sustained release nanoparticle such as those described in International Patent Publication No. WO201415221 1 (Attorney Docket No. M030.20), the contents of which are herein incorporated by reference in its entirety, such as in paragraphs [000531] - [000533].

[000891] In one embodiment, the polynucleotides of the present invention may be encapsulated in a synthetic nanocarrier. Synthetic nanocarriers may be formulated by methods described herein and known in the art such as, but not limited to, International Patent Publication No. WO201415221 1 (Attorney Docket No. M030.20), the contents of which are herein incorporated by reference in its entirety, such as in paragraphs [000552] - [000563].

[000892] In one embodiment, the nanoparticles of the present invention may comprise a polymeric matrix. As a non-limiting example, the nanoparticle may comprise two or more polymers such as, but not limited to, polyethylenes, polycarbonates, polyanhydrides,

polyhydroxyacids, polypropylfumerates, polycaprolactones, polyamides, polyacetals, polyethers, polyesters, poly(orthoesters), polycyanoacrylates, polyvinyl alcohols, polyurethanes, polyphosphazenes, polyacrylates, polymethacrylates, polycyanoacrylates, polyureas, polystyrenes, polyamines, polylysine, poly(ethylene imine), poly(serine ester), poly(L-lactide-co-L-lysine), poly(4-hydroxy-L-proline ester) or combinations thereof.

[000893] In one embodiment, the therapeutic nanoparticle comprises a diblock copolymer. In one embodiment, the diblock copolymer may include PEG in combination with a polymer such as, but not limited to, polyethylenes, polycarbonates, polyanhydrides, polyhydroxyacids, polypropylfumerates, polycaprolactones, polyamides, polyacetals, polyethers, polyesters, poly(orthoesters), polycyanoacrylates, polyvinyl alcohols, polyurethanes, polyphosphazenes, polyacrylates, polymethacrylates, polycyanoacrylates, polyureas, polystyrenes, polyamines, polylysine, poly(ethylene imine), poly(serine ester), poly(L-lactide-co-L-lysine), poly(4-hydroxy-L-proline ester) or combinations thereof. In another embodiment, the diblock copolymer may comprise the diblock copolymers described in European Patent Publication No. the contents of which are herein incorporated by reference in its entirety. In yet another embodiment, the diblock copolymer may be a high-X diblock copolymer such as those described in International Patent Publication No. WO20 13 120052, the contents of which are herein incorporated by reference in its entirety.

[000894] In one embodiment, the nanoparticle (e.g., therapeutic nanoparticle) may comprise a multiblock copolymer (See e.g., U.S. Pat. No. 8,263,665 and 8,287,910 and US Patent Pub. No. US20130195987; the contents of each of which are herein incorporated by reference in its entirety). As a non-limiting example, the multiblock copolymer which may be used in the nanoparticles described herein may be a non-linear multiblock copolymer such as those described in US Patent Publication No. 20130272994, the contents of which are herein incorporated by reference in its entirety.

[000895] In one embodiment, the polynucleotides may be formulated in colloid nanocarriers as described in US Patent Publication No. US20130197100, the contents of which are herein incorporated by reference in its entirety.

[000896] In one embodiment, the nanoparticle may be optimized for oral administration. The nanoparticle may comprise at least one cationic biopolymer such as, but not limited to, chitosan or a derivative thereof. As a non-limiting example, the nanoparticle may be formulated by the methods described in U.S. Pub. No. 20120282343; herein incorporated by reference in its entirety.

[000897] In some embodiments, LNPs comprise the lipid KL52 (an amino-lipid disclosed in U.S. Application Publication No. 2012/0295832 expressly incorporated herein by reference in its entirety). Activity and/or safety (as measured by examining one or more of ALT/AST, white blood cell count and cytokine induction) of LNP administration may be improved by incorporation of such lipids. LNPs comprising KL52 may be administered intravenously and/or in one or more doses. In some embodiments, administration of LNPs comprising KL52 results in equal or improved mRNA and/or protein expression as compared to LNPs comprising MC3.

[000898] In some embodiments, LNPs may comprise linear amino-lipids as described in US Patent No. 8,691,750, the contents of which is herein incorporated by reference in its entirety.

[000899] In one embodiment, polynucleotides may be delivered using LNPs which may comprise a diameter from about 1 nm to about 100 nm, from about 1 nm to about 10 nm, about 1 nm to about 20 nm, from about 1 nm to about 30 nm, from about 1 nm to about 40 nm, from about 1 nm to about 50 nm, from about 1 nm to about 60 nm, from about 1 nm to about 70 nm, from about 1 nm to about 80 nm, from about 1 nm to about 90 nm, from about 5 nm to about from 100 nm, from about 5 nm to about 10 nm, about 5 nm to about 20 nm, from about 5 nm to about 30 nm, from about 5 nm to about 40 nm, from about 5 nm to about 50 nm, from about 5 nm to about 60 nm, from about 5 nm to about 70 nm, from about 5 nm to about 80 nm, from about 5 nm to about 90 nm, from about 10 nm to about from 100 nm, about 10 nm to about 20 nm, from about 10 nm to about 30 nm, from about 10 nm to about 40 nm, from about 10 nm to about 50 nm, from about 10 nm to about 60 nm, from about 10 nm to about 70 nm, from about 10 nm to about 80 nm, from about 10 nm to about 90 nm, from about 20 nm to about from 100 nm, from about 20 nm to about 30 nm, from about 20 nm to about 40 nm, from about 20 nm to about 50 nm, from about 20 nm to about 60 nm, from about 20 nm to about 70 nm, from about 20 nm to about 80 nm, from about 20 nm to about 90 nm, from about 30 nm to about from 100 nm, from about 30 nm to about 40 nm, from about 30 nm to about 50 nm, from about 30 nm to about 60 nm, from about 30 nm to about 70 nm, from about 30 nm to about 80 nm, from about 30 nm to about 90 nm, from about 40 nm to about from 100 nm, from about 40 nm to about 50 nm, from about 40 nm to about 60 nm, from about 40 nm to about 70 nm, from about 40 nm to about 80 nm, from about 40 nm to about 90 nm, from about 50 nm to about from 100 nm, from about 50 nm to about 60 nm, from about 50 nm to about 70 nm, from about 50 nm to about 80 nm, from about 50 nm to about 90 nm, from about 60 nm to about from 100 nm, from about 60 nm to about 70 nm, from about 60 nm to about 80 nm, from about 60 nm to about 90 nm, from about 70 nm to about from 100 nm, from about 70 nm to about 80 nm, from about 70 nm to

about 90 nm, from about 80 nm to about from 100 nm, from about 80 nm to about 90 nm or from about 90 nm to about from 100 nm.

[000900] In some embodiments, such LNPs are synthesized using methods comprising microfluidic mixers. Exemplary microfluidic mixers may include, but are not limited to a slit interdigitial micromixer including, but not limited to those manufactured by Microinnova (Allerheiligen bei Wildon, Austria) and/or a staggered herringbone micromixer (SHM) (Zhigaltsev, I.V. et al, Bottom-up design and synthesis of limit size lipid nanoparticle systems with aqueous and triglyceride cores using millisecond microfluidic mixing have been published (Langmuir. 2012. 28:3633-40; Belliveau, N.M. et al, Microfluidic synthesis of highly potent limit-size lipid nanoparticles for in vivo delivery of siRNA. Molecular Therapy-Nucleic Acids. 2012. I:e37; Chen, D. et al, Rapid discovery of potent siRNA-containing lipid nanoparticles enabled by controlled microfluidic formulation. J Am Chem Soc. 2012. 134(16):6948-51; each of which is herein incorporated by reference in its entirety). In some embodiments, methods of LNP generation comprising SHM, further comprise the mixing of at least two input streams wherein mixing occurs by microstructure-induced chaotic advection (MICA). According to this method, fluid streams flow through channels present in a herringbone pattern causing rotational flow and folding the fluids around each other. This method may also comprise a surface for fluid mixing wherein the surface changes orientations during fluid cycling. Methods of generating LNPs using SHM include those disclosed in U.S. Application Publication Nos. 2004/0262223 and 2012/0276209, each of which is expressly incorporated herein by reference in their entirety.

[000901] In one embodiment, the polynucleotides of the present invention may be formulated in lipid nanoparticles created using a micromixer such as, but not limited to, a Slit Interdigital Microstructured Mixer (SIMM-V2) or a Standard Slit Interdigital Micro Mixer (SSIMM) or Caterpillar (CPMM) or Impinging-jet (IJMM)from the Institut fur Mikrotechnik Mainz GmbH, Mainz Germany).

[000902] In one embodiment, the polynucleotides of the present invention may be formulated in lipid nanoparticles created using microfluidic technology (see Whitesides, George M. The Origins and the Future of Microfluidics. Nature, 2006 442: 368-373; Abraham et al. Chaotic Mixer for Microchannels. Science, 2002 295: 647-651; and Valencia et al. Microfluidic Platform for Combinatorial Synthesis and Optimization of Targeted Nanoparticles for Cancer Therapy. ACS Nano 2013 (DOI/10.1021/nn403370e); the contents of each of which is herein incorporated by reference in their entirety). As a non-limiting example, controlled microfluidic formulation includes a passive method for mixing streams of steady pressure-driven flows in micro channels

at a low Reynolds number (See e.g., Abraham et al. Chaotic Mixer for Microchannels. Science, 2002 295: 647-651; which is herein incorporated by reference in its entirety).

[000903] In one embodiment, the polynucleotides of the present invention may be formulated in lipid nanoparticles created using a micromixer chip such as, but not limited to, those from Harvard Apparatus (Holliston, MA) or Dolomite Microfluidics (Royston, UK). A micromixer chip can be used for rapid mixing of two or more fluid streams with a split and recombine mechanism.

[000904] In one embodiment, the polynucleotides of the present invention may be formulated in lipid nanoparticles created using NanoAssemblr Y-mixer chip technology.

[000905] In one embodiment, the polynucleotides may be formulated in nanoparticles created using a microfluidic device such as the methods for making nanoparticles described in International Patent Publication No. WO2014016439, the contents of which are herein incorporated by reference in its entirety. As a non-limiting example, the nanoparticles may be created by adding a nanoparticle precursor to the microfluidic device through one or more flow channels, generating microplasma in the microfluidic device, causing the microplasma to interact with the nanoparticle precursor to generate nanoparticles, adding a conjugate material into the microfluidic device through one or more flow channels and causing the nanoparticles to mixwith the conjugate material in a continuous flow to form conjugated nanoparticles (see e.g., International Patent Publication No. WO2014016439, the contents of which are herein incorporated by reference in its entirety).

[000906] In one embodiment, the polynucleotides of the invention may be formulated for delivery using the drug encapsulating microspheres described in International Patent Publication No. WO2013063468 or U.S. Patent No. 8,440,614, each of which is herein incorporated by reference in its entirety. The microspheres may comprise a compound of the formula (I), (II), (III), (IV), (V) or (VI) as described in International patent application No. WO2013063468, the contents of which are herein incorporated by reference in its entirety. In another aspect, the amino acid, peptide, polypeptide, lipids (APPL) are useful in delivering the polynucleotides of the invention to cells (see International Patent Publication No. WO2013063468, herein incorporated by reference in its entirety).

[000907] In one embodiment, the polynucleotides of the invention may be formulated in lipid nanoparticles having a diameter from about 10 to about 100 nm such as, but not limited to, about 10 to about 20 nm, about 10 to about 30 nm, about 10 to about 40 nm, about 10 to about 50 nm, about 10 to about 60 nm, about 10 to about 70 nm, about 10 to about 80 nm, about 10 to about 90 nm, about 20 to about 30 nm, about 20 to about 40 nm, about 20 to about 50 nm, about 20 to

about 60 nm, about 20 to about 70 nm, about 20 to about 80 nm, about 20 to about 90 nm, about 20 to about 100 nm, about 30 to about 40 nm, about 30 to about 50 nm, about 30 to about 60 nm, about 30 to about 70 nm, about 30 to about 80 nm, about 30 to about 90 nm, about 30 to about 100 nm, about 40 to about 50 nm, about 40 to about 60 nm, about 40 to about 70 nm, about 40 to about 80 nm, about 40 to about 90 nm, about 40 to about 100 nm, about 50 to about 60 nm, about 50 to about 70 nm about 50 to about 80 nm, about 50 to about 90 nm, about 50 to about 100 nm, about 60 to about 70 nm, about 60 to about 80 nm, about 60 to about 90 nm, about 60 to about 100 nm, about 70 to about 80 nm, about 70 to about 90 nm, about 70 to about 100 nm, about 80 to about 90 nm, about 80 to about 100 nm and/or about 90 to about 100 nm.

[000908] In one embodiment, the lipid nanoparticles may have a diameter from about 10 to 500 nm.

[000909] In one embodiment, the lipid nanoparticle may have a diameter greater than 100 nm, greater than 150 nm, greater than 200 nm, greater than 250 nm, greater than 300 nm, greater than 350 nm, greater than 400 nm, greater than 450 nm, greater than 500 nm, greater than 550 nm, greater than 600 nm, greater than 650 nm, greater than 700 nm, greater than 750 nm, greater than 800 nm, greater than 850 nm, greater than 900 nm, greater than 950 nm or greater than 1000 nm.

[000910] In one aspect, the lipid nanoparticle may be a limit size lipid nanoparticle described in International Patent Publication No. WO2013059922, the contents of which are herein incorporated by reference in its entirety. The limit size lipid nanoparticle may comprise a lipid bilayer surrounding an aqueous core or a hydrophobic core; where the lipid bilayer may comprise a phospholipid such as, but not limited to, diacylphosphatidylcholine, a diacylphosphatidylethanolamine, a ceramide, a sphingomyelin, a dihydrosphingomyelin, a cephalin, a cerebroside, a C8-C20 fatty acid diacylphophatidylcholine, and l-palmitoyl-2-oleoyl phosphatidylcholine (POPC). In another aspect the limit size lipid nanoparticle may comprise a polyethylene glycol-lipid such as, but not limited to, DLPE-PEG, DMPE-PEG, DPPC-PEG and DSPE-PEG.

[000911] In one embodiment, the polynucleotides may be delivered, localized and/or concentrated in a specific location using the delivery methods described in International Patent Publication No. WO2013063530, the contents of which are herein incorporated by reference in its entirety. As a non-limiting example, a subject may be administered an empty polymeric particle prior to, simultaneously with or after delivering the polynucleotides to the subject. The empty polymeric particle undergoes a change in volume once in contact with the subject and becomes lodged, embedded, immobilized or entrapped at a specific location in the subject.

[000912] In one embodiment, the polynucleotides may be formulated in an active substance release system (See e.g., US Patent Publication No. US20130102545, herein incorporated by reference in its entirety). The active substance release system may comprise 1) at least one nanoparticle bonded to an oligonucleotide inhibitor strand which is hybridized with a catalytically active nucleic acid and 2) a compound bonded to at least one substrate molecule bonded to a therapeutically active substance (e.g., polynucleotides described herein), where the therapeutically active substance is released by the cleavage of the substrate molecule by the catalytically active nucleic acid.

[000913] In one embodiment, the polynucleotides may be formulated in a nanoparticle comprising an inner core comprising a non-cellular material and an outer surface comprising a cellular membrane. The cellular membrane may be derived from a cell or a membrane derived from a virus. As a non-limiting example, the nanoparticle may be made by the methods described in International Patent Publication No. WO20 13052 167, herein incorporated by reference in its entirety. As another non-limiting example, the nanoparticle described in International Patent Publication No. WO2013052167, herein incorporated by reference in its entirety, may be used to deliver the polynucleotides described herein.

[000914] In one embodiment, the polynucleotides may be formulated in porous nanoparticle-supported lipid bilayers (protocells). Protocells are described in International Patent Publication Nos. WO2012149376, WO2013056132 and US Patent Publication 20140079774, the contents of each of which are herein incorporated by reference in their entirety, and can be used for targeted delivery, including but not limited to hepatocellular or other cancer cells.

[000915] In one embodiment, the polynucleotides described herein may be formulated in polymeric nanoparticles as described in or made by the methods described in US Patent No. 8,420,123, 8,518,963 and 8,618,240 and European Patent No. EP2073848B1 and US Patent Publication No. US20130273 117, the contents of each of which are herein incorporated by reference in their entirety. As a non-limiting example, the polymeric nanoparticle may have a high glass transition temperature such as the nanoparticles described in or nanoparticles made by the methods described in US Patent No. 8,5 18,963 and US Patent Publication Nos. US20 14003035 1 and US201 10294717, the contents of each of which are herein incorporated by reference in their entirety. As another non-limiting example, the polymer nanoparticle for oral, parenteral and topical formulations may be made by the methods described in European Patent No. EP2073848B1, the contents of which are herein incorporated by reference in its entirety. As yet another non-limiting example, the polynucleotides may be formulated in a population of polymeric nanoparticles comprising a plurality of polymeric nanoparticles approximately the

same size and having an amphiphilic co-polymer (e.g., PLA) as described in US Patent No. 8,618,240, the contents of which are herein incorporated by reference in its entirety.

[000916] In one embodiment, the polynucleotides described herein may be formulated in lyophilized pharmaceutical compositions comprising polymeric nanoparticles such as the compositions described in US Patent No. 8,603,535 and 8,637,083 (BIND Therapeutics) and US Patent Publication Nos. US20130295191 and US20 13 0295 183, the contents of each ofwhich are herein incorporated by reference in its entirety. As a non-limiting example, the lyophilized composition may include polymeric nanoparticles which may comprise a poly(lactic) acid-block-poly(ethylene)glycol copolymer or poly(lactic)-co-poly(glycolic) acid-block-poly(ethylene)glycol copolymer, and a therapeutic agent (e.g., polynucleotides).

[000917] In another embodiment, the polynucleotides described herein may be formulated in nanoparticles used in imaging. The nanoparticles may be liposome nanoparticles such as those described in US Patent Publication No US20 130129636, herein incorporated by reference in its entirety. As a non-limiting example, the liposome may comprise gadolinium(III)2-{4,7-bis-carboxymethyl-10-[(N,N-distearylamidomethyl-N'-amido-methyl]-l, 4,7,1 0-tetra-azacyclododec-l-yl}-acetic acid and a neutral, fully saturated phospholipid component (see e.g., US Patent Publication No US20 130129636, the contents of which is herein incorporated by reference in its entirety).

[000918] In one embodiment, the polynucleotides described herein may be formulated in pH-sensitive liposome nanoparticles, including but not limited to nanoparticles that contain a photosensitive compound, which releases protons upon photolysis, as described in US Patent No. 8,663,599, the contents of which is herein incorporated by reference in its entirety.

[000919] In one embodiment, the nanoparticles which may be used in the present invention are formed by the methods described in U.S. Patent Application No. US20130130348, the contents of which is herein incorporated by reference in its entirety.

[000920] The nanoparticles of the present invention may further include nutrients such as, but not limited to, those which deficiencies can lead to health hazards from anemia to neural tube defects (see e.g, the nanoparticles described in International Patent Publication No WO2013072929, the contents of which is herein incorporated by reference in its entirety). As a non-limiting example, the nutrient may be iron in the form of ferrous, ferric salts or elemental iron, iodine, folic acid, vitamins or micronutrients.

[000921] In one embodiment, the polynucleotides of the present invention may be formulated in a swellable nanoparticle. The swellable nanoparticle may be, but is not limited to, those described in U.S. Patent No. 8,440,23 1 and US Patent Publication No. 20130323 10, the contents

of which are herein incorporated by reference in their entirety. As a non-limiting embodiment, the swellable nanoparticle may be used for delivery of the polynucleotides of the present invention to the pulmonary system (see e.g., U.S. Patent No. 8,440,231 and US Patent Publication No. 20130323 10, the contents of each of which are herein incorporated by reference in their entirety).

[000922] The polynucleotides of the present invention may be formulated in polyanhydride nanoparticles such as, but not limited to, those described in U.S. Patent No. 8,449,916, the contents of which is herein incorporated by reference in its entirety.

[000923] The nanoparticles and microparticles of the present invention may be geometrically engineered to modulate macrophage and/or the immune response. In one aspect, the geometrically engineered particles may have varied shapes, sizes and/or surface charges in order to incorporated the polynucleotides of the present invention for targeted delivery such as, but not limited to, pulmonary delivery (see e.g., International Publication No WO20130821 11, the contents of which is herein incorporated by reference in its entirety). Other physical features the geometrically engineering particles may have include, but are not limited to, fenestrations, angled arms, asymmetry and surface roughness, charge which can alter the interactions with cells and tissues. As a non-limiting example, nanoparticles of the present invention may be made by the methods described in International Publication No WO20130821 11, the contents of which is herein incorporated by reference in its entirety.

[000924] In one embodiment, the nanoparticles of the present invention may be water soluble nanoparticles such as, but not limited to, those described in International Publication No. WO20 13090601, the contents of which is herein incorporated by reference in its entirety. The nanoparticles may be inorganic nanoparticles which have a compact and zwitterionic ligand in order to exhibit good water solubility. The nanoparticles may also have small hydrodynamic diameters (HD), stability with respect to time, pH, and salinity and a low level of non-specific protein binding.

[000925] In one embodiment the nanoparticles of the present invention may be developed by the methods described in US Patent Publication No. US20 1301 72406 (Bind), US20130251817 (Bind), US2013251816 (Bind) and US20130251766 (Bind), the contents of each of which are herein incorporated by reference in its entirety. The stealth nanoparticles may comprise a diblock copolymer and a chemotherapeutic agent. These stealth nanoparticles may be made by the methods described in US Patent Publication Nos. US20130172406, US20130251817, US2013251816 and US20130251766, the contents of each of which are herein incorporated by reference in its entirety. As a non-limiting example, the stealth nanoparticles may target cancer

cells such as the nanoparticles described in US Patent Publication Nos. US20 1301 72406, US20130251817, US2013251816 and US20130251766, the contents of each of which are herein incorporated by reference in its entirety.

[000926] In one embodiment, the nanoparticles of the present invention are stealth nanoparticles or target-specific stealth nanoparticles such as, but not limited to, those described in US Patent Publication No. US20130172406; the contents of which is herein incorporated by reference in its entirety. The nanoparticles of the present invention may be made by the methods described in US Patent Publication No. US20130172406, the contents of which are herein incorporated by reference in its entirety.

[000927] In another embodiment, the stealth or target-specific stealth nanoparticles may comprise a polymeric matrix. The polymeric matrix may comprise two or more polymers such as, but not limited to, polyethylenes, polycarbonates, polyanhydrides, polyhydroxyacids, polypropylfumerates, polycaprolactones, polyamides, polyacetals, polyethers, polyesters, poly(orthoesters), polycyanoacrylates, polyvinyl alcohols, polyurethanes, polyphosphazenes, polyacrylates, polymethacrylates, polycyanoacrylates, polyureas, polystyrenes, polyamines, polyesters, polyanhydrides, polyethers, polyurethanes, polymethacrylates, polyacrylates, polycyanoacrylates or combinations thereof.

[000928] In one embodiment, the nanoparticle may be a nanoparticle-nucleic acid hybrid structure having a high density nucleic acid layer. As a non-limiting example, the nanoparticle-nucleic acid hybrid structure may made by the methods described in US Patent Publication No. US20130171646, the contents of which are herein incorporated by reference in its entirety. The nanoparticle may comprise a nucleic acid such as, but not limited to, polynucleotides described herein and/or known in the art.

[000929] At least one of the nanoparticles of the present invention may be embedded in the of core a nanostructure or coated with a low density porous **3-D** structure or coating which is capable of carrying or associating with at least one payload within or on the surface of the nanostructure. Non-limiting examples of the nanostructures comprising at least one nanoparticle are described in International Patent Publication No. WO20 13 123523, the contents of which are herein incorporated by reference in its entirety.

[000930] In one embodiment, the nanoparticle may comprise self-assembling peptides described in International Publication Nos. WO2014014613 and WO2014018675, the contents of each of which are herein incorporated by reference in their entirety. As a non-limiting example, the polynucleotides may be formulated in a self-assembled peptide nanostructure as described in International Publication No. WO2014014613, the contents of which are herein

incorporated by reference in its entirety. As another non-limiting example, the polynucleotides may be formulated in a self-assembled nucleic acid nanostructure as described in International Publication No. WO2014018675, the contents of which are herein incorporated by reference in its entirety.

[000931] In one embodiment, the nanoparticle described herein may be a lipid-polymer hybrid particle as described in US Patent Publication No. US201303 1583 1, the contents of which are herein incorporated by reference in its entirety. The lipid-polymer hybrid particles may have an aqueous core, a first amphiphilic layer surrounding the aqueous core and a polymeric matrix surrounding the amphiphilic layer. As a non-limiting example, the polynucleotides described herein may be formulated in a lipid-polymer hybrid particle. As another non-limiting example, the lipid-polymer hybrid nanoparticle may have heterogenous surface functional groups such as lipid-PEG-COOH, lipid-PEG-NH2 and lipid-PEG-OCH3.

[000932] In one embodiment, the polynucleotides may be formulated in and/or delivered in a lipid nanoparticle as described in International Patent Publication No. WO2012170930, the contents of which are herein incorporated by reference in its entirety. The lipid nanoparticle may comprise one or more cationic lipids, one or more non-cationic lipids and one or more PEG-modified lipids. As a non-limiting example, the lipid nanoparticle comprises DLin-KC2-DMA, Cholesterol (CHOL), DOPE and DMG-PEG-2000. As another non-limiting example, the lipid nanoparticle comprises C12-200, DOPE, cholesterol (CHOL) and DMGPEG2K.

[000933] In one embodiment, the polynucleotides may be formulated in and/or delivered in highly concentrated lipid nanoparticle dispersions as described in or made by the method described in US Patent 8,663,692, the contents of which is herein incorporated by reference in its entirety, for example in an ointment or lotion.

[000934] In one embodiment, the polynucleotides may be formulated in and/or delivered in a nanoparticle coated with a polymer for reversible immobilization and/or controlled release of the polynucleotides as described in International Patent Publication No. WO2013 174409, the contents of which is herein incorporated by reference in its entirety. As a non-limiting example, the nanoparticle is coated with a biodegradable polymer as described in International Patent Publication No. WO2013 174409, the contents of which is herein incorporated by reference in its entirety.

[000935] In one embodiment, the polynucleotides may be formulated in and/or delivered in a hydrophobic nanoparticle. The hydrophobic nanoparticle may further comprise a liver targeting moiety such as, but not limited to, the hydrophobic nanoparticles described in US Patent

Publication No. US20140017329, the contents of which are herein incorporated by reference in its entirety.

[000936] In one embodiment, the polynucleotides may be formulated in and/or delivered in a milled nanoparticle. As a non-limiting example, the milled nanoparticles may be those described in or made by the methods described in US Patent No. US8568784, the contents of which are herein incorporated by reference in its entirety. The milled nanoparticles may comprise a biologically active agent, at least one biopolymer and a polymer or ligand coating.

[000937] In one embodiment, the polynucleotides may be formulated in compositions which induce an immune response such as, but not limited to the formulations described in International Patent Publication Nos. WO2013 143555 and WO2013 143683, the contents of each of which are herein incorporated by reference in their entirety. As a non-limiting example, the formulations may induce an immune response after systemic administration of the polynucleotides. As another non-limiting example, the formulation which may induce the immune response may include nanoparticles comprising at least one nucleic acid molecule as described in International Patent Publication Nos. WO2013 143555 and WO2013 143683, the contents of each of which are herein incorporated by reference in their entirety.

[000938] In one embodiment, the polynucleotides may be formulated in and/or delivered in neutral nanoparticles. As a non-limiting example, the neutral nanoparticles may be those described in or made by the methods described in International Patent Publication No. WO2013 149141, the contents of which are herein incorporated by reference in its entirety.

[000939] In one embodiment, the nanoparticles may be neutralized by the methods described in International Patent Publication No. WO201 3149141, the contents of which are herein incorporated by reference in its entirety.

[000940] In one embodiment, the polynucleotides may be formulated in and/or delivered in a nanoparticle having a nucleic acid nanostructure core and a lipid coating such as, but not limited to, the nanoparticles described in International Patent Publication No. WO2013 148186, the contents of which are herein incorporated by reference in its entirety.

[000941] In one embodiment, the polynucleotides may be formulated in a particle comprising a conjugate for delivering nucleic acid agents such as the particles described in US Patent Publication No. US20140037573, the contents of which are herein incorporated by reference in its entirety. As a non-limiting example, the particle comprising a plurality of hydrophobic moieties, a plurality of hydrophilic-hydrophobic polymers and nucleic acid agents.

[000942] In one embodiment, the nanoparticles which may be used to formulate and/or deliver the polynucleotides described herein may comprise a cationic lipid such as, but not limited to,

the cationic lipids of formula (I) described in US Patent Publication NO. US20 1400459 13, the contents of which are herein incorporated by reference in its entirety.

[000943] In one embodiment, the nanoparticle may be a polyethylene glycolated (PEGylated) nanoparticle such as, but not limited to, the PEGylated nanoparticles described in US Patent Publication No. US20 140044791, the contents of which are herein incorporated by reference in its entirety. The PEGylated nanoparticle may comprise at least one targeting moiety coupled to the polyethylene glycol of the nanoparticle in order to target the composition to a specific cell. Non-limiting examples, of PEGylated nanoparticles and targeting moieties are described in US Patent Publication No. US20140044791, the contents of which are herein incorporated by reference in its entirety.

[000944] In one embodiment, the nanoparticle may be a mesoporous nanoparticle such as, but not limited to, those described in International Patent Publication No. WO20 12 142240, the contents of which are herein incorporated by reference in its entirety. The mesoporous nanoparticle may be loaded with the polynucleotide and may release the load in a controlled manner for a desired period of time such as, but not limited to an extended period of time.

Polymers, Biodegradable Nanoparticles, and Core-Shell Nanoparticles

[000945] The polynucleotides of the invention can be formulated using natural and/or synthetic polymers. Non-limiting examples of polymers which may be used for delivery include, but are not limited to, DYNAMIC POLYCONJUGATE® (Arrowhead Research Corp., Pasadena, CA) formulations from MIRUS® Bio (Madison, WI) and Roche Madison (Madison, WI), PHASERX™ polymer formulations such as, without limitation, SMARTT POLYMER TECHNOLOGY™ (PHASERX®, Seattle, WA), DMRI/DOPE, poloxamer, VAXFECTIN® adjuvant from Vical (San Diego, CA), chitosan, cyclodextrin from Calando Pharmaceuticals (Pasadena, CA), dendrimers and poly(lactic-co-glycolic acid) (PLGA) polymers. RONDEL™ (RNAi/Oligonucleotide Nanoparticle Delivery) polymers (Arrowhead Research Corporation, Pasadena, CA) and pH responsive co-block polymers such as, but not limited to, PHASERX® (Seattle, WA).

[000946] The polynucleotides of the invention can be formulated using polyconjugate systems with multiple reversible or biologically labile linkages connecting component parts to provide for physiologically responsive activity modulation. For example, the polynucleotides of the invention may be delivered to a cell with a reversibly masked membrane active polyamine as a delivery polymer reversibly conjugated to the polynucleotides, as described in US Patent No. 8,658,21 1, the contents of which is herein incorporated by reference in its entirety.

[000947] In another non-limiting example, the polynucleotides of the invention can be formulated with a delivery system, which delivers compositions to a targeted intracellual location by using endogenous processes that occur ubiquitously within all cells. For example, polynucleotides may be formulated with a the delivery system may further essentially consist of at least one module that mediates cell targeting and facilitates cellular uptake, at least one module that facilitates transport to the endoplasmic reticulum (ER), at least one module that mediates translocation from the ER to the cytosol, linked with the polynucleotides and each module in any arrangement, as described in US Patent Publication No. 20140065 172, the contents of which is incorporated herein by reference in its entirety.

[000948] A non-limiting example of chitosan formulation includes a core of positively charged chitosan and an outer portion of negatively charged substrate (U.S. Pub. No. 20120258176; herein incorporated by reference in its entirety). Chitosan includes, but is not limited to N-trimethyl chitosan, mono-N-carboxymethyl chitosan (MCC), N-palmitoyl chitosan (NPCS), EDTA-chitosan, low molecular weight chitosan, chitosan derivatives, or combinations thereof.

[000949] In one embodiment, the polymers used in the present invention have undergone processing to reduce and/or inhibit the attachment of unwanted substances such as, but not limited to, bacteria, to the surface of the polymer. The polymer may be processed by methods known and/or described in the art and/or described in International Pub. No. WO2012150467, herein incorporated by reference in its entirety.

[000950] A non-limiting example of PLGA formulations include, but are not limited to, PLGA injectable depots (e.g., ELIGARD® which is formed by dissolving PLGA in 66% N-methyl-2-pyrrolidone (NMP) and the remainder being aqueous solvent and leuprolide. Once injected, the PLGA and leuprolide peptide precipitates into the subcutaneous space).

[000951] Many of these polymer approaches have demonstrated efficacy in delivering oligonucleotides *in vivo* into the cell cytoplasm (reviewed in deFougerolles *Hum Gene Ther.* 2008 19:125-132; herein incorporated by reference in its entirety). Two polymer approaches that have yielded robust *in vivo* delivery of nucleic acids, in this case with small interfering RNA (siRNA), are dynamic polyconjugates and cyclodextrin-based nanoparticles (see e.g., US Patent Publication No. US20130156721, herein incorporated by reference in its entirety). The first of these delivery approaches uses dynamic polyconjugates and has been shown *in vivo* in mice to effectively deliver siRNA and silence endogenous target mRNA in hepatocytes (Rozema et al, Proc Natl Acad Sci U S A. 2007 104:12982-12887; herein incorporated by reference in its entirety). This particular approach is a multicomponent polymer system whose key features include a membrane-active polymer to which nucleic acid, in this case siRNA, is covalently

coupled via a disulfide bond and where both PEG (for charge masking) and *N*-acetylgalactosamine (for hepatocyte targeting) groups are linked via pH-sensitive bonds (Rozema et al, Proc Natl Acad Sci U S A. 2007 104:12982-12887; herein incorporated by reference in its entirety). On binding to the hepatocyte and entry into the endosome, the polymer complex disassembles in the low-pH environment, with the polymer exposing its positive charge, leading to endosomal escape and cytoplasmic release of the siRNA from the polymer. Through replacement of the *N*-acetylgalactosamine group with a mannose group, it was shown one could alter targeting from asialoglycoprotein receptor-expressing hepatocytes to sinusoidal endothelium and Kupffer cells. Another polymer approach involves using transferrin-targeted cyclodextrin-containing polycation nanoparticles. These nanoparticles have demonstrated targeted silencing of the *EWS-FLI1* gene product in transferrin receptor-expressing Ewing's sarcoma tumor cells (Hu-Lieskovan *et al,* Cancer Res.2005 65: 8984-8982; herein incorporated by reference in its entirety) and siRNA formulated in these nanoparticles was well tolerated in non-human primates (Heidel *et al.,* Proc Natl Acad Sci USA 2007 104:5715-21; herein incorporated by reference in its entirety). Both of these delivery strategies incorporate rational approaches using both targeted delivery and endosomal escape mechanisms.

[000952] The polymer formulation can permit the sustained or delayed release of polynucleotides (e.g., following intramuscular or subcutaneous injection). The altered release profile for the polynucleotide can result in, for example, translation of an encoded protein over an extended period of time. The polymer formulation may also be used to increase the stability of the polynucleotide. Biodegradable polymers have been previously used to protect nucleic acids other than polynucleotide from degradation and been shown to result in sustained release of payloads in vivo (Rozema et al, Proc Natl Acad Sci U S A. 2007 104:12982-12887; Sullivan et al, Expert Opin Drug Deliv. 2010 7:1433-1446; Convertine et al., Biomacromolecules. 2010 Oct 1; Chu et al, Acc Chem Res. 2012 Jan 13; Manganiello et al, Biomaterials. 2012 33:2301-2309; Benoit et al, Biomacromolecules. 201 1 12:2708-2714; Singha et al, Nucleic Acid Ther. 201 1 2:133-147; deFougerolles Hum Gene Ther. 2008 19:125-132; Schaffert and Wagner, Gene Ther. 2008 16:1131-1 138; Chaturvedi et al, Expert Opin Drug Deliv. 201 1 8:1455-1468; Davis, Mol Pharm. 2009 6:659-668; Davis, Nature 2010 464:1067-1070; each of which is herein incorporated by reference in its entirety).

[000953] In one embodiment, the pharmaceutical compositions may be sustained release formulations. In a further embodiment, the sustained release formulations may be for subcutaneous delivery. Sustained release formulations may include, but are not limited to, PLGA microspheres, ethylene vinyl acetate (EVAc), poloxamer, GELSITE® (Nanotherapeutics,

Inc. Alachua, FL), HYLENEX® (Halozyme Therapeutics, San Diego CA), surgical sealants such as fibrinogen polymers (Ethicon Inc. Cornelia, GA), TISSELL® (Baxter International, Inc Deerfield, IL), PEG-based sealants, and COSEAL® (Baxter International, Inc Deerfield, IL).

[000954] As a non-limiting example modified mRNA may be formulated in PLGA microspheres by preparing the PLGA microspheres with tunable release rates (e.g., days and weeks) and encapsulating the modified mRNA in the PLGA microspheres while maintaining the integrity of the modified mRNA during the encapsulation process. EVAc are non-biodegradable, biocompatible polymers which are used extensively in pre-clinical sustained release implant applications (e.g., extended release products Ocusert a pilocarpine ophthalmic insert for glaucoma or progestasert a sustained release progesterone intrauterine deivce; transdermal delivery systems Testoderm, Duragesic and Selegiline; catheters). Poloxamer F-407 NF is a hydrophilic, non-ionic surfactant triblock copolymer of polyoxyethylene-polyoxypropylene-polyoxyethylene having a low viscosity at temperatures less than 5°C and forms a solid gel at temperatures greater than 15°C. PEG-based surgical sealants comprise two synthetic PEG components mixed in a delivery device which can be prepared in one minute, seals in 3 minutes and is reabsorbed within 30 days. GELSITE® and natural polymers are capable of in-situ gelation at the site of administration. They have been shown to interact with protein and peptide therapeutic candidates through ionic interaction to provide a stabilizing effect.

[000955] Polymer formulations can also be selectively targeted through expression of different ligands as exemplified by, but not limited by, folate, transferrin, and N-acetylgalactosamine (GalNAc) (Benoit et al, Biomacromolecules. 201 1 12:2708-2714; Rozema et al, Proc Natl Acad Sci U S A. 2007 104:12982-12887; Davis, Mol Pharm. 2009 6:659-668; Davis, Nature 2010 464:1067-1070; each of which is herein incorporated by reference in its entirety).

[000956] The polynucleotides of the invention may be formulated with or in a polymeric compound. The polymer may include at least one polymer such as, but not limited to, polyethenes, polyethylene glycol (PEG), poly(l-lysine)(PLL), PEG grafted to PLL, cationic lipopolymer, biodegradable cationic lipopolymer, polyethyleneimine (PEI), cross-linked branched poly(alkylene imines), a polyamine derivative, a modified poloxamer, a biodegradable polymer, elastic biodegradable polymer, biodegradable block copolymer, biodegradable random copolymer, biodegradable polyester copolymer, biodegradable polyester block copolymer, biodegradable polyester block random copolymer, multiblock copolymers, linear biodegradable copolymer, poly[a-(4-aminobutyl)-L-glycolic acid) (PAGA), biodegradable cross-linked cationic multi-block copolymers, polycarbonates, polyanhydrides, polyhydroxyacids,

polypropylfumerates, polycaprolactones, polyamides, polyacetals, polyethers, polyesters, poly(orthoesters), polycyanoacrylates, polyvinyl alcohols, polyurethanes, polyphosphazenes, polyacrylates, polymethacrylates, polycyanoacrylates, polyureas, polystyrenes, polyamines, polylysine, poly(ethylene imine), poly(serine ester), poly(L-lactide-co-L-lysine), poly(4-hydroxy-L-proline ester), acrylic polymers, amine-containing polymers, dextran polymers, dextran polymer derivatives or or combinations thereof .

[000957] As a non-limiting example, the polynucleotides of the invention may be formulated with the polymeric compound of PEG grafted with PLL as described in U.S. Pat. No. 6,177,274; herein incorporated by reference in its entirety. The formulation may be used for transfecting cells *in vitro* or for *in vivo* delivery of polynucleotide. In another example, the polynucleotide may be suspended in a solution or medium with a cationic polymer, in a dry pharmaceutical composition or in a solution that is capable of being dried as described in U.S. Pub. Nos. 20090042829 and 20090042825; each of which are herein incorporated by reference in their entireties.

[000958] As another non-limiting example the polynucleotides of the invention may be formulated with a PLGA-PEG block copolymer (see US Pub. No. US20120004293 and US Pat No. 8,236,330, herein incorporated by reference in their entireties) or PLGA-PEG-PLGA block copolymers (See U.S. Pat. No. 6,004,573, herein incorporated by reference in its entirety). As a non-limiting example, the polynucleotides of the invention may be formulated with a diblock copolymer of PEG and PLA or PEG and PLGA (see US Pat No 8,246,968, herein incorporated by reference in its entirety).

[000959] In one embodiment, a polymer combination may be used for the formulation and/or delivery of the polynucleotides described herein. As a non-limiting example, the polymer combination may be two polymers used at a ratio of 1:1, 1:2, 1:2.5, 1:3, 1:4, 1:5, 1:6, 1:7, 1:8, 1:9, 1:10, 1:12.5, 1:15, 1:20, 1:25, 1:30, 1:40 or at least 1:50. In order to reduce the shear stress on the lipids during the delivery of the polynucleotides a polymer may be used to stabilize the polymers sensitive to degradation during delivery.

[000960] In one embodiment, a polymer combination of PLGA and PEG may be used for the formulation and/or delivery of the polynucleotides described herein. As a non-limiting example, PEG may be used with PLGA in the delivery and/or formulation of the polynucleotides to reduce the degradation of PLGA during delivery. As another non-limiting example, the PLGA and PEG lipids used in the formulation and/or delivery of the polynucleotides may be in a 50:50 ratio. As yet another non-limiting example, the PLGA has a size of approximately 15K and the PEG has a

size of approximately 2K and used in the formulation and/or delivery of the polynucleotides in a 50:50 ratio.

[000961] A polyamine derivative may be used to deliver nucleic acids or to treat and/or prevent a disease or to be included in an implantable or injectable device (U.S. Pub. No. 20100260817 (now U.S. Patent Nos. 8,460,696) and 20140050775, are the contents of each of which is herein incorporated by reference in its entirety). As a non-limiting example, a pharmaceutical composition may include the polynucleotide and the polyamine derivative described in U.S. Pub. No. 20100260817 (now U.S. Patent No. 8,460,696; the contents of which are incorporated herein by reference in its entirety. As a non-limiting example the polynucleotides of the present invention may be delivered using a polyamine polymer such as, but not limited to, a polymer comprising a 1,3-dipolar addition polymer prepared by combining a carbohydrate diazide monomer with a dilkyne unite comprising oligoamines (U.S. Pat. No. 8,236,280; herein incorporated by reference in its entirety). As another non-limiting example, the modified nucleic acids and/or mmRNA of the present invention may be delivered using or formulated in compositions comprising polyamine derivatives such as those described in formulas I-VI described in US Patent Publication No. US20140050775, the contents of which are herein incorporated by reference in its entirety.

[000962] In one embodiment, the polynucleotides of the invention may be delivered in a formulation with branched polyamines, such carbamate functionalized branched polyethylenimines comprising hydrophobic carbamate end groups, as described in International Patent Publication No. WO20 14042920, the contents of which is herein incorporated by reference in its entirety.

[000963] The polynucleotides of the invention may be formulated with at least one acrylic polymer. Acrylic polymers include but are not limited to, acrylic acid, methacrylic acid, acrylic acid and methacrylic acid copolymers, methyl methacrylate copolymers, ethoxyethyl methacrylates, cyanoethyl methacrylate, amino alkyl methacrylate copolymer, poly(acrylic acid), poly(methacrylic acid), polycyanoacrylates and combinations thereof. As a non-limiting example, the polynucleotides may be formulated with at least one poly(acrylate) copolymer as described in US Patent Publication No. US201303 17079, the contents of which are herein incorporated by reference in its entirety. As another non-limiting example, the polynucleotides may be formulated with at least one poly(acrylate) polymer as described in International Patent Publication No. WO2013 158141, the contents of which are herein incorporated by reference in its entirety.

[000964] In one embodiment, the polynucleotides of the present invention may be formulated with at least one polymer and/or derivatives thereof described in International Publication Nos. WO201 1115862, WO2012082574 and WO2012068187 and U.S. Pub. No. 20120283427, each of which are herein incorporated by reference in their entireties. In another embodiment, the polynucleotides of the present invention may be formulated with a polymer of formula Z as described in WO201 1115862, herein incorporated by reference in its entirety. In yet another embodiment, the polynucleotides may be formulated with a polymer of formula Z, Z' or Z" as described in International Pub. Nos. WO2012082574 or WO2012068187 and U.S. Pub. No. 2012028342, each of which are herein incorporated by reference in their entireties. The polymers formulated with the modified RNA of the present invention may be synthesized by the methods described in International Pub. Nos. WO20 12082574 or WO20 12068 187, each of which are herein incorporated by reference in their entireties.

[000965] The polynucleotides of the invention may be formulated with at least one acrylic polymer. Acrylic polymers include but are not limited to, acrylic acid, methacrylic acid, acrylic acid and methacrylic acid copolymers, methyl methacrylate copolymers, ethoxyethyl methacrylates, cyanoethyl methacrylate, amino alkyl methacrylate copolymer, poly(acrylic acid), poly(methacrylic acid), polycyanoacrylates and combinations thereof.

[000966] Formulations of polynucleotides of the invention may include at least one amine-containing polymer such as, but not limited to polylysine, polyethylene imine, poly(amidoamine) dendrimers, poly(amine-co-esters) or combinations thereof. As a non-limiting example, the poly(amine-co-esters) may be the polymers described in and/or made by the methods described in International Publication No WO20 13082529, the contents of which are herein incorporated by reference in its entirety. As another non-limiting example, the poly(amido amine) polymer may be the polymers described in and/or made by the methods described in US Publication No US20130289207, the contents of which are herein incorporated by reference in its entirety.

[000967] Formulations of the polynucleotides of the invention may include at least one of the cationic lipids described in International Patent Publication Nos. WO2013 158127 and WO2013 148541, the contents of each of which are herein incorporated by reference in its entirety. As a non-limiting example, the cationic lipid has the structure (I) as described in International Patent Publication No. WO2013 158127, the contents of which are herein incorporated by reference in its entirety. As another non-limiting example, the cationic lipid has the structure formula A as described in International Patent Publication No. WO201 3148541, the contents of each of which are herein incorporated by reference in its entirety.

[000968] Formulations of the polynucleotides of the invention may include at least one of the diester and trimester based low molecular weight, biodegradable cationic lipids described in International Patent Publication No. WO2013 158579, the contents of which are herein incorporated by reference in its entirety. As a non-limiting example, the cationic lipid has the formula A as described in International Patent Publication No. WO2013 158579, the contents of which are herein incorporated by reference in its entirety.

[000969] In one embodiment, formulations of the polynucleotides of the invention may include cationic lipids and lipid particles comprising amino lipids or salts thereof as described in European Patent Publication No. EP2350043, the contents of which is herein incorporated by reference in its entirety.

[000970] In one embodiment, polymers described herein may be synthesized using reversible addition-fragmentation chain transfer (RAFT) polymerization. RAFT is a controlled radical polymerization that may allow synthesis of monodisperse and polymers with block or other architectures and telechelic end chemistries providing opportunities for site-specific bioconjugation (see e.g., Nelson et al. *Tunable Delivery of siRNA from a Biodegradable Scaffold to Promote Angiogenesis In Vivo.* Adv. Mater. 2013; the contents of which are herein incorporated by reference in its entirety).

[000971] For example, the polynucleotides of the invention may be formulated in a pharmaceutical compound including a poly(alkylene imine), a biodegradable cationic lipopolymer, a biodegradable block copolymer, a biodegradable polymer, or a biodegradable random copolymer, a biodegradable polyester block copolymer, a biodegradable polyester polymer, a biodegradable polyester random copolymer, a linear biodegradable copolymer, PAGA, a biodegradable cross-linked cationic multi-block copolymer or combinations thereof. The biodegradable cationic lipopolymer may be made by methods known in the art and/or described in U.S. Pat. No. 6,696,038, U.S. App. Nos. 20030073619 and 20040142474 each of which is herein incorporated by reference in their entireties. The poly(alkylene imine) may be made using methods known in the art and/or as described in U.S. Pub. No. 201000043 15, herein incorporated by reference in its entirety. The biodegradabale polymer, biodegradable block copolymer, the biodegradable random copolymer, biodegradable polyester block copolymer, biodegradable polyester polymer, or biodegradable polyester random copolymer may be made using methods known in the art and/or as described in U.S. Pat. Nos. 6,517,869 and 6,267,987, the contents of which are each incorporated herein by reference in their entirety. The linear biodegradable copolymer may be made using methods known in the art and/or as described in U.S. Pat. No. 6,652,886. The PAGA polymer may be made using methods known in the art

and/or as described in U.S. Pat. No. 6,217,912 herein incorporated by reference in its entirety. The PAGA polymer may be copolymerized to form a copolymer or block copolymer with polymers such as but not limited to, poly-L-lysine, polyargine, polyornithine, histones, avidin, protamines, polylactides and poly(lactide-co-glycolides). The biodegradable cross-linked cationic multi-block copolymers may be made my methods known in the art and/or as described in U.S. Pat. No. 8,057,821, 8,444,992 or U.S. Pub. No. 2012009145 each of which are herein incorporated by reference in their entireties. For example, the multi-block copolymers may be synthesized using linear polyethyleneimine (LPEI) blocks which have distinct patterns as compared to branched polyethyleneimines. Further, the composition or pharmaceutical composition may be made by the methods known in the art, described herein, or as described in U.S. Pub. No. 20100004315 or U.S. Pat. Nos. 6,267,987 and 6,217,912 each of which are herein incorporated by reference in their entireties.

[000972] The polynucleotides of the invention may be formulated with at least one degradable polyester which may contain polycationic side chains. Degradable polyesters include, but are not limited to, poly(serine ester), poly(L-lactide-co-L-lysine), poly(4-hydroxy-L-proline ester), and combinations thereof. In another embodiment, the degradable polyesters may include a PEG conjugation to form a PEGylated polymer.

[000973] The polynucleotides of the invention may be formulated with at least one crosslinkable polyester. Crosslinkable polyesters include those known in the art and described in US Pub. No. 20120269761, the contents of which is herein incorporated by reference in its entirety.

[000974] The polynucleotides of the invention may be formulated in or with at least one cyclodextrin polymer. Cyclodextrin polymers and methods of making cyclodextrin polymers include those known in the art and described in US Pub. No. 20130184453, the contents of which are herein incorporated by reference in its entirety.

[000975] The polynucleotides of the invention may be formulated in or with at least one delivery polymers, such as polyamides, dendritic macromolecules and carbohydrate-containing degradable polyesters. For example, where the polymer may be a cyclodextrin-based dendritic macromolecule comprising a cyclodextrin core and an oligoamine shell attached to the cyclodextrin core, as described in US Patent No. 8,685,368, the contents of which is herein incorporated by reference in its entirety.

[000976] In one embodiment, the polynucleotides of the invention may be formulated in or with at least one crosslinked cation-binding polymers. Crosslinked cation-binding polymers and methods of making crosslinked cation-binding polymers include those known in the art and

described in International Patent Publication No. WO2013 106072, WO201 3106073 and WO2013 106086, the contents of each of which are herein incorporated by reference in its entirety.

[000977] In one embodiment, the polynucleotides of the invention may be formulated in or with at least one branched polymer. Branched polymers and methods of making branched polymers include those known in the art and described in International Patent Publication No. WO2013 113071, the contents of each of which are herein incorporated by reference in its entirety.

[000978] In one embodiment, the polynucleotides may be formulated with a polymer comprising a plurality of polymeric branches, wherein at least one branch comprises at least one disulfide group and at least one vinyl group, which may an unsubstituted vinyl or a functionalized vinyl group, as described in International Patent Publication No. WO2014053654, the contents of which is herein incorporated by reference in its entirety.

[000979] In one embodiment, the polynucleotides of the invention may be formulated in or with at least PEGylated albumin polymer. PEGylated albumin polymer and methods of making PEGylated albumin polymer include those known in the art and described in US Patent Publication No. US2013023 1287, the contents of each of which are herein incorporated by reference in its entirety.

[000980] In one embodiment, the polymers described herein may be conjugated to a lipid-terminating PEG. As a non-limiting example, PLGA may be conjugated to a lipid-terminating PEG forming PLGA-DSPE-PEG. As another non-limiting example, PEG conjugates for use with the present invention are described in International Publication No. WO2008 103276, herein incorporated by reference in its entirety. The polymers may be conjugated using a ligand conjugate such as, but not limited to, the conjugates described in U.S. Pat. No. 8,273,363, herein incorporated by reference in its entirety.

[000981] In one embodiment, the polynucleotides disclosed herein may be mixed with the PEGs or the sodium phosphate/sodium carbonate solution prior to administration. In another embodiment, polynucleotides encoding a protein of interest may be mixed with the PEGs and also mixed with the sodium phosphate/sodium carbonate solution. In yet another embodiment, polynucleotides encoding a protein of interest may be mixed with the PEGs and polynucleotides encoding a second protein of interest may be mixed with the sodium phosphate/sodium carbonate solution.

[000982] In one embodiment, the polynucleotides described herein may be conjugated with another compound. Non-limiting examples of conjugates are described in US Patent Nos.

7,964,578 and 7,833,992, each of which are herein incorporated by reference in their entireties. In another embodiment, modified RNA of the present invention may be conjugated with conjugates of formula 1-122 as described in US Patent Nos. 7,964,578 and 7,833,992, each of which are herein incorporated by reference in their entireties. The polynucleotides described herein may be conjugated with a metal such as, but not limited to, gold. (See e.g., Giljohann et al. Journ. Amer. Chem. Soc. 2009 13 1(6): 2072-2073; herein incorporated by reference in its entirety). In another embodiment, the polynucleotides described herein may be conjugated and/or encapsulated in gold-nanoparticles. (International Pub. No. WO201216269 and U.S. Pub. No. 20120302940 and US20130177523; the contents of each of which is herein incorporated by reference in its entirety).

[000983] As described in U.S. Pub. No. 201000043 13, herein incorporated by reference in its entirety, a gene delivery composition may include a nucleotide sequence and a poloxamer. For example, the polynucleotides of the present invention may be used in a gene delivery composition with the poloxamer described in U.S. Pub. No. 20100004313.

[000984] In one embodiment, the polymer formulation of the present invention may be stabilized by contacting the polymer formulation, which may include a cationic carrier, with a cationic lipopolymer which may be covalently linked to cholesterol and polyethylene glycol groups. The polymer formulation may be contacted with a cationic lipopolymer using the methods described in U.S. Pub. No. 20090042829 herein incorporated by reference in its entirety. The cationic carrier may include, but is not limited to, polyethylenimine, poly(trimethylenimine), poly(tetramethylenimine), polypropylenimine, aminoglycoside-polyamine, dideoxy-diamino-b-cyclodextrin, spermine, spermidine, poly(2-dimethylamino)ethyl methacrylate, poly(lysine), poly(histidine), poly(arginine), cationized gelatin, dendrimers, chitosan, l,2-Dioleoyl-3-Trimethylammonium-Propane(DOTAP), N-[l-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), l-[2-(oleoyloxy)ethyl]-2-oleyl-3-(2-hydroxyethyl)imidazolinium chloride (DOTIM), 2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-l-propanaminium trifluoroacetate (DOSPA), 3B-[N—(N',N'-Dimethylaminoethane)-carbamoyl]Cholesterol Hydrochloride (DC-Cholesterol HC1) diheptadecylamidoglycyl spermidine (DOGS), N,N-distearyl-N,N-dimethylammonium bromide (DDAB), N-(l,2-dimyristyloxyprop-3-yl)-N,N-dimethyl-N-hydroxyethyl ammonium bromide (DMRIE), N,N-dioleyl-N,N-dimethylammonium chloride DODAC) and combinations thereof. As a non-limiting example, the polynucleotides may be formulated with a cationic lipopolymer such as those described in U.S. Patent Application No. 20130065942, herein incorporated by reference in its entirety.

[000985] The polynucleotides of the invention may be formulated in a polyplex of one or more polymers (See e.g., U.S. Pat. No. 8,501,478, U.S. Pub. No. 20120237565, 20120270927, 20130149783 and 20130344117 and International Patent Pub. No. WO2013090861; the contents of each of which is herein incorporated by reference in its entirety). As a non-limiting example, the polyplex may be formed using the novel alpha-aminoamidine polymers described in International Publication No. WO2013090861, the contents of which are herein incorporated by reference in its entirety. As another non-limiting example, the polyplex may be formed using the click polymers described in US Patent No. 8,501,478, the contents of which is herein incorporated by reference in its entirety. As yet another non-limiting example, the polyplex may comprise a cationic polymer having formula I, I-a, I-b, II, III, Ill-a, Ill-b, IV, V, V-a, V-b, VI, Vl-b, Vl-a, VII as described in US Patent Publication No. US20130344117, the contents of which are herein incorporated by reference in its entirety.

[000986] In one embodiment, the polyplex comprises two or more cationic polymers. The catioinic polymer may comprise a poly(ethylene imine) (PEI) such as linear PEL In another embodiment, the polyplex comprises p(TETA/CBA) its PEGylated analog p(TETA/CBA)-g-PEG2k and mixtures thereof (see e.g., US Patent Publication No. US20130149783, the contents of which are herein incorporated by reference in its entirety.

[000987] The polynucleotides of the invention can also be formulated as a nanoparticle using a combination of polymers, lipids, and/or other biodegradable agents, such as, but not limited to, calcium phosphate. Components may be combined in a core-shell, hybrid, and/or layer-by-layer architecture, to allow for fine-tuning of the nanoparticle so to delivery of the polynucleotide, polynucleotides may be enhanced (Wang et al, Nat Mater. 2006 5:791-796; Fuller et al, Biomaterials. 2008 29:1526-1532; DeKoker et al, Adv Drug Deliv Rev. 2011 63:748-761; Endres et al, Biomaterials. 2011 32:7721-7731; Su et al, Mol Pharm. 2011 Jun 6;8(3):774-87; herein incorporated by reference in its entirety). As a non-limiting example, the nanoparticle may comprise a plurality of polymers such as, but not limited to hydrophilic-hydrophobic polymers (e.g., PEG-PLGA), hydrophobic polymers (e.g., PEG) and/or hydrophilic polymers (International Pub. No. WO20120225129; the contents of which is herein incorporated by reference in its entirety).

[000988] In one embodiment, the polynucleotides of the invention may be formulated in stably layer-by-layer coated particles, as described in or made by the methods described in US Patent Publication No. 20140093575, the contents of which are herein incorporated by reference in their entirety.

[000989] As another non-limiting example the nanoparticle comprising hydrophilic polymers for the polynucleotides may be those described in or made by the methods described in International Patent Publication No. WO2013 119936, the contents of which are herein incorporated by reference in its entirety.

[000990] In one embodiment, the biodegradable polymers which may be used in the present invention are poly(ether-anhydride) block copolymers. As a non-limiting example, the biodegradable polymers used herein may be a block copolymer as described in International Patent Publication No WO2006063249, herein incorporated by reference in its entirety, or made by the methods described in International Patent Publication No WO2006063249, herein incorporated by reference in its entirety.

[000991] In another embodiment, the biodegradable polymers which may be used in the present invention are alkyl and cycloalkyl terminated biodegradable lipids. As a non-limiting example, the alkyl and cycloalkyl terminated biodegradable lipids may be those described in International Publication No. WO2013086322 and/or made by the methods described in International Publication No. WO2013086322; the contents of which are herein incorporated by reference in its entirety.

[000992] In yet another embodiment, the biodegradable polymers which may be used in the present invention are cationic lipids having one or more biodegradable group located in a lipid moiety. As a non-limiting example, the biodegradable lipids may be those described in US Patent Publication No. US20130195920, the contents of which are herein incorporated by reference in its entirety.

[000993] In another embodiment, the biodegradable polymers which may be used in the present invention are described in US Patent No. US8535655, the contents of which are herein incorporated by reference in its entirety. The biodegradable polymer may comprise at least one bioactive moiety such as, but not limited to the polynucleotides described herein. The bioactive moieties may be pendant from and/or covalently bonded to the biodegradable polymer backbone and the bioactive moieties may be released at a rate equal to or faster than the rate of the biodegradation of the polymer backbone.

[000994] In one embodiment, biodegradable polymers described herein and/or those known in the art may be used in nanoparticles to deliver the polynucleotides described herein. As a non-limiting example, nanoparticles comprising biodegradable polymers which may be used to deliver nucleic acids such as the polynucleotides are described in US Patent No. US8628801, the contents of which are herein incorporated by reference in its entirety.

[000995] Biodegradable calcium phosphate nanoparticles in combination with lipids and/or polymers have been shown to deliver polynucleotides *in vivo.* In one embodiment, a lipid coated calcium phosphate nanoparticle, which may also contain a targeting ligand such as anisamide, may be used to deliver the polynucleotide, polynucleotides of the present invention. For example, to effectively deliver siRNA in a mouse metastatic lung model a lipid coated calcium phosphate nanoparticle was used (Li et al, J Contr Rel. 2010 142: 416-421; Li et al, J Contr Rel. 2012 158:108-114; Yang et al, Mol Ther. 2012 20:609-615; herein incorporated by reference in its entirety). This delivery system combines both a targeted nanoparticle and a component to enhance the endosomal escape, calcium phosphate, in order to improve delivery of the siRNA.

[000996] In one embodiment, calcium phosphate with a PEG-polyanion block copolymer may be used to delivery polynucleotides (Kazikawa et al, J Contr Rel. 2004 97:345-356; Kazikawa et al, J Contr Rel. 2006 111:368-370; the contents of each of which are herein incorporated by reference in its entirety).

[000997] In one embodiment, a PEG-charge-conversional polymer (Pitella et al, Biomaterials. 201 1 32:3 106-3 114; the contents of which are herein incorporated by reference in its entirety) may be used to form a nanoparticle to deliver the polynucleotides of the present invention. The PEG-charge-conversional polymer may improve upon the PEG-polyanion block copolymers by being cleaved into a polycation at acidic pH, thus enhancing endosomal escape.

[000998] In one embodiment, a polymer used in the present invention may be a pentablock polymer such as, but not limited to, the pentablock polymers described in International Patent Publication No. WO201305533 1, herein incorporated by reference in its entirety. As a non-limiting example, the pentablock polymer comprises PGA-PCL-PEG-PCL-PGA, wherein PEG is polyethylene glycol, PCL is poly(E-caprolactone), PGA is poly(glycolic acid), and PLA is poly(lactic acid). As another non-limiting example, the pentablock polymer comprises PEG-PCL- PLA-PCL-PEG, wherein PEG is polyethylene glycol, PCL is poly(E-caprolactone), PGA is poly(glycolic acid), and PLA is poly(lactic acid).

[000999] In one embodiment, a polymer which may be used in the present invention comprises at least one diepoxide and at least one aminoglycoside (See e.g., International Patent Publication No. WO2013055971, the contents of which are herein incorporated by reference in its entirety). The diepoxide may be selected from, but is not limited to, 1,4 butanediol diglycidyl ether (1,4 B), 1,4-cyclohexanedimethanol diglycidyl ether (1,4 C), 4-vinylcyclohexene diepoxide (4VCD), ethyleneglycol diglycidyl ether (EDGE), glycerol diglycidyl ether (GDE), neopentylglycol diglycidyl ether (NPDGE), poly(ethyleneglycol) diglycidyl ether (PEGDE), poly(propyleneglycol) diglycidyl ether (PPGDE) and resorcinol diglycidyl ether (RDE). The

aminoglycoside may be selected from, but is not limited to, streptomycin, neomycin, framycetin, paromomycin, ribostamycin, kanamycin, amikacin, arbekacin, bekanamycin, dibekacin, tobramycin, spectinomycin, hygromycin, gentamicin, netilmicin, sisomicin, isepamicin, verdamicin, astromicin, and apramycin. As a non-limiting example, the polymers may be made by the methods described in International Patent Publication No. WO2013055971, the contents of which are herein incorporated by reference in its entirety. As another non-limiting example, compositions comprising any of the polymers comprising at least one least one diepoxide and at least one aminoglycoside may be made by the methods described in International Patent Publication No. WO2013055971, the contents of which are herein incorporated by reference in its entirety.

[0001000] In one embodiment, a polymer which may be used in the present invention may be a cross-linked polymer. As a non-limiting example, the cross-linked polymers may be used to form a particle as described in US Patent No. 8,414,927, the contents of which are herein incorporated by reference in its entirety. As another non-limiting example, the cross-linked polymer may be obtained by the methods described in US Patent Publication No. US20 1301 72600, the contents of which are herein incorporated by reference in its entirety.

[0001001] In another embodiment, a polymer which may be used in the present invention may be a cross-linked polymer such as those described in US Patent No. 8,461,132, the contents of which are herein incorporated by reference in its entirety. As a non-limiting example, the cross-linked polymer may be used in a therapeutic composition for the treatment of a body tissue. The therapeutic composition may be administered to damaged tissue using various methods known in the art and/or described herein such as injection or catheterization.

[0001002] In one embodiment, a polymer which may be used in the present invention may be a di-alphatic substituted pegylated lipid such as, but not limited to, those described in International Patent Publication No. WO2013049328, the contents of which are herein incorporated by reference in its entirety.

[0001003] In another embodiment, a polymer which may be used in the delivery and/or formulation of polynucleotides is a pegylated polymer such as, but not limited to, those described in International Patent Publication No. WO2012099755, the contents of which are herein incorporated by reference in its entirety.

[0001004] In one embodiment, a block copolymer is PEG-PLGA-PEG (see e.g., the thermos ensitive hydrogel (PEG-PLGA-PEG) was used as a TGF-betal gene delivery vehicle in Lee et al. Thermosensitive Hydrogel as a Tgf-βï Gene Delivery Vehicle Enhances Diabetic Wound Healing. Pharmaceutical Research, 2003 20(12): 1995-2000; as a controlled gene

delivery system in Li et al. Controlled Gene Delivery System Based on Thermosensitive Biodegradable Hydrogel. Pharmaceutical Research 2003 20(6):884-888; and Chang et al., Non-ionic amphiphilic biodegradable PEG-PLGA-PEG copolymer enhances gene delivery efficiency in rat skeletal muscle. J Controlled Release. 2007 118:245-253; each of which is herein incorporated by reference in its entirety) may be used in the present invention. The present invention may be formulated with PEG-PLGA-PEG for administration such as, but not limited to, intramuscular and subcutaneous administration.

[0001005] In another embodiment, the PEG-PLGA-PEG block copolymer is used in the present invention to develop a biodegradable sustained release system. In one aspect, the polynucleotides of the present invention are mixed with the block copolymer prior to administration. In another aspect, the polynucleotides acids of the present invention are co-administered with the block copolymer.

[0001006] In one embodiment, the polymer used in the present invention may be a multi-functional polymer derivative such as, but not limited to, a multi-functional N-maleimidyl polymer derivatives as described in US Patent No US8454946, the contents of which are herein incorporated by reference in its entirety. In another embodiment, the polymer used in the present invention may be a multi-functional copolymer as described in US Patent Publication No. US20130236968, the contents of which are herein incorporated by reference in its entirety. As a non-limiting example, the multi-functional copolymer may have the formula (I), (II), (III), (IV), (V) or (VI) as described in US Patent Publication No. US20130236968, the contents of which are herein incorporated by reference in its entirety.

[0001007] In one embodiment, the polymer which may be used in the present invention is a co-polymer having formula A-L-D (A is a linear, branched or dendritic polyamine; D is a lipid; and L is a linker comprising a water soluble polymer) as described in International Patent Publication No. WO20 14025795, the contents of which are herein incorporated by reference in its entirety.

[0001008] The use of core-shell nanoparticles has additionally focused on a high-throughput approach to synthesize cationic cross-linked nanogel cores and various shells (Siegwart et al, Proc Natl Acad Sci U S A. 201 1 108:12996-13001; the contents of which are herein incorporated by reference in its entirety). The complexation, delivery, and internalization of the polymeric nanoparticles can be precisely controlled by altering the chemical composition in both the core and shell components of the nanoparticle. For example, the core-shell nanoparticles may efficiently deliver siRNA to mouse hepatocytes after they covalently attach cholesterol to the nanoparticle.

[0001009] In one embodiment, a hollow lipid core comprising a middle PLGA layer and an outer neutral lipid layer containing PEG may be used to delivery of the polynucleotide, polynucleotides of the present invention. As a non-limiting example, in mice bearing a luciferease-expressing tumor, it was determined that the lipid-polymer-lipid hybrid nanoparticle significantly suppressed luciferase expression, as compared to a conventional lipoplex (Shi et al, Angew Chem Int Ed. 201 1 50:7027-7031; herein incorporated by reference in its entirety).

[0001010] In one embodiment, the lipid nanoparticles may comprise a core of the polynucleotides disclosed herein and a polymer shell. The polymer shell may be any of the polymers described herein and are known in the art. In an additional embodiment, the polymer shell may be used to protect the polynucleotides in the core.

[0001011] Core-shell nanoparticles for use with the polynucleotides of the present invention are described and may be formed by the methods described in U.S. Pat. No. 8,3 13,777 or International Patent Publication No. WO201 3124867, the contents of each of which are herein incorporated by reference in their entirety.

[0001012] In one embodiment, the core-shell nanoparticles may comprise a core of the polynucleotides disclosed herein and a polymer shell. The polymer shell may be any of the polymers described herein and are known in the art. In an additional embodiment, the polymer shell may be used to protect the polynucleotides in the core.

[0001013] In one embodiment, the polymer used with the formulations described herein may be a modified polymer (such as, but not limited to, a modified polyacetal) as described in International Publication No. WO201 1120053, the contents of which are herein incorporated by reference in its entirety.

[0001014] In one embodiment, the formulation may be a polymeric carrier cargo complex comprising a polymeric carrier and at least one nucleic acid molecule. Non-limiting examples of polymeric carrier cargo complexes are described in International Patent Publications Nos. WO2013 113326, WO2013 113501, WO2013 113325, WO2013 113502 and WO2013 113736 and European Patent Publication No. EP2623 121, the contents of each of which are herein incorporated by reference in their entireties. In one aspect the polymeric carrier cargo complexes may comprise a negatively charged nucleic acid molecule such as, but not limited to, those described in International Patent Publication Nos. WO2013 113325 and WO2013 113502, the contents of each of which are herein incorporated by reference in its entirety.

[0001015] In one embodiment, a pharmaceutical composition may comprise polynucleotides of the invention and a polymeric carrier cargo complex. The polynucleotides may encode a protein of interest such as, but not limited to, an antigen from a pathogen associated with infectious

disease, an antigen associated with allergy or allergic disease, an antigen associated with autoimmune disease or an antigen associated with cancer or tumor disease (See e.g., the antigens described in International Patent Publications Nos. WO20131 13326, WO20131 13501, WO2013 113325, WO2013 113502 and WO2013 113736 and European Patent Publication No. EP2623 121, the contents of each of which are herein incorporated by reference in their entireties).

[0001016] As a non-limiting example, the core-shell nanoparticle may be used to treat an eye disease or disorder (See e.g. US Publication No. 20120321719, the contents of which are herein incorporated by reference in its entirety).

[0001017] In one embodiment, the polymer used with the formulations described herein may be a modified polymer (such as, but not limited to, a modified polyacetal) as described in International Publication No. WO201 1120053, the contents of which are herein incorporated by reference in its entirety.

Peptides and Proteins

[0001018] The polynucleotides of the invention can be formulated with peptides and/or proteins in order to increase transfection of cells by the polynucleotide. Peptides and/or proteins which may be used in the present invention are described in paragraphs [000540] - [000543] of co-pending International Publication No. WO2015034925, the contents of which is herein incorporated by reference in its entirety.

Cells

[0001019] The polynucleotides of the invention can be transfected *ex vivo* into cells, which are subsequently transplanted into a subject. As non-limiting examples, the pharmaceutical compositions may include red blood cells to deliver modified RNA to liver and myeloid cells, virosomes to deliver modified RNA in virus-like particles (VLPs), and electroporated cells such as, but not limited to, those described in paragraphs [000544] - [000546] of co-pending International Publication No. WO2015034925, the contents of which is herein incorporated by reference in its entirety.

Introduction Into Cells

[0001020] A variety of methods are known in the art and suitable for introduction of nucleic acid into a cell, including viral and non-viral mediated techniques. Examples of introduction methods which may be used in the present invention are described in paragraphs [000547] - [000549] of co-pending International Publication No. WO2015034925, the contents of which is herein incorporated by reference in its entirety. Micro-Organ

[0001021] The polynucleotides may be contained in a micro-organ which can then express an encoded polypeptide of interest in a long-lasting therapeutic formulation. Micro-organs and formulations thereof are described in International Patent Publication No. WO201415221 1, the contents of which are herein incorporated by reference in its entirety, such as in paragraphs [000701] - [000705].

Hyaluronidase

[0001022] The intramuscular or subcutaneous localized injection of polynucleotides of the invention can include hyaluronidase, which catalyzes the hydrolysis of hyaluronan. By catalyzing the hydrolysis of hyaluronan, a constituent of the interstitial barrier, hyaluronidase lowers the viscosity of hyaluronan, thereby increasing tissue permeability (Frost, Expert Opin. Drug Deliv. (2007) 4:427-440; herein incorporated by reference in its entirety). It is useful to speed their dispersion and systemic distribution of encoded proteins produced by transfected cells. Alternatively, the hyaluronidase can be used to increase the number of cells exposed to a polynucleotide of the invention administered intramuscularly or subcutaneously.

Nanoparticle Mimics

[0001023] The polynucleotides of the invention may be encapsulated within and/or absorbed to a nanoparticle mimic. A nanoparticle mimic can mimic the delivery function organisms or particles such as, but not limited to, pathogens, viruses, bacteria, fungus, parasites, prions and cells. As a non-limiting example the polynucleotides of the invention may be encapsulated in a non-viron particle which can mimic the delivery function of a virus (see International Pub. No. WO2012006376 and US Patent Publication No. US20130171241 and US20130195968, the contents of each of which are herein incorporated by reference in its entirety).

Nanotubes

[0001024] The polynucleotides of the invention can be attached or otherwise bound to at least one nanotube such as, but not limited to, rosette nanotubes, rosette nanotubes having twin bases with a linker, carbon nanotubes and/or single-walled carbon nanotubes, The polynucleotides may be bound to the nanotubes through forces such as, but not limited to, steric, ionic, covalent and/or other forces. Nanotubes and nanotube formulations comprising polynucleotides are described in International Patent Publication No. WO201415221 1, the contents of which are herein incorporated by reference in its entirety, such as in paragraphs [000708] - [000714].

Conjugates

[0001025] The polynucleotides of the invention include conjugates, such as a polynucleotide covalently linked to a carrier or targeting group, or including two encoding regions that together produce a fusion protein (e.g., bearing a targeting group and therapeutic protein or peptide).

[0001026] The conjugates of the invention include a naturally occurring substance, such as a protein (e.g., human serum albumin (HSA), low-density lipoprotein (LDL), high-density lipoprotein (HDL), or globulin); an carbohydrate (e.g., a dextran, pullulan, chitin, chitosan, inulin, cyclodextrin or hyaluronic acid); or a lipid. The ligand may also be a recombinant or synthetic molecule, such as a synthetic polymer, e.g., a synthetic polyamino acid, an oligonucleotide (e.g. an aptamer). Examples of polyamino acids include polyamino acid is a polylysine (PLL), poly L-aspartic acid, poly L-glutamic acid, styrene-maleic acid anhydride copolymer, poly(L-lactide-co-glycolied) copolymer, divinyl ether-maleic anhydride copolymer, N-(2-hydroxypropyl)methacrylamide copolymer (HMPA), polyethylene glycol (PEG), polyvinyl alcohol (PVA), polyurethane, poly(2-ethylacryllic acid), N-isopropylacrylamide polymers, or polyphosphazine. Example of polyamines include: polyethylenimine, polylysine (PLL), spermine, spermidine, polyamine, pseudopeptide-polyamine, peptidomimetic polyamine, dendrimer polyamine, arginine, amidine, protamine, cationic lipid, cationic porphyrin, quaternary salt of a polyamine, or an alpha helical peptide.

[0001027] Representative U.S. patents that teach the preparation of polynucleotide conjugates, particularly to RNA, include, but are not limited to, U.S. Pat. Nos. 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717, 5,580,731; 5,591,584; 5,109,124; 5,118,802; 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830; 5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241, 5,391,723; 5,416,203, 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and 5,688,941; 6,294,664; 6,320,017; 6,576,752; 6,783,931; 6,900,297; 7,037,646; each of which is herein incorporated by reference in their entireties.

[0001028] In one embodiment, the conjugate of the present invention may function as a carrier for the polynucleotides of the present invention. The conjugate may comprise a cationic polymer such as, but not limited to, polyamine, polylysine, polyalkylenimine, and polyethylenimine which may be grafted to with poly(ethylene glycol). As a non-limiting example, the conjugate may be similar to the polymeric conjugate and the method of synthesizing the polymeric conjugate described in U.S. Pat. No. 6,586,524 herein incorporated by reference in its entirety.

[0001029] A non-limiting example of a method for conjugation to a substrate is described in US Patent Publication No. US2013021 1249, the contents of which are herein incorporated by

reference in its entirety. The method may be used to make a conjugated polymeric particle comprising a polynucleotide.

[0001030] The conjugates can also include targeting groups, e.g., a cell or tissue targeting agent, e.g., a lectin, glycoprotein, lipid or protein, e.g., an antibody, that binds to a specified cell type such as a kidney cell. A targeting group can be a thyrotropin, melanotropin, lectin, glycoprotein, surfactant protein A, Mucin carbohydrate, multivalent lactose, multivalent galactose, N-acetyl-galactosamine, N-acetyl-D-glucosasamine, N-acetyl-glucosamine multivalent mannose, multivalent fucose, glycosylated polyaminoacids, multivalent galactose, transferrin, bisphosphonate, polyglutamate, polyaspartate, a lipid, cholesterol, a steroid, bile acid, folate, vitamin B12, biotin, an RGD peptide, an RGD peptide mimetic or an aptamer.

[0001031] Targeting groups can be proteins, e.g., glycoproteins, or peptides, e.g., molecules having a specific affinity for a co-ligand, or antibodies e.g., an antibody, that binds to a specified cell type such as a cancer cell, endothelial cell, or bone cell. Targeting groups may also include hormones and hormone receptors. They can also include non-peptidic species, such as lipids, lectins, carbohydrates, vitamins, cofactors, multivalent lactose, multivalent galactose, N-acetyl-galactosamine, N-acetyl-D-glucosasamine, N-acetyl-glucosamine multivalent mannose, multivalent fucose, or aptamers. The ligand can be, for example, a lipopolysaccharide, or an activator of p38 MAP kinase.

[0001032] The targeting group can be any ligand that is capable of targeting a specific receptor. Examples include, without limitation, folate, GalNAc, galactose, mannose, mannose-6P, apatamers, integrin receptor ligands, chemokine receptor ligands, transferrin, biotin, serotonin receptor ligands, PSMA, endothelin, GCPII, somatostatin, LDL, and HDL ligands. In particular embodiments, the targeting group is an aptamer. The aptamer can be unmodified or have any combination of modifications disclosed herein.

[0001033] As a non-limiting example, the targeting group may be a glutathione receptor (GR)-binding conjugate for targeted delivery across the blood-central nervious system barrier (See e.g., US Patent Publication No. US2013021661012, the contents of which are herein incorporated by reference in its entirety.

[0001034] In one embodiment, the conjugate of the present invention may be a synergistic biomolecule-polymer conjugate. The synergistic biomolecule-polymer conjugate may be long-acting continuous-release system to provide a greater therapeutic efficacy. The synergistic biomolecule-polymer conjugate may be those described in US Patent Publication No. US20130195799, the contents of which are herein incorporated by reference in its entirety.

[0001035] In one embodiment, the formulation may comprise a polymer conjugate which may be formulated into a nanoparticle, as described in US Patent No. 8,668,926, the contents of which is herein incorporated by reference in its entirety.

[0001036] In another embodiment, the conjugate which may be used in the present invention may be an aptamer conjugate. Non-limiting examples of apatamer conjugates are described in International Patent Publication No. WO20 12040524, the contents of which are herein incorporated by reference in its entirety. The aptamer conjugates may be used to provide targeted delivery of formulations comprising polynucleotides.

[0001037] In one embodiment, the conjugate which may be used in the present invention may be an amine containing polymer conjugate. Non-limiting examples of amine containing polymer conjugate are described in US Patent No. US 8,507,653, the contents of which are herein incorporated by reference in its entirety. The factor IX moiety polymer conjugate may comprise releasable linkages to release the polynucleotides upon and/or after delivery to a subject.

[0001038] In one embodiment, the pharmaceutical compositions of the present invention may include polymeric backbone having attached a therapeutically active agent and a bone targeting moiety, for example to treat or monitor bone-related diseases or disorders, as described in International Patent Publication WO2012153297, the contents of which is herein incorporated by reference in its entirety.

[0001039] In one embodiment, pharmaceutical compositions of the present invention may include chemical modifications such as, but not limited to, modifications similar to locked nucleic acids.

[0001040] Representative U.S. Patents that teach the preparation of locked nucleic acid (LNA) such as those from Santaris, include, but are not limited to, the following: U.S. Pat. Nos. 6,268,490; 6,670,461; 6,794,499; 6,998,484; 7,053,207; 7,084,125; and 7,399,845, each of which is herein incorporated by reference in its entirety.

[0001041] Representative U.S. patents that teach the preparation of PNA compounds include, but are not limited to, U.S. Pat. Nos. 5,539,082; 5,714,33 1; and 5,719,262, each of which is herein incorporated by reference. Further teaching of PNA compounds can be found, for example, in Nielsen *et ah,* Science, 1991, 254, 1497-1500.

[0001042] Some embodiments featured in the invention include polynucleotides with phosphorothioate backbones and oligonucleosides with other modified backbones, and in particular —CH_2—NH—CH_2--, --CH_2--N(CH_3)--0--CH_2--[known as a methylene (methylimino) or MMI backbone], ~CH_2~0--N(CH_3)--CH2~, ~CH2~N(CH_3)~N(CH_3)~CH2~ and ~N(CH_3)~CH2-CH2— [wherein the native phosphodiester backbone is represented as —O—P(0)$_2$~0~CH_2-

-] of the above-referenced U.S. Pat. No. 5,489,677, and the amide backbones of the above-referenced U.S. Pat. No. 5,602,240. In some embodiments, the polynucleotides featured herein have morpholino backbone structures of the above-referenced U.S. Pat. No. 5,034,506.

[0001043] Modifications at the 2' position may also aid in delivery. Preferably, modifications at the 2' position are not located in a polypepti de-coding sequence, i.e., not in a translatable region. Modifications at the 2' position may be located in a 5'UTR, a 3'UTR and/or a tailing region. Modifications at the 2' position can include one of the following at the 2' position: H (i.e., 2'-deoxy); F; 0-, S-, or N-alkyl; 0-, S-, or N-alkenyl; 0-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted Ci to Cio alkyl or C_2 to Cio alkenyl and alkynyl. Exemplary suitable modifications include $0[(CH_2)_nO]_mCH_3$, $0(CH_2)_nOCH_3$, $0(CH_2)_nNH_2$, $0(CH_2)_nCH_3$, $0(CH_2)_nONH_2$, and $0(CH_2)_nON[(CH_2)_nCH_3)]_2$, where n and m are from 1 to about 10. In other embodiments, the polynucleotides include one of the following at the 2' position: Ci to Cio lower alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH_3, OCN, CI, Br, CN, CF_3, OCF_3, $SOCH_3$, $S0_2CH_3$, $ON0_2$, $N0_2$, N_3, NH_2, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties, or a group for improving the pharmacodynamic properties, and other substituents having similar properties. In some embodiments, the modification includes a 2'-methoxyethoxy (2'-0-$CH_2CH_2OCH_3$, also known as 2'-0-(2-methoxyethyl) or 2'-MOE) (Martin *etal, Helv. Chim. Acta,* 1995, 78:486-504) *i.e.,* an alkoxy-alkoxy group. Another exemplary modification is 2'-dimethylaminooxyethoxy, *i.e.,* a $0(CH_2)_2ON(CH_3)_2$ group, also known as 2'-DMAOE, as described in examples herein below, and 2'-dimethylaminoethoxyethoxy (also known in the art as 2'-0-dimethylaminoethoxyethyl or 2'-DMAEOE), *i.e.,* 2'-0-CH_2-0-CH_2-$N(CH_2)_2$, also described in examples herein below. Other modifications include 2'-methoxy (2'-OCH_3), 2'-aminopropoxy (2'-$OCH_2CH_2CH_2NH_2$) and 2'-fluoro (2'-F). Similar modifications may also be made at other positions, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked dsRNAs and the 5' position of 5' terminal nucleotide. Polynucleotides of the invention may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative U.S. patents that teach the preparation of such modified sugar structures include, but are not limited to, U.S. Pat. Nos. 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; and 5,700,920; the contents of each of which is herein incorporated by reference in their entirety.

[0001044] In still other embodiments, the polynucleotide is covalently conjugated to a cell penetrating polypeptide. The cell-penetrating peptide may also include a signal sequence. The conjugates of the invention can be designed to have increased stability; increased cell transfection; and/or altered the biodistribution (e.g., targeted to specific tissues or cell types).

[0001045] In one embodiment, the polynucleotides may be conjugated to an agent to enhance delivery. As a non-limiting example, the agent may be a monomer or polymer such as a targeting monomer or a polymer having targeting blocks as described in International Publication No. WO201 1062965, herein incorporated by reference in its entirety. In another non-limiting example, the agent may be a transport agent covalently coupled to the polynucleotides of the present invention (See e.g., U.S. Pat. Nos. 6,835.393 and 7,374,778, each of which is herein incorporated by reference in its entirety). In yet another non-limiting example, the agent may be a membrane barrier transport enhancing agent such as those described in U.S. Pat. Nos. 7,737,108 and 8,003,129, each of which is herein incorporated by reference in its entirety.

[0001046] In another embodiment, polynucleotides may be conjugated to SMARTT POLYMER TECHNOLOGY® (PHASERX®, Inc. Seattle, WA).

[0001047] In another aspect, the conjugate may be a peptide that selectively directs the nanoparticle to neurons in a tissue or organism. As a non-limiting example, the peptide used may be, but is not limited to, the peptides described in US Patent Publication No US20 130129627, herein incorporated by reference in its entirety.

[0001048] In yet another aspect, the conjugate may be a peptide that can assist in crossing the blood-brain barrier.

[0001049] In one embodiment, the conjugate may be an aptamer-mRNA conjugate which may be used for targeted expression. As a non-limiting example, the aptamer-mRNA conjugate may include any of the aptamers and/or conjugates described in US Patent Publication No. US20130022538, the contents of which is herein incorporated by reference in its entirety. The aptamer-mRNA conjugate may include an aptamer component that can bind to a membrane associated protein on a target cell.

[0001050] In one embodiment, the conjugate may be a water-soluble polymer conjugate such as the conjugates described in US Patent No. US8636994, the contents of which are herein incorporated by reference in its entirety. As a non-limiting example, the water-soluble polymer conjugate may comprise at least one residue of an antimicrobial agent (see e.g., the conjugates described in US Patent No. US8636994, the contents of which are herein incorporated by reference in its entirety).

[0001051] In one embodiment, the conjugate may be a targeting amino acid chain bound to a biocompatiable polymer such as, but not limited to, the targeting amino acids and biocompatiable polymers described in International Patent Publication No. WO2014025890, the contents of which are herein incorporated by reference in its entirety. As a non-limiting example, the targeting amino acid may be any of the targeting amino acid chains described in SEQ ID NO: 1-62 of International Patent Publication No. WO2014025890, the contents of which are herein incorporated by reference in its entirety. In one embodiment, the targeting amino acid chain is smaller than 50 amino acids in length.

[0001052] In one embodiment, the conjugate may be a targeted poly amino-acid subunits which contains a targeting amino acid chain conjugated to a carboxylic acid such as, but not limited to, the targeting amino acids and carboxylic acids described in US Patent Publication No. US20 140045950, the contents of which are herein incorporated by reference in its entirety. In one embodiment, the targeted drug delivery vehicle comprises 5 to 50 targeted amino acid subunits. As a non-limiting example, the targeting amino acid may be any of the targeting amino acid chains described in SEQ ID NO: 1-62 of US Patent Publication No. US20140045950, the contents of which are herein incorporated by reference in its entirety.

Self-Assembled Nanoparticles

[0001053] The polynucleotides described herein may be formulated in self-assembled nanoparticles. Nucleic acid self-assembled nanoparticles are described in in International Patent Publication No. WO201415221 1 the contents of which are herein incorporated by reference in its entirety, such as in paragraphs [000740] - [000743]. Polymer-based self-assembled nanoparticles are described in International Patent Publication No. WO201415221 1, the contents of which are herein incorporated by reference in its entirety, such as in paragraphs [000744] - [000749]. *Self-Assembled Macromolecules*

[0001054] The polynucleotides may be formulated in amphiphilic macromolecules (AMs) for delivery. AMs comprise biocompatible amphiphilic polymers which have an alkylated sugar backbone covalently linked to poly(ethylene glycol). In aqueous solution, the AMs self-assemble to form micelles. Non-limiting examples of methods of forming AMs and AMs are described in US Patent Publication No. US20130217753, the contents of which are herein incorporated by reference in its entirety.

Inorganic Nanoparticles

[0001055] The polynucleotides of the present invention may be formulated in inorganic nanoparticles (U.S. Pat. No. 8,257,745, herein incorporated by reference in its entirety). The inorganic nanoparticles may include, but are not limited to, clay substances that are water

swellable. As a non-limiting example, the inorganic nanoparticle may include synthetic smectite clays which are made from simple silicates (See e.g., U.S. Pat. No. 5,585,108 and 8,257,745 each of which are herein incorporated by reference in their entirety).

[0001056] In one embodiment, the inorganic nanoparticles may comprise a core of the polynucleotides disclosed herein and a polymer shell. The polymer shell may be any of the polymers described herein and are known in the art. In an additional embodiment, the polymer shell may be used to protect the polynucleotides in the core.

Semi-conductive and Metallic Nanoparticles

[0001057] The polynucleotides of the present invention may be formulated in water-dispersible nanoparticle comprising a semiconductive or metallic material (U.S. Pub. No. 20120228565; herein incorporated by reference in its entirety) or formed in a magnetic nanoparticle (U.S. Pub. No. 20120265001 and 20120283503; each of which is herein incorporated by reference in its entirety). The water-dispersible nanoparticles may be hydrophobic nanoparticles or hydrophilic nanoparticles.

[0001058] In one embodiment, the semi-conductive and/or metallic nanoparticles may comprise a core of the polynucleotides disclosed herein and a polymer shell. The polymer shell may be any of the polymers described herein and are known in the art. In an additional embodiment, the polymer shell may be used to protect the polynucleotides in the core.

Micelles

[0001059] In one embodiment, the polynucleotides may be formulated in a micelle or coated on a micelle for delivery. As a non-limiting example, the micelle may be any of the micelles described in International Patent Publication No. WO2013 154774 and US Patent Publication No. US20130243867, the contents of each of which are herein incorporated by reference in its entirety. As a non-limiting example, the micelle may comprise polyethylene glycol-phosphatidyl ethanolamine (PEG-PE), a DC-cholesterol and a dioleoylphosphatidly-ethanolamine (DOPE). As another non-limiting example, the micelle may comprise at least one multiblock copolymer such as those described in International Patent Publication No. WO20 13 154774, the contents of which are herein incorporated by reference in its entirety.

[0001060] In one embodiment, the polynucleotides may be encapsulated in the polymeric micelles described in US Patent Publication No. US20130266617, the contents of which are herein incorporated by reference in its entirety.

Surgical Sealants: Gels and Hydrogels

[0001061] In one embodiment, the polynucleotides disclosed herein may be encapsulated into any hydrogel known in the art which may form a gel when injected into a subject. Surgical

sealants such as gels and hydrogels are described in International Patent Publication No. WO201415221 1, the contents of which are herein incorporated by reference in its entirety, such as in paragraphs [000762] - [000809].

Nanolipogel

[0001062] In one embodiment, the polynucleotides may be formulated in and/or delivered using a nanolipogel. A nanolipogel is a delivery vehicle which may include one or more lipid layers surrounding a hydrogel core. Nanolipogel formulation may be used to release the polynucleotides in a controlled fashion. Non-limiting examples of nanolipogels are described in International Patent Publication No. WO201 3155487, the contents of which are herein incorporated by reference in its entirety. As a non-limiting example, nanolipogels which may be used in the treatment of inflammatory and autoimmune disease and disorders are described in International Patent Publication No. WO2013 155493, the contents of which are herein incorporated by reference in its entirety.

Suspension formulations

[0001063] In some embodiments, suspension formulations are provided comprising polynucleotides, water immiscible oil depots, surfactants and/or co-surfactants and/or co-solvents. Combinations of oils and surfactants may enable suspension formulation with polynucleotides. Delivery of polynucleotides in a water immiscible depot may be used to improve bioavailability through sustained release of mRNA from the depot to the surrounding physiologic environment and prevent polynucleotides degradation by nucleases. Suspension formulations which may be used in the present invention are described in paragraphs [000640] - [000646] of co-pending International Publication No. WO20 15034925, the contents of which is herein incorporated by reference in its entirety

Cations and Anions

[0001064] Formulations of polynucleotides disclosed herein may include cations or anions. In one embodiment, the formulations include metal cations such as, but not limited to, Zn2+, Ca2+, Cu2+, Mg+ and combinations thereof. As a non-limiting example, formulations may include polymers and polynucleotides complexed with a metal cation (See e.g., U.S. Pat. Nos. 6,265,389 and 6,555,525, each of which is herein incorporated by reference in its entirety).

[0001065] In some embodiments, cationic nanoparticles comprising combinations of divalent and monovalent cations may be formulated with polynucleotides. Such nanoparticles may form spontaneously in solution over a give period (e.g. hours, days, etc). Such nanoparticles do not form in the presence of divalent cations alone or in the presence of monovalent cations alone.

The delivery of polynucleotides in cationic nanoparticles or in one or more depot comprising cationic nanoparticles may improve polynucleotide bioavailability by acting as a long-acting depot and/or reducing the rate of degradation by nucleases.

Molded Nanoparticles and Microparticles

[0001066] The polynucleotides disclosed herein may be formulated in nanoparticles and/or microparticles. These nanoparticles and/or microparticles may be molded into any size shape and chemistry. As an example, the nanoparticles and/or microparticles may be made using the PRINT® technology by LIQUIDA TECHNOLOGIES® (Morrisville, NC) (See e.g., International Pub. No. WO2007024323; the contents of which are herein incorporated by reference in its entirety).

[0001067] In one embodiment, the molded nanoparticles may comprise a core of the polynucleotides disclosed herein and a polymer shell. The polymer shell may be any of the polymers described herein and are known in the art. In an additional embodiment, the polymer shell may be used to protect the polynucleotides in the core.

[0001068] In one embodiment, the polynucleotides of the present invention may be formulated in microparticles. The microparticles may contain a core of the polynucleotides and a cortext of a biocompatible and/or biodegradable polymer. As a non-limiting example, the microparticles which may be used with the present invention may be those described in U.S. Patent No. 8,460,709, U.S. Patent Publication No. US20130129830 and International Patent Publication No WO20 13075068, each of which is herein incorporated by reference in its entirety. As another non-limiting example, the microparticles may be designed to extend the release of the polynucleotides of the present invention over a desired period of time (see e.g, extended release of a therapeutic protein in U.S. Patent Publication No. US20130129830, herein incorporated by reference in its entirety).

[0001069] In another non-limiting example, the microparticles may be polymer microparticles containing multi-block vinylic polymers, as described in European Patent Publication EP2038320, the contents of which is herein incorporated by reference in its entirety.

[0001070] The microparticle for use with the present invention may have a diameter of at least 1 micron to at least 100 microns (e.g., at least 1 micron, at least 5 micron, at least 10 micron, at least 15 micron, at least 20 micron, at least 25 micron, at least 30 micron, at least 35 micron, at least 40 micron, at least 45 micron, at least 50 micron, at least 55 micron, at least 60 micron, at least 65 micron, at least 70 micron, at least 75 micron, at least 80 micron, at least 85 micron, at least 90 micron, at least 95 micron, at least 97 micron, at least 99 micron, and at least 100 micron).

[0001071] The micorparticle may be a hydrogel microparticle. In one embodiment, the hydrogel microparticle may be made using the methods described in International Patent publication No. WO20 140253 12, the contents of which are herein incorporated by reference in its entirety. The hydrogel microparticles may include one or more species of living cells attached thereon and/or encapsulated therein such as the hydrogel microparticles described in International Patent publication No. WO2014025312, the contents of which are herein incorporated by reference in its entirety. As a non-limiting example, the polynucleotides described herein may be formulated in or delivered using hydrogel microparticles

NanoJackets and NanoLiposomes

[0001072] The polynucleotides disclosed herein may be formulated in NanoJackets and NanoLiposomes by Keystone Nano (State College, PA). NanoJackets are made of compounds that are naturally found in the body including calcium, phosphate and may also include a small amount of silicates. Nanojackets may range in size from 5 to 50 nm and may be used to deliver hydrophilic and hydrophobic compounds such as, but not limited to, polynucleotides.

[0001073] NanoLiposomes are made of lipids such as, but not limited to, lipids which naturally occur in the body. NanoLiposomes may range in size from 60-80 nm and may be used to deliver hydrophilic and hydrophobic compounds such as, but not limited to, polynucleotides. In one aspect, the polynucleotides disclosed herein are formulated in a NanoLiposome such as, but not limited to, Ceramide NanoLiposomes.

Pseudovirions

[0001074] In one embodiment, the polynucleotides disclosed herein may be formulated in Pseudovirions (e.g., pseudo-virions). Pseudovirions which may be used in the present invention are described in paragraphs [000655] - [000660] of co-pending International Publication No. WO2015034925, the contents of which is herein incorporated by reference in its entirety.

Minicells

[0001075] In one aspect, the polynucleotides may be formulated in bacterial minicells. As a non-limiting example, bacterial minicells may be those described in International Publication No. WO2013088250 or US Patent Publication No. US20130177499, the contents of each of which are herein incorporated by reference in its entirety. The bacterial minicells comprising therapeutic agents such as polynucleotides described herein may be used to deliver the therapeutic agents to brain tumors. In one embodiment, the polynucleotides may be formulated in a composition of at least two separate types of minicells differing in their cargo. In one aspect, the polynucleotides of the invention may be formulated in a minicell and encode a polypeptide that contributes to resistance to a cancer chemotherapy agent in tumor cells, and may be

formulated together with a minicell that contains said cancer chemotherapy agent, along with a pharmaceutically acceptable carrier for the minicells, as described in US Patent No. 8,691,963, the contents of which is herein incorporated by reference in its entirety.

Semi-solid Compositions

[0001076] In one embodiment, the polynucleotides may be formulated with a hydrophobic matrix to form a semi-solid composition. As a non-limiting example, the semi-solid composition or paste-like composition may be made by the methods described in International Patent Publication No WO2013 07604, herein incorporated by reference in its entirety. The semi-solid composition may be a sustained release formulation as described in International Patent Publication No WO2013 07604, herein incorporated by reference in its entirety.

[0001077] In another embodiment, the semi-solid composition may further have a micro-porous membrane or a biodegradable polymer formed around the composition (see e.g., International Patent Publication No WO201307604, herein incorporated by reference in its entirety).

[0001078] The semi-solid composition using the polynucleotides of the present invention may have the characteristics of the semi-solid mixture as described in International Patent Publication No WO201307604, herein incorporated by reference in its entirety (e.g., a modulus of elasticity of at least 10^{-4} $N \cdot mm^{-2}$, and/or a viscosity of at least 100mPa-s).

Exosomes

[0001079] In one embodiment, the polynucleotides may be formulated in exosomes. The exosomes may be loaded with at least one polynucleotide and delivered to cells, tissues and/or organisms. As a non-limiting example, the polynucleotides may be loaded in the exosomes described in International Publication No. WO2013 084000, herein incorporated by reference in its entirety.

[0001080] In one embodiment, the exosome are obtained from cells that have been induced to undergo oxidative stress such as, but not limited to, the exosomes described in International Patent Publication No. WO2014028763, the contents of which are herein incorporated by reference in its entirety.

Silk-Based Delivery

[0001081] In one embodiment, the polynucleotides may be formulated in a sustained release silk-based delivery system. The silk-based delivery system may be formed by contacting a silk fibroin solution with a therapeutic agent such as, but not limited to, the polynucleotides described herein and/or known in the art. As a non-limiting example, the sustained release silk-based delivery system which may be used in the present invention and methods of making such

system are described in US Patent No 8530625 and US Patent Publication No. US20 1301 7761 1, the contents of each of which are herein incorporated by reference in their entirety.

Microparticles

[0001082] In one embodiment, formulations comprising polynucleotides may comprise microparticles. The microparticles may comprise a polymer described herein and/or known in the art such as, but not limited to, poly(a-hydroxy acid), a polyhydroxy butyric acid, a polycaprolactone, a polyorthoester and a polyanhydride. The microparticle may have adsorbent surfaces to adsorb biologically active molecules such as polynucleotides. As a non-limiting example microparticles for use with the present invention and methods of making microparticles are described in US Patent Publication No. US2013 195923, US20130195898 and US 20130236550 and US Patent No. 8,309,139 and 8,206,749, the contents of each of which are herein incorporated by reference in its entirety. As a non-limiting example, the formulations comprising polynucleotides may comprise any of the microparticles described in or made by the methods described in US Patent Publication No. US20130236550, the contents of which are herein incorporated by reference in its entirety.

[0001083] In another embodiment, the formulation may be a microemulsion comprising microparticles and polynucleotides. As a non-limiting example, microemulsions comprising microparticles are described in US Patent Publication No. US2013 195923 and US20130195898 and US Patent No. 8,309,139 and 8,206,749, the contents of each of which are herein incorporated by reference in its entirety.

Amino Acid Lipids

[0001084] In one embodiment, the polynucleotides may be formulated in amino acid lipids. Amino acid lipids are lipophilic compounds comprising an amino acid residue and one or more lipophilic tails. Non-limiting examples of amino acid lipids and methods of making amino acid lipids are described in US Patent No. 8,501,824 and US Patent Publication No. US20140037714, the contents of each of which are herein incorporated by reference in their entirety.

[0001085] In one embodiment, the amino acid lipids have a hydrophilic portion and a lipophilic portion. The hydrophilic portion may be an amino acid residue and a lipophilic portion may comprise at least one lipophilic tail.

[0001086] In one embodiment, the amino acid lipid formulations may be used to deliver the polynucleotides to a subject.

[0001087] In another embodiment, the amino acid lipid formulations may deliver a polynucleotide in releasable form which comprises an amino acid lipid that binds and releases the polynucleotides. As a non-limiting example, the release of the polynucleotides may be

provided by an acid-labile linker such as, but not limited to, those described in U.S. Patent Nos. 7,098,032, 6,897,196, 6,426,086, 7,138,382, 5,563,250, and 5,505,931, the contents of each of which are herein incorporated by reference in its entirety.

[0001088] In one embodiment, the amino acid lipid is a targeting amino acid lipid as described in International Publication No., WO2013 135359, the contents of which are herein incorporated by reference in its entirety, such as but not limited to an amino acid lipid having formula I. As a non-limiting example, the targeting amino acid may target specific tissues and/or cells.

[0001089] In another embodiment, the amino acid lipid is an ether-lipid having the general formula I as described in WO2013 135360, the contents of which are herein incorporated by reference in its entirety.

[0001090] In one embodiment, the amino acid lipid is an amino acid lipid of Formula I as described in US Patent Publication No., US20140037714, the contents of which are herein incorporated by reference in its entirety.

Microvesicles

[0001091] In one embodiment, polynucleotides may be formulated in microvesicles. Non-limiting examples of microvesicles include those described in US Patent Publication No. US20 13 0209544, the contents of which are herein incorporated by reference in its entirety.

[0001092] In one embodiment, the microvesicle is an ARRDC1-mediated microvesicles (ARMMs). Non-limiting examples of ARMMs and methods of making ARMMs are described in International Patent Publication No. WO2013 119602, the contents of which are herein incorporated by reference in its entirety.

[0001093] In one embodiment, the microvesicles which may be used to formulate polynucleotides may be made by the methods described in International Publication No. WO2013 138427, the contents of which are herein incorporated by reference in its entirety. As a non-limiting example, microvesicles comprising polynucleotides may be used to treat diseases such as cancer as described in International Publication No. WO201 3138427, the contents of which are herein incorporated by reference in its entirety.

[0001094] In one embodiment, the microvesicles which may be used to formulate polynucleotides may be cell-derived microvesicles described in US Publication No. US20130195765, the contents of which are herein incorporated by reference in its entirety. As a non-limiting example, microvesicles comprising polynucleotides may be used to treat diseases such as cancer as described in US Publication No. US20130195765, the contents of which are herein incorporated by reference in its entirety.

Interpolyelectrolyte Complexes

[0001095] In one embodiment, the polynucleotides may be formulated in an interpolyelectrolyte complex. Interpolyelectrolyte complexes are formed when charge-dynamic polymers are complexed with one or more anionic molecules. Non-limiting examples of charge-dynamic polymers and interpolyelectrolyte complexes and methods of making interpolyelectrolyte complexes are described in US Patent No. 8,524,368, the contents of which is herein incorporated by reference in its entirety.

Cyrstalline Polymeric Systems

[0001096] In one embodiment, the polynucleotides may be formulated in crystalline polymeric systems. Crystalline polymeric systems are polymers with crystalline moieties and/or terminal units comprising crystalline moieties. Non-limiting examples of polymers with crystalline moieties and/or terminal units comprising crystalline moieties termed "CYC polymers," crystalline polymer systems and methods of making such polymers and systems are described in US Patent No. US 8,524,259, the contents of which are herein incorporated by reference in its entirety.

Polymer and Synthetic Scaffolds

[0001097] In one embodiment, the polynucleotides may be formulated in, delivered by or associated with polymer scaffolds. In one embodiment, the polymer scaffold may be a polyester urethane polymer scaffold (PEUR).

[0001098] In one embodiment, the polynucleotides may be formulated in, delivered by or associated with biodegradable, synthetic scaffolds such as, but not limited to, prefabricated ε-caprolactone and ethyl ethylene phosphate copolymer (PCLEEP) nanofibers, poly(lactic-co-glycolic acid) (PLGA) nanofibers, and porous polyester urethane (PEUR) scaffold design (see e.g., Nelson et al. *Tunable Delivery of siRNA from a Biodegradable Scaffold to Promote Angiogenesis In Vivo.* Adv. Mater. 2013; the contents of which are herein incorporated by reference in its entirety).

Polymer Implant

[0001099] In one embodiment, the polynucleotides may be formulated in or delivered using polymer implants. As a non-limiting example, the polymer implant is inserted into or onto damaged human tissue and the polynucleotides are released from the polymer implant. (See e.g., MariGen Omega3 from Kerecis for the treatment of damaged tissue).

[0001100] In one embodiment, the polynucleotides may be formulated in or delivered using delivery devices comprising polymer implants.

Lipomers

[0001101] In one embodiment, the polynucleotides may be formulated in or delivered using a conjugated lipomer. As a non-limiting example, the conjugated lipomer may be a conjugated polyethyleneimine (PEI) polymer or a conjugated aza-macrocycle which contains one or more groups of the formula (iii) as described in International Patent Publication No. WO2012135025, the contents of which are herein incorporated by reference in its entirey.

Poloxamer Delivery

[0001102] In one embodiment, the polynucleotides may be formulated in or delivered using a pharmaceutical vehicle comprising at least one poloxamer. In one embodiment, the pharmaceutical vehicle may be suitable for the delivery of drugs to the mucosal surfaces such as, but not limited to, the pharmaceutical vehicles described in International Patent Publication No. WO20 14027006, the contents of which are herein incorporated by reference in its entirety. As a non-limiting example the poloxamers used in the pharmaceutical vehicles are Poloxamer 407 and Poloxamer 188.

Excipients

[0001103] Pharmaceutical formulations may additionally comprise a pharmaceutically acceptable excipient, which, as used herein, includes, but are not limited to, any and all solvents, dispersion media, diluents, or other liquid vehicles, dispersion or suspension aids, surface active agents, isotonic agents, thickening or emulsifying agents, preservatives, solid binders, lubricants, flavoring agents, stabilizers, antioxidants, osmolality adjusting agents, pH adjusting agents and the like, as suited to the particular dosage form desired. Various excipients for formulating pharmaceutical compositions and techniques for preparing the composition are known in the art (see *Remington: The Science and Practice of Pharmacy*, 21st Edition, A. R. Gennaro (Lippincott, Williams & Wilkins, Baltimore, MD, 2006; incorporated herein by reference in its entirety). The use of a conventional excipient medium may be contemplated within the scope of the present disclosure, except insofar as any conventional excipient medium is incompatible with a substance or its derivatives, such as by producing any undesirable biological effect or otherwise interacting in a deleterious manner with any other component(s) of the pharmaceutical composition, its use is contemplated to be within the scope of this invention.

[0001104] In some embodiments, a pharmaceutically acceptable excipient may be at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% pure. In some embodiments, an excipient is approved for use for humans and for veterinary use. In some embodiments, an excipient may be approved by United States Food and Drug Administration. In some embodiments, an excipient may be of pharmaceutical grade. In some embodiments, an excipient

may meet the standards of the United States Pharmacopoeia (USP), the European Pharmacopoeia (EP), the British Pharmacopoeia, and/or the International Pharmacopoeia.

[0001105] Pharmaceutically acceptable excipients used in the manufacture of pharmaceutical compositions include, but are not limited to, inert diluents, dispersing and/or granulating agents, surface active agents and/or emulsifiers, disintegrating agents, binding agents, preservatives, buffering agents, lubricating agents, and/or oils. Such excipients may optionally be included in pharmaceutical compositions. The composition may also include excipients such as cocoa butter and suppository waxes, coloring agents, coating agents, sweetening, flavoring, and/or perfuming agents.

[0001106] Exemplary diluents, granulating and/or dispersing agents, surface active agents and/or emulsifiers, binding agents, preservatives, buffers, lubricating agents, oils, additives, cocoa butter and suppository waxes, coloring agents, coating agents, sweetening, flavoring, and/or perfuming agents are described in co-pending International Patent Publication No. WO2015038892, the contents of which is incorporated by reference in its entirety, such as, but not limited to, in paragraphs [000828] - [000838].

Cryoprotectants for mRNA

[0001107] In some embodiments, polynucleotide formulations may comprise cyroprotectants. As used herein, there term "cryoprotectant" refers to one or more agent that when combined with a given substance, helps to reduce or eliminate damage to that substance that occurs upon freezing. In some embodiments, cryoprotectants are combined with polynucleotides in order to stabilize them during freezing. Frozen storage of mRNA between -20°C and -80°C may be advantageous for long term (e.g. 36 months) stability of polynucleotide. In some embodiments, cryoprotectants are included in polynucleotide formulations to stabilize polynucleotide through freeze/thaw cycles and under frozen storage conditions. Cryoprotectants of the present invention may include, but are not limited to sucrose, trehalose, lactose, glycerol, dextrose, raffinose and/or mannitol. Trehalose is listed by the Food and Drug Administration as being generally regarded as safe (GRAS) and is commonly used in commercial pharmaceutical formulations.

Bulking agents

[0001108] In some embodiments, polynucleotide formulations may comprise bulking agents. As used herein, ther term "bulking agent" refers to one or more agents included in formulations to impart a desired consistency to the formulation and/or stabilization of formulation components. In some embodiments, bulking agents are included in lyophilized polynucleotide formulations to yield a "pharmaceutically elegant" cake, stabilizing the lyophilized polynucleotides during long

term (e.g. 36 month) storage. Bulking agents of the present invention may include, but are not limited to sucrose, trehalose, mannitol, glycine, lactose and/or raffinose. In some embodiments, combinations of cryoprotectants and bulking agents (for example, sucrose/glycine or trehalose/mannitol) may be included to both stabilize polynucleotides during freezing and provide a bulking agent for lyophilization.

[0001109] Non-limiting examples of formulations and methods for formulating the polynucleotides of the present invention are also provided in International Publication No WO2013090648 filed December 14, 2012, the contents of which are incorporated herein by reference in their entirety.

Inactive Ingredients

[0001110] In some embodiments, polynucleotide formulations may comprise at least one excipient which is an inactive ingredient. As used herein, ther term "inactive ingredient" refers to one or more inactive agents included in formulations. In some embodiments, all, none or some of the inactive ingredients which may be used in the formulations of the present invention may be approved by the US Food and Drug Administration (FDA). A non-exhaustive list of inactive ingredients and the routes of administration the inactive ingredients may be formulated in are described in Table 4 of co-pending International Application No. WO201415221 1 (Attorney Docket No. M030).

Delivery

[0001111] The present disclosure encompasses the delivery of polynucleotides for any of therapeutic, pharmaceutical, diagnostic or imaging by any appropriate route taking into consideration likely advances in the sciences of drug delivery. Delivery may be naked or formulated.

Naked Delivery

[0001112] The polynucleotides of the present invention may be delivered to a cell naked. As used herein in, "naked" refers to delivering polynucleotides free from agents which promote transfection. For example, the polynucleotides delivered to the cell may contain no modifications. The naked polynucleotides may be delivered to the cell using routes of administration known in the art and described herein.

Formulated Delivery

[0001113] The polynucleotides of the present invention may be formulated, using the methods described herein. The formulations may contain polynucleotides which may be modified and/or unmodified. The formulations may further include, but are not limited to, cell penetration agents, a pharmaceutically acceptable carrier, a delivery agent, a bioerodible or biocompatible

polymer, a solvent, and a sustained-release delivery depot. The formulated polynucleotides may be delivered to the cell using routes of administration known in the art and described herein.

[0001114] The compositions may also be formulated for direct delivery to an organ or tissue in any of several ways in the art including, but not limited to, direct soaking or bathing, via a catheter, by gels, powder, ointments, creams, gels, lotions, and/or drops, by using substrates such as fabric or biodegradable materials coated or impregnated with the compositions, and the like.

Administration

[0001115] The polynucleotides of the present invention may be administered by any route which results in a therapeutically effective outcome. These include, but are not limited to enteral (into the intestine), gastroenteral, epidural (into the dura matter), oral (by way of the mouth), transdermal, peridural, intracerebral (into the cerebrum), intracerebroventricular (into the cerebral ventricles), epicutaneous (application onto the skin), intradermal, (into the skin itself), subcutaneous (under the skin), nasal administration (through the nose), intravenous (into a vein), intravenous bolus, intravenous drip, intraarterial (into an artery), intramuscular (into a muscle), intracardiac (into the heart), intraosseous infusion (into the bone marrow), intrathecal (into the spinal canal), intraperitoneal, (infusion or injection into the peritoneum), intravesical infusion, intravitreal, (through the eye), intracavernous injection (into a pathologic cavity) intracavitary (into the base of the penis), intravaginal administration, intrauterine, extra-amniotic administration, transdermal (diffusion through the intact skin for systemic distribution), transmucosal (diffusion through a mucous membrane), transvaginal, insufflation (snorting), sublingual, sublabial, enema, eye drops (onto the conjunctiva), in ear drops, auricular (in or by way of the ear), buccal (directed toward the cheek), conjunctival, cutaneous, dental (to a tooth or teeth), electro-osmosis, endocervical, endosinusial, endotracheal, extracorporeal, hemodialysis, infiltration, interstitial, intra-abdominal, intra-amniotic, intra-articular, intrabiliary, intrabronchial, intrabursal, intracartilaginous (within a cartilage), intracaudal (within the cauda equine), intracisternal (within the cisterna magna cerebellomedularis), intracorneal (within the cornea), dental intracornal, intracoronary (within the coronary arteries), intracorporus cavernosum (within the dilatable spaces of the corporus cavernosa of the penis), intradiscal (within a disc), intraductal (within a duct of a gland), intraduodenal (within the duodenum), intradural (within or beneath the dura), intraepidermal (to the epidermis), intraesophageal (to the esophagus), intragastric (within the stomach), intragingival (within the gingivae), intraileal (within the distal portion of the small intestine), intralesional (within or introduced directly to a localized lesion), intraluminal (within a lumen of a tube), intralymphatic (within the lymph), intramedullary (within the marrow cavity of a bone), intrameningeal (within the meninges),

intramyocardial (within the myocardium), intraocular (within the eye), intraovarian (within the ovary), intrapericardial (within the pericardium), intrapleural (within the pleura), intraprostatic (within the prostate gland), intrapulmonary (within the lungs or its bronchi), intrasinal (within the nasal or periorbital sinuses), intraspinal (within the vertebral column), intrasynovial (within the synovial cavity of a joint), intratendinous (within a tendon), intratesticular (within the testicle), intrathecal (within the cerebrospinal fluid at any level of the cerebrospinal axis), intrathoracic (within the thorax), intratubular (within the tubules of an organ), intratumor (within a tumor), intratympanic (within the aurus media), intravascular (within a vessel or vessels), intraventricular (within a ventricle), iontophoresis (by means of electric current where ions of soluble salts migrate into the tissues of the body), irrigation (to bathe or flush open wounds or body cavities), laryngeal (directly upon the larynx), nasogastric (through the nose and into the stomach), occlusive dressing technique (topical route administration which is then covered by a dressing which occludes the area), ophthalmic (to the external eye), oropharyngeal (directly to the mouth and pharynx), parenteral, percutaneous, periarticular, peridural, perineural, periodontal, rectal, respiratory (within the respiratory tract by inhaling orally or nasally for local or systemic effect), retrobulbar (behind the pons or behind the eyeball), intramyocardial (entering the myocardium), soft tissue, subarachnoid, subconjunctival, submucosal, topical, transplacental (through or across the placenta), transtracheal (through the wall of the trachea), transtympanic (across or through the tympanic cavity), ureteral (to the ureter), urethral (to the urethra), vaginal, caudal block, diagnostic, nerve block, biliary perfusion, cardiac perfusion, photopheresis or spinal. In specific embodiments, compositions may be administered in a way which allows them cross the blood-brain barrier, vascular barrier, or other epithelial barrier. As a non-limiting example, formulations of the polynucleotides described herein may be delivered by intramyocardial injection. As another non-limiting example, formulations of the polynucleotides described herein may be delivered by intramyocardial injection into the ischemic region prior to, during or after coronary artery ligation.

[0001116] In one embodiment, a formulation for a route of administration may include at least one inactive ingredient. Non-limiting examples of routes of administration and inactive ingredients which may be included in formulations for the specific route of administration is shown in Table 9 of copending International Publication No. WO2015038892, the contents of which are herein incorporated by reference in its entirety. In the table, "AN" means anesthetic, "CNBLK" means cervical nerve block, "NBLK" means nerve block, "IV" means intravenous, "IM" means intramuscular and "SC" means subcutaneous.

[0001117] Non-limiting routes of administration for the polynucleotides of the present invention are described below.

Parenteral and Injectable Administration

[0001118] Liquid dosage forms for parenteral administration include, but are not limited to, pharmaceutically acceptable emulsions, microemulsions, solutions, suspensions, syrups, and/or elixirs. In addition to active ingredients, liquid dosage forms may comprise inert diluents commonly used in the art such as, for example, water or other solvents, solubilizing agents and emulsifiers such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, dimethylformamide, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor, and sesame oils), glycerol, tetrahydrofurfuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof. Besides inert diluents, oral compositions can include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, and/or perfuming agents. In certain embodiments for parenteral administration, compositions are mixed with solubilizing agents such as CREMOPHOR®, alcohols, oils, modified oils, glycols, polysorbates, cyclodextrins, polymers, and/or combinations thereof.

[0001119] A pharmaceutical composition for parenteral administration may comprise at least one inactive ingredient. Any or none of the inactive ingredients used may have been approved by the US Food and Drug Administration (FDA). A non-exhaustive list of inactive ingredients for use in pharmaceutical compositions for parenteral administration includes hydrochloric acid, mannitol, nitrogen, sodium acetate, sodium chloride and sodium hydroxide.

[0001120] Injectable preparations, for example, sterile injectable aqueous or oleaginous suspensions may be formulated according to the known art using suitable dispersing agents, wetting agents, and/or suspending agents. Sterile injectable preparations may be sterile injectable solutions, suspensions, and/or emulsions in nontoxic parenterally acceptable diluents and/or solvents, for example, as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, U.S.P., and isotonic sodium chloride solution. Sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil can be employed including synthetic mono- or diglycerides. Fatty acids such as oleic acid can be used in the preparation of injectables. The sterile formulation may also comprise adjuvants such as local anesthetics, preservatives and buffering agents.

[0001121] Injectable formulations can be sterilized, for example, by filtration through a bacterial-retaining filter, and/or by incorporating sterilizing agents in the form of sterile solid

compositions which can be dissolved or dispersed in sterile water or other sterile injectable medium prior to use.

[0001122] Injectable formulations may be for direct injection into a region of a tissue, organ and/or subject. As a non-limiting example, a tissue, organ and/or subject may be directly injected a formulation by intramyocardial injection into the ischemic region. (See e.g., Zangi et al. Nature Biotechnology 2013; the contents of which are herein incorporated by reference in its entirety).

[0001123] In order to prolong the effect of an active ingredient, it is often desirable to slow the absorption of the active ingredient from subcutaneous or intramuscular injection. This may be accomplished by the use of a liquid suspension of crystalline or amorphous material with poor water solubility. The rate of absorption of the drug then depends upon its rate of dissolution which, in turn, may depend upon crystal size and crystalline form. Alternatively, delayed absorption of a parenterally administered drug form is accomplished by dissolving or suspending the drug in an oil vehicle. Injectable depot forms are made by forming microencapsule matrices of the drug in biodegradable polymers such as polylactide-polyglycolide. Depending upon the ratio of drug to polymer and the nature of the particular polymer employed, the rate of drug release can be controlled. Examples of other biodegradable polymers include poly(orthoesters) and poly(anhydrides). Depot injectable formulations are prepared by entrapping the drug in liposomes or microemulsions which are compatible with body tissues.

[0001124] In one embodiment, injectable formulations may comprise an excipient in addition to the polynucleotides described herein. As a non-limiting example the excipient may be N-acetyl-D-glucosasamine.

[0001125] In one embodiment, formulations comprising the polynucleotides described herein may be formulated for intramuscular delivery may comprise an excipient. As a non-limiting example the excipient may be N-acetyl-D-glucosasamine.

Rectal and VaginalAdministration

[0001126] In one embodiment, the polynucleotides described here may be formulated for rectal and vaginal administration by the methods or compositions described in International Patent Publication No. WO201415221 1, the contents of which are incorporated by reference in its entirety, such as in paragraphs [000910] - [000913].

Oral Administration

[0001127] In one embodiment, the polynucleotides described here may be formulated for oral administration by the methods or compositions described in International Patent Publication No.

WO201415221 1, the contents of which are incorporated by reference in its entirety, such as in paragraphs [000914] - [000924].

Topical or Transdermal Administration

In one embodiment, the polynucleotides described here may be formulated for topical or transdermal administration by the methods or compositions described in International Patent Publication No. WO201415221 1, the contents of which are incorporated by reference in its entirety, such as in paragraphs [000925] - [000941].

Depot Administration

[0001128] In one embodiment, the polynucleotides described here may be formulated for depot administration by the methods or compositions described in International Patent Publication No. WO201415221 1, the contents of which are incorporated by reference in its entirety, such as in paragraphs [000942] - [000948].

Pulmonary Administration

[0001129] In one embodiment, the polynucleotides described here may be formulated for pulmonary administration by the methods or compositions described in International Patent Publication No. WO201415221 1, the contents of which are incorporated by reference in its entirety, such as in paragraphs [000949] - [000954].

Intranasal, Nasal and Buccal Administration

[0001130] In one embodiment, the polynucleotides described here may be formulated for intranasal, nasal or buccal administration by the methods or compositions described in International Patent Publication No. WO201415221 1, the contents of which are incorporated by reference in its entirety, such as in paragraphs [000955] - [000958].

Ophthalmic and Auricular (Otic) Administration

[0001131] In one embodiment, the polynucleotides described here may be formulated for ophthalmic or auricular (otic) administration by the methods or compositions described in International Patent Publication No. WO201415221 1, the contents of which are incorporated by reference in its entirety, such as in paragraphs [000959] - [000965].

Payload Administration: Detectable Agents and Therapeutic Agents

[0001132] The polynucleotides described herein can be used in a number of different scenarios in which delivery of a substance (the "payload") to a biological target is desired, for example delivery of detectable substances for detection of the target, or delivery of a therapeutic agent. Detection methods can include, but are not limited to, both imaging *in vitro* and *in vivo* imaging methods, *e.g.*, immunohistochemistry, bioluminescence imaging (BLI), Magnetic Resonance Imaging (MRI), positron emission tomography (PET), electron microscopy, X-ray computed

tomography, Raman imaging, optical coherence tomography, absorption imaging, thermal imaging, fluorescence reflectance imaging, fluorescence microscopy, fluorescence molecular tomographic imaging, nuclear magnetic resonance imaging, X-ray imaging, ultrasound imaging, photoacoustic imaging, lab assays, or in any situation where tagging/staining/imaging is required.

[0001133] The polynucleotides can be designed to include both a linker and a payload in any useful orientation. For example, a linker having two ends is used to attach one end to the payload and the other end to the nucleobase, such as at the C-7 or C-8 positions of the deaza-adenosine or deaza-guanosine or to the N-3 or C-5 positions of cytosine or uracil. The polynucleotide of the invention can include more than one payload (e.g., a label and a transcription inhibitor), as well as a cleavable linker. In one embodiment, the modified nucleotide is a modified 7-deaza-adenosine triphosphate, where one end of a cleavable linker is attached to the C7 position of 7-deaza-adenine, the other end of the linker is attached to an inhibitor (e.g., to the C5 position of the nucleobase on a cytidine), and a label (e.g., Cy5) is attached to the center of the linker (see, e.g., compound 1 of A*pCp C5 Parg Capless in Fig. 5 and columns 9 and 10 of U.S. Pat. No. 7,994,304, incorporated herein by reference). Upon incorporation of the modified 7-deaza-adenosine triphosphate to an encoding region, the resulting polynucleotide having a cleavable linker attached to a label and an inhibitor (e.g., a polymerase inhibitor). Upon cleavage of the linker (e.g., with reductive conditions to reduce a linker having a cleavable disulfide moiety), the label and inhibitor are released. Additional linkers and payloads (e.g., therapeutic agents, detectable labels, and cell penetrating payloads) are described herein and in International Publication No. WO2013151666 filed March 9, 2013 (Attorney Docket Number M300), the contents of which are incorporated herein by reference in their entirety.

[0001134] For example, the polynucleotides described herein can be used in reprogramming induced pluripotent stem cells (iPS cells), which can directly track cells that are transfected compared to total cells in the cluster. In another example, a drug that may be attached to the polynucleotides via a linker and may be fluorescently labeled can be used to track the drug *in vivo, e.g.* intracellularly. Other examples include, but are not limited to, the use of polynucleotides in reversible drug delivery into cells.

[0001135] The polynucleotides described herein can be used in intracellular targeting of a payload, *e.g.*, detectable or therapeutic agent, to specific organelle. Exemplary intracellular targets can include, but are not limited to, the nuclear localization for advanced mRNA

processing, or a nuclear localization sequence (NLS) linked to the mRNA containing an inhibitor.

[0001136] In addition, the polynucleotides described herein can be used to deliver therapeutic agents to cells or tissues, *e.g.,* in living animals. For example, the polynucleotides described herein can be used to deliver highly polar chemotherapeutics agents to kill cancer cells. The polynucleotides attached to the therapeutic agent through a linker can facilitate member permeation allowing the therapeutic agent to travel into a cell to reach an intracellular target. As a non-limiting example, a peptide or peptide composition may be used to facilitate delivery through the stratum corneum and/or the cellular membrane of viable cells such as the skin permeating and cell entering (SPACE) peptides described in WO20 12064429, the contents of which are herein incorporated by reference in its entirety. As another non-limiting example, nanoparticles designed to have enhanced entry into cancerous cells may be used to deliver the polynucleotides described herein (see e.g., the nanoparticles with a first shell comprising a first shell substance, a therapeutic agent and an endocytosis-enhancing agent (different from the therapeutic agent) described in International Patent Publication No. WO2013 173693, the contents of which are herein incorporated by reference in its entirety).

[0001137] In one example, the linker is attached at the 2'-position of the ribose ring and/or at the 3' and/or 5' position of the polynucleotides (See e.g., International Pub. No. WO2012030683, herein incorporated by reference in its entirety). The linker may be any linker disclosed herein, known in the art and/or disclosed in International Pub. No. WO2012030683, herein incorporated by reference in its entirety.

[0001138] In another example, the polynucleotides can be attached to the polynucleotides a viral inhibitory peptide (VIP) through a cleavable linker. The cleavable linker can release the VIP and dye into the cell. In another example, the polynucleotides can be attached through the linker to an ADP-ribosylate, which is responsible for the actions of some bacterial toxins, such as cholera toxin, diphtheria toxin, and pertussis toxin. These toxin proteins are ADP-ribosyltransferases that modify target proteins in human cells. For example, cholera toxin ADP-ribosylates G proteins modifies human cells by causing massive fluid secretion from the lining of the small intestine, which results in life-threatening diarrhea.

[0001139] In some embodiments, the payload may be a therapeutic agent such as a cytotoxin, radioactive ion, chemotherapeutic, or other therapeutic agent. A cytotoxin or cytotoxic agent includes any agent that may be detrimental to cells. Examples include, but are not limited to, taxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, teniposide, vincristine, vinblastine, colchicine, doxorubicin, daunorubicin,

dihydroxyanthracinedione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, puromycin, maytansinoids, *e.g.,* maytansinol (see U.S. Pat. No. 5,208,020 incorporated herein in its entirety), rachelmycin (CC-1065, see U.S. Pat. Nos. 5,475,092, 5,585,499, and 5,846,545, all of which are incorporated herein by reference), and analogs or homologs thereof. Radioactive ions include, but are not limited to iodine *(e.g.,* iodine 125 or iodine 131), strontium 89, phosphorous, palladium, cesium, iridium, phosphate, cobalt, yttrium 90, samarium 153, and praseodymium. Other therapeutic agents include, but are not limited to, antimetabolites (e.g., methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine), alkylating agents (e.g., mechlorethamine, thiotepa chlorambucil, rachelmycin (CC-1065), melphalan, carmustine (BSNU), lomustine (CCNU), cyclophosphamide, busulfan, dibromomannitol, streptozotocin, mitomycin C, and cis-dichlorodiamine platinum (II) (DDP) cisplatin), anthracyclines (e.g., daunorubicin (formerly daunomycin) and doxorubicin), antibiotics (e.g., dactinomycin (formerly actinomycin), bleomycin, mithramycin, and anthramycin (AMC)), and anti-mitotic agents (e.g., vincristine, vinblastine, taxol and maytansinoids).

[0001140] In some embodiments, the payload may be a detectable agent, such as various organic small molecules, inorganic compounds, nanoparticles, enzymes or enzyme substrates, fluorescent materials, luminescent materials (e.g., luminol), bioluminescent materials (e.g., luciferase, luciferin, and aequorin), chemiluminescent materials, radioactive materials (e.g., ^{18}F, ^{67}Ga, ^{81m}Kr, ^{82}Rb, ^{111}In, ^{123}1, ^{133}Xe, ^{201}T1, ^{125}1, ^{35}S, ^{14}C, ^{3}H, or ^{99m}Tc (e.g., as pertechnetate (technetate(VII), TcO_4^-)), and contrast agents (e.g., gold *(e.g.,* gold nanoparticles), gadolinium (e.g., chelated Gd), iron oxides (e.g., superparamagnetic iron oxide (SPIO), monocrystalline iron oxide nanoparticles (MIONs), and ultrasmall superparamagnetic iron oxide (USPIO)), manganese chelates (e.g., Mn-DPDP), barium sulfate, iodinated contrast media (iohexol), microbubbles, or perfluorocarbons). Such optically-detectable labels include for example, without limitation, 4-acetamido-4'-isothiocyanatostilbene-2,2'disulfonic acid; acridine and derivatives (e.g., acridine and acridine isothiocyanate); 5-(2'-aminoethyl)aminonaphthalene-l-sulfonic acid (EDANS); 4-amino-N-[3-vinylsulfonyl)phenyl]naphthalimide-3,5 disulfonate; N-(4-anilino-l-naphthyl)maleimide; anthranilamide; BODIPY; Brilliant Yellow; coumarin and derivatives (e.g., coumarin, 7-amino-4-methylcoumarin (AMC, Coumarin 120), and 7-amino-4-trifluoromethylcoumarin (Coumarin 151)); cyanine dyes; cyanosine; 4',6-diaminidino-2-phenylindole (DAPI); 5' 5''-dibromopyrogallol-sulfonaphthalein (Bromopyrogallol Red); 7-diethylamino-3-(4'-isothiocyanatophenyl)-4-methylcoumarin; diethylenetriamine pentaacetate; 4,4'-diisothiocyanatodihydro-stilbene-2,2'-disulfonic acid; 4,4'-diisothiocyanatostilbene-2,2'-

disulfonic acid; 5-[dimethylamino]-naphthalene-l-sulfonyl chloride (DNS, dansylchloride); 4-dimethylaminophenylazophenyl-4' -isothiocyanate (DABITC); eosin and derivatives (e.g., eosin and eosin isothiocyanate); erythrosin and derivatives (e.g., erythrosin B and erythrosin isothiocyanate); ethidium; fluorescein and derivatives (e.g., 5-carboxyfluorescein (FAM), 5-(4,6-dichlorotriazin-2-yl)aminofluorescein (DTAF), 2',7'-dimethoxy-4'5'-dichloro-6-carboxyfluorescein, fluorescein, fluorescein isothiocyanate, X-rhodamine-5-(and-6)-isothiocyanate (QFITC or XRITC), and fluorescamine); 2-[2-[3-[[l,3-dihydro-l,l-dimethyl-3-(3-sulfopropyl)-2H-benz[e]indol-2-ylidene]ethylidene]-2-[4-(ethoxycarbonyl)-l-piperazinyl]-l-cyclopenten- 1-yl]ethenyl] -1,1 -dimethyl-3 -(3 -sulforpropyl)- 1H-benz[e] indolium hydroxide, inner salt, compound with n,n-diethylethanamine(l:l) (IR144); 5-chloro-2-[2-[3-[(5-chloro-3-ethyl-2(3H)-benzothiazol- ylidene)ethylidene]-2-(dipheny lamino)-1-cyclopenten- 1-y 1] etheny 1] -3-ethyl benzothiazolium perchlorate (IR140); Malachite Green isothiocyanate; 4-methylumbelliferone orthocresolphthalein; nitrotyrosine; pararosaniline; Phenol Red; B-phycoerythrin; o-phthaldialdehyde; pyrene and derivatives(e.g., pyrene, pyrene butyrate, and succinimidyl 1-pyrene); butyrate quantum dots; Reactive Red 4 (CIBACRON™ Brilliant Red 3B-A); rhodamine and derivatives (e.g., 6-carboxy-X-rhodamine (ROX), 6-carboxyrhodamine (R6G), lissamine rhodamine B sulfonyl chloride rhodarnine (Rhod), rhodamine B, rhodamine 123, rhodamine X isothiocyanate, sulforhodamine B, sulforhodamine 101, sulfonyl chloride derivative of sulforhodamine 101 (Texas Red), N,N,N ',N 'tetramethyl-6-carboxyrhodamine (TAMRA) tetramethyl rhodamine, and tetramethyl rhodamine isothiocyanate (TRITC)); riboflavin; rosolic acid; terbium chelate derivatives; Cyanine-3 (Cy3); Cyanine-5 (Cy5); cyanine-5.5 (Cy5.5), Cyanine-7 (Cy7); IRD 700; IRD 800; Alexa 647; La Jolta Blue; phthalo cyanine; and naphthalo cyanine.

[0001141] In some embodiments, the detectable agent may be a non-detectable pre-cursor that becomes detectable upon activation (e.g., fluorogenic tetrazine-fluorophore constructs (e.g., tetrazine-BODIPY FL, tetrazine-Oregon Green 488, or tetrazine-BODIPY TMR-X) or enzyme activatable fluorogenic agents (e.g., PROSENSE® (VisEn Medical))). In vitro assays in which the enzyme labeled compositions can be used include, but are not limited to, enzyme linked immunosorbent assays (ELISAs), immunoprecipitation assays, immunofluorescence, enzyme immunoassays (EIA), radioimmunoassays (RIA), and Western blot analysis.

Combinations

[0001142] The polynucleotides may be used in combination with one or more other therapeutic, prophylactic, diagnostic, or imaging agents. By "in combination with," it is not intended to imply that the agents must be administered at the same time and/or formulated for delivery

together, although these methods of delivery are within the scope of the present disclosure. Compositions can be administered concurrently with, prior to, or subsequent to, one or more other desired therapeutics or medical procedures. In general, each agent will be administered at a dose and/or on a time schedule determined for that agent. In some embodiments, the present disclosure encompasses the delivery of pharmaceutical, prophylactic, diagnostic, or imaging compositions in combination with agents that may improve their bioavailability, reduce and/or modify their metabolism, inhibit their excretion, and/or modify their distribution within the body.

[0001143] In one embodiment, the polynucleotides described here may be used in combination with one or more other agents as described in International Patent Application No. PCT/US2014/027077, the contents of which are incorporated by reference in its entirety, such as in paragraphs [000978] - [001023].

[0001144] In one embodiment, polynucleotides may be co-administered with at least one amino acid and/or at least one small molecule additive. As used herein, "co-administered" means the administration of two or more components. These components for co-administration include, but are not limited to active ingredients, polynucleotides, amino acids, inactive ingredients and excipients.

[0001145] Non-limiting example of amino acids and other moieties for co-administration and methods and use thereof are described in co-pending International Application No. PCT/US2014/055394, filed September 12, 2014 (Attorney Docket No. M060.20), the contents of which are herein incorporated by reference in their entirety, specifically at pargraphs [000967] - [0001031].

[0001146] It will further be appreciated that therapeutically, prophylactically, diagnostically, or imaging active agents utilized in combination may be administered together in a single composition or administered separately in different compositions. In general, it is expected that agents utilized in combination with be utilized at levels that do not exceed the levels at which they are utilized individually. In some embodiments, the levels utilized in combination will be lower than those utilized individually. In one embodiment, the combinations, each or together may be administered according to the split dosing regimens described herein.

Dosing

[0001147] The present invention provides methods comprising administering modified mRNAs and their encoded proteins or complexes in accordance with the invention to a subject in need thereof. Nucleic acids, proteins or complexes, or pharmaceutical, imaging, diagnostic, or prophylactic compositions thereof, may be administered to a subject using any amount and any route of administration effective for preventing, treating, diagnosing, or imaging a disease,

disorder, and/or condition *(e.g.,* a disease, disorder, and/or condition relating to working memory deficits). The exact amount required will vary from subject to subject, depending on the species, age, and general condition of the subject, the severity of the disease, the particular composition, its mode of administration, its mode of activity, and the like. Compositions in accordance with the invention are typically formulated in dosage unit form for ease of administration and uniformity of dosage. It will be understood, however, that the total daily usage of the compositions of the present invention may be decided by the attending physician within the scope of sound medical judgment. The specific therapeutically effective, prophylactically effective, or appropriate imaging dose level for any particular patient will depend upon a variety of factors including the disorder being treated and the severity of the disorder; the activity of the specific compound employed; the specific composition employed; the age, body weight, general health, sex and diet of the patient; the time of administration, route of administration, and rate of excretion of the specific compound employed; the duration of the treatment; drugs used in combination or coincidental with the specific compound employed; and like factors well known in the medical arts.

[0001148] In certain embodiments, compositions in accordance with the present invention may be administered at dosage levels sufficient to deliver from about 0.0001 mg/kg to about 100 mg/kg, from about 0.001 mg/kg to about 0.05 mg/kg, from about 0.005 mg/kg to about 0.05 mg/kg, from about 0.001 mg/kg to about 0.005 mg/kg, from about 0.05 mg/kg to about 0.5 mg/kg, from about 0.01 mg/kg to about 50 mg/kg, from about 0.1 mg/kg to about 40 mg/kg, from about 0.5 mg/kg to about 30 mg/kg, from about 0.01 mg/kg to about 10 mg/kg, from about 0.1 mg/kg to about 10 mg/kg, or from about 1 mg/kg to about 25 mg/kg, of subject body weight per day, one or more times a day, to obtain the desired therapeutic, diagnostic, prophylactic, or imaging effect (see e.g., the range of unit doses described in International Publication No WO2013078199, herein incorporated by reference in its entirety). The desired dosage may be delivered three times a day, two times a day, once a day, every other day, every third day, every week, every two weeks, every three weeks, or every four weeks. In certain embodiments, the desired dosage may be delivered using multiple administrations *(e.g.,* two, three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, or more administrations). When multiple administrations are employed, split dosing regimens such as those described herein may be used.

[0001149] According to the present invention, it has been discovered that administration of polynucleotides in split-dose regimens produce higher levels of proteins in mammalian subjects. As used herein, a "split dose" is the division of single unit dose or total daily dose into two or

more doses, e.g, two or more administrations of the single unit dose. As used herein, a "single unit dose" is a dose of any therapeutic administed in one dose/at one time/single route/single point of contact, i.e., single administration event. As used herein, a "total daily dose" is an amount given or prescribed in 24 hr period. It may be administered as a single unit dose. In one embodiment, the polynucleotides of the present invention are administed to a subject in split doses. The polynucleotides may be formulated in buffer only or in a formulation described herein.

Dosage Forms

[0001150] A pharmaceutical composition described herein can be formulated into a dosage form described herein, such as a topical, intranasal, intratracheal, or injectable (e.g., intravenous, intraocular, intravitreal, intramuscular, intracardiac, intraperitoneal, subcutaneous).

Liquid dosageforms

[0001151] Liquid dosage forms for parenteral administration are described in co-pending International Patent Publication No. WO20 1503 8892, the contents of which is incorporated by reference in its entirety, such as, but not limited to, in paragraph [0001037].

Injectable

[0001152] Injectable preparations, for example, sterile injectable aqueous or oleaginous suspensions may be formulated according to the known art and may include suitable dispersing agents, wetting agents, and/or suspending agents. Sterile injectable preparations may be sterile injectable solutions, suspensions, and/or emulsions in nontoxic parenterally acceptable diluents and/or solvents, for example, a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed include, but are not limited to, water, Ringer's solution, U.S.P., and isotonic sodium chloride solution. Sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil can be employed including synthetic mono- or diglycerides. Fatty acids such as oleic acid can be used in the preparation of injectables.

[0001153] Injectable formulations can be sterilized, for example, by filtration through a bacterial-retaining filter, and/or by incorporating sterilizing agents in the form of sterile solid compositions which can be dissolved or dispersed in sterile water or other sterile injectable medium prior to use.

[0001154] In order to prolong the effect of an active ingredient, it may be desirable to slow the absorption of the active ingredient from subcutaneous or intramuscular injection. This may be accomplished by the use of a liquid suspension of crystalline or amorphous material with poor water solubility. The rate of absorption of the polynucleotides then depends upon its rate of

dissolution which, in turn, may depend upon crystal size and crystalline form. Alternatively, delayed absorption of a parenterally administered polynucleotides may be accomplished by dissolving or suspending the polynucleotides in an oil vehicle. Injectable depot forms are made by forming microencapsule matrices of the polynucleotides in biodegradable polymers such as polylactide-polyglycolide. Depending upon the ratio of polynucleotides to polymer and the nature of the particular polymer employed, the rate of polynucleotides release can be controlled. Examples of other biodegradable polymers include, but are not limited to, poly(orthoesters) and poly(anhydrides). Depot injectable formulations may be prepared by entrapping the polynucleotides in liposomes or microemulsions which are compatible with body tissues.

[0001155] Localized injection of naked DNA was demonstrated intramuscularly in 1990 and later was injected into several other tissues including liver, skin and brain. The uptake of the DNA was mostly localized in the area of the needle track. Different agents may be used to enhance overall gene expression. In one embodiment, the polynucleotides may be administered with an agent to enhance expression. Non-limiting examples of agents include transferrin, water-immiscible solvents, nonionic polymers, surfactants, and nuclease inhibitors.

[0001156] A needle-free delivery method known as jet injection may be used to deliver a drug to a tissue. The jet injection method uses a high-speed ultrafine stream of solution driven by a pressurized gas. The penetration power of this method may be adjusted by altering the gas pressure and the mechanical properties of the target tissue. The fluid being administered travels through the path of least resistance and may facilitate transport outside the traditional zone of delivery. As a non-limiting example, the solution may include the polynucleotides described herein. The solution (approximately 3-5 ul) may be loaded into the jet injection device and administered to a tissue at a pressure of approximately 1-3 bars. Commerical liquid jet injectors include, but are not limited to, Vitaject and bioject 2000 (Bioject), Advantagect (Activa systems), Injex 30 (Injex equidyne) and Mediject VISION (Antares Pharma).

[0001157] Microneedles may be used to inject the polynucleotides and formulations thereof described herein. Microneedles are an array of microstructured projections which can be coated with a drug that can be administered to a subject to provide delivery of therapeutic agents (e.g., polynucleotides) within the epidermis. Microneedles can be approximately 1 um in diameter and from about 1 um to about 100 um (e.g., about 1 um, about 2 um, about 3 um, about 4 um, about 5 um, about 6 um, about 7 um, about 8 um, about 9 um, about 10 um, about 12 um, about 14 um, about 15 um, about 16 um, about 18 um, about 20 um, about 25 um, about 30 um, about 35 um, about 40 um, about 45 um, about 50 um, about 55 um, about 60 um, about 65 um, about 70 um about 75 um, about 80 um, about 85 um, about 90 um, about 95 um, or about 100 um) in length.

The material used to make microneedles may be, but is not limited to, metals, silicon, silicon dioxide, polymers, glass and other materials and the material selected may depend on the type of agent to be delivered and the tissue contacted. In one embodiment, the miconeedles may be solid and may either be straight, bend or filtered. In one embodiment, the miconeedles may be hollow and may either be straight, bend or filtered.

[0001158] In one embodiment, the polynucleotides and formulations thereof may be administered using a microneedle drug delivery system. The microneedles may be hollow, solid or a combination thereof. As a non-limiting example, the microneedle drug delivery system may be the 3M Hollow Microstructured Transdermal System (hMTS). As another non-limiting example, the microneedle drug delivery system may be a microneedle patch comprising solid microneedle technology from 3M (3M Drug Delivery Systems).

[0001159] In one embodiment, the formulations described herein may be administered using a multi-prong needle device. As a non-limiting example, the device may administer more than one formulation in a single delivery. The formulations may be delivered at the same time or the formulations may have a pre-determined interval between each formulation delivery.

[0001160] In one embodiment, the formulations described herein may be administered to more than one location to a tissue, organ or subject at the same time using a multi-prong needle device. The formulations may be administered at the same time or the formulations may have a pre-determined interval between each administration of a formulation.

[0001161] In one embodiment, the amount of formulation comprising the polynucleotides administered may be varied depending on the type of injection and/or the cell, tissue or organ administered the formulation. As a non-limiting example, for intramuscular injection the formulation may be more concentrated to produce a polypeptide of interest as compared to a formulation for intravenous delivery.

Pulmonary

[0001162] Pulmonary and intranasal formulations for delivery and administration are described in co-pending International Patent Publication No. WO2013 15 1666, the contents of which is incorporated by reference in its entirety, such as, but not limited to, in paragraphs [000766] - [000781].

Coatings or Shells

[0001163] Solid dosage forms of tablets, dragees, capsules, pills, and granules can be prepared with coatings and shells such as enteric coatings and other coatings well known in the pharmaceutical formulating art. They may optionally comprise opacifying agents and can be of a composition that they release the active ingredient(s) only, or preferentially, in a certain part of

the intestinal tract, optionally, in a delayed manner. Examples of embedding compositions which can be used include polymeric substances and waxes. Solid compositions of a similar type may be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugar as well as high molecular weight polyethylene glycols and the like.

Multi-dose and repeat-dose administration

[0001164] In some embodiments, compounds and/or compositions of the present invention may be administered in two or more doses (referred to herein as "multi-dose administration"). Such doses may comprise the same components or may comprise components not included in a previous dose. Such doses may comprise the same mass and/or volume of components or an altered mass and/or volume of components in comparison to a previous dose. In some embodiments, multi-dose administration may comprise repeat-dose administration. As used herein, the term "repeat-dose administration" refers to two or more doses administered consecutively or within a regimen of repeat doses comprising substantially the same components provided at substantially the same mass and/or volume. In some embodiments, subjects may display a repeat-dose response. As used herein, the term "repeat-dose response" refers to a response in a subject to a repeat-dose that differs from that of another dose administered within a repeat-dose administration regimen. In some embodiments, such a response may be the expression of a protein in response to a repeat-dose comprising mRNA. In such embodiments, protein expression may be elevated in comparison to another dose administered within a repeat-dose administration regimen or protein expression may be reduced in comparison to another dose administered within a repeat-dose administration regimen. Alteration of protein expression may be from about 1% to about 20%, from about 5% to about 50% from about 10% to about 60%, from about 25% to about 75%, from about 40% to about 100% and/or at least 100%. A reduction in expression of mRNA administered as part of a repeat-dose regimen, wherein the level of protein translated from the administered RNA is reduced by more than 40% in comparison to another dose within the repeat-dose regimen is referred to herein as "repeat-dose resistance."

Properties of the Pharmaceutical Compositions

[0001165] The pharmaceutical compositions described herein can be characterized by one or more of the following properties:

Bioavailability

[0001166] The polynucleotides, when formulated into a composition with a delivery agent as described herein, can exhibit an increase in bioavailability as compared to a composition lacking a delivery agent as described herein. As used herein, the term "bioavailability" refers to the systemic availability of a given amount of polynucleotides administered to a mammal.

Bioavailability can be assessed by measuring the area under the curve (AUC) or the maximum serum or plasma concentration (C_{max}) of the unchanged form of a compound following administration of the compound to a mammal. AUC is a determination of the area under the curve plotting the serum or plasma concentration of a compound along the ordinate (Y-axis) against time along the abscissa (X-axis). Generally, the AUC for a particular compound can be calculated using methods known to those of ordinary skill in the art and as described in G. S. Banker, Modern Pharmaceutics, Drugs and the Pharmaceutical Sciences, v. 72, Marcel Dekker, New York, Inc., 1996, herein incorporated by reference in its entirety.

[0001167] The C_{max} value is the maximum concentration of the compound achieved in the serum or plasma of a mammal following administration of the compound to the mammal. The C_{max} value of a particular compound can be measured using methods known to those of ordinary skill in the art. The phrases "increasing bioavailability" or "improving the pharmacokinetics," as used herein mean that the systemic availability of a first polynucleotides, measured as AUC, C_{max}, or C_{min} in a mammal is greater, when co-administered with a delivery agent as described herein, than when such co-administration does not take place. In some embodiments, the bioavailability of the polynucleotides can increase by at least about 2%, at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or about 100%.

[0001168] In some embodiments, liquid formulations of polynucleotides may have varying in vivo half-life, requiring modulation of doses to yield a therapeutic effect. To address this, in some embodiments of the present invention, polynucleotides formulations may be designed to improve bioavailability and/or therapeutic effect during repeat administrations. Such formulations may enable sustained release of polynucleotides and/or reduce polynucleotide degradation rates by nucleases. In some embodiments, suspension formulations are provided comprising polynucleotides, water immiscible oil depots, surfactants and/or co-surfactants and/or co-solvents. Combinations of oils and surfactants may enable suspension formulation with polynucleotides. Delivery of polynucleotides in a water immiscible depot may be used to improve bioavailability through sustained release of polynucleotides from the depot to the surrounding physiologic environment and/or prevent polynucleotide degradation by nucleases.

[0001169] In some embodiments, cationic nanoparticles comprising combinations of divalent and monovalent cations may be formulated with polynucleotides. Such nanoparticles may form spontaneously in solution over a given period (e.g. hours, days, etc). Such nanoparticles do not

form in the presence of divalent cations alone or in the presence of monovalent cations alone. The delivery of polynucleotides in cationic nanoparticles or in one or more depot comprising cationic nanoparticles may improve polynucleotide bioavailability by acting as a long-acting depot and/or reducing the rate of degradation by nucleases.

Therapeutic Window

[0001170] The polynucleotides, when formulated into a composition with a delivery agent as described herein, can exhibit an increase in the therapeutic window of the administered polynucleotides composition as compared to the therapeutic window of the administered polynucleotides composition lacking a delivery agent as described herein. As used herein "therapeutic window" refers to the range of plasma concentrations, or the range of levels of therapeutically active substance at the site of action, with a high probability of eliciting a therapeutic effect. In some embodiments, the therapeutic window of the polynucleotides when co-administered with a delivery agent as described herein can increase by at least about 2%, at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or about 100%.

Volume of Distribution

[0001171] The polynucleotides, when formulated into a composition with a delivery agent as described herein, can exhibit an improved volume of distribution **(Vdi_st),** e.g., reduced or targeted, relative to a composition lacking a delivery agent as described herein. The volume of distribution (Vdist) relates the amount of the drug in the body to the concentration of the drug in the blood or plasma. As used herein, the term "volume of distribution" refers to the fluid volume that would be required to contain the total amount of the drug in the body at the same concentration as in the blood or plasma: Vdist equals the amount of drug in the body/concentration of drug in blood or plasma. For example, for a 10 mg dose and a plasma concentration of 10 mg/L, the volume of distribution would be 1 liter. The volume of distribution reflects the extent to which the drug is present in the extravascular tissue. A large volume of distribution reflects the tendency of a compound to bind to the tissue components compared with plasma protein binding. In a clinical setting, Vdist can be used to determine a loading dose to achieve a steady state concentration. In some embodiments, the volume of distribution of the polynucleotides when co-administered with a delivery agent as described herein can decrease at least about 2%, at least about 5%, at least about 10%, at least about 15%,

at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%.

Biological Effect

[0001172] In one embodiment, the biological effect of the modified mRNA delivered to the animals may be categorized by analyzing the protein expression in the animals. The protein expression may be determined from analyzing a biological sample collected from a mammal administered the modified mRNA of the present invention. In one embodiment, the expression protein encoded by the modified mRNA administered to the mammal of at least 50 pg/ml may be preferred. For example, a protein expression of 50-200 pg/ml for the protein encoded by the modified mRNA delivered to the mammal may be seen as a therapeutically effective amount of protein in the mammal.

Detection of Polynucleotides Acids by Mass Spectrometry

[0001173] Mass spectrometry (MS) is an analytical technique that can provide structural and molecular mass/concentration information on molecules after their conversion to ions. The molecules are first ionized to acquire positive or negative charges and then they travel through the mass analyzer to arrive at different areas of the detector according to their mass/charge (m/z) ratio. Methods of detecting polynucleotides are described in co-pending International Patent Publication No. WO2015038892, the contents of which is incorporated by reference in its entirety, such as, but not limited to, in paragraphs [0001055] - [0001067].

V. Uses of polynucleotides of the Invention

[0001174] The polynucleotides of the present invention are designed, in preferred embodiments, to provide for avoidance or evasion of deleterious bio-responses such as the immune response and/or degradation pathways, overcoming the threshold of expression and/or improving protein production capacity, improved expression rates or translation efficiency, improved drug or protein half life and/or protein concentrations, optimized protein localization, to improve one or more of the stability and/or clearance in tissues, receptor uptake and/or kinetics, cellular access by the compositions, engagement with translational machinery, secretion efficiency (when applicable), accessibility to circulation, and/or modulation of a cell's status, function and/or activity.

Therapeutics

Therapeutic Agents

[0001175] The polynucleotides of the present invention, such as, but not limited to, IVT polynucleotides, chimeric polynucleotides, modified nucleic acids and modified RNAs, and the

proteins translated from them described herein can be used as therapeutic or prophylactic agents. They are provided for use in medicine. For example, a polynucleotide described herein can be administered to a subject, wherein the polynucleotides is translated *in vivo* to produce a therapeutic or prophylactic polypeptide in the subject. Provided are compositions, methods, kits, and reagents for diagnosis, treatment or prevention of a disease or condition in humans and other mammals. The active therapeutic agents of the invention include polynucleotides, cells containing polynucleotides or polypeptides translated from the polynucleotides.

[0001176] In certain embodiments, provided herein are combination therapeutics containing one or more polynucleotides containing translatable regions that encode for a protein or proteins that boost a mammalian subject's immunity along with a protein that induces antibody-dependent cellular toxicity. For example, provided herein are therapeutics containing one or more nucleic acids that encode trastuzumab and granulocyte-colony stimulating factor (G-CSF). In particular, such combination therapeutics are useful in Her2+ breast cancer patients who develop induced resistance to trastuzumab. (See, e.g., Albrecht, Immunotherapy. 2(6):795-8 (2010)).

[0001177] Provided herein are methods of inducing translation of a recombinant polypeptide in a cell population using the polynucleotides described herein. Such translation can be *in vivo, ex vivo, in culture,* or *in vitro.* The cell population is contacted with an effective amount of a composition containing a nucleic acid that has at least one nucleoside modification, and a translatable region encoding the recombinant polypeptide. The population is contacted under conditions such that the nucleic acid is localized into one or more cells of the cell population and the recombinant polypeptide is translated in the cell from the nucleic acid.

[0001178] An "effective amount" of the composition is provided based, at least in part, on the target tissue, target cell type, means of administration, physical characteristics of the nucleic acid (e.g., size, and extent of modified nucleosides), and other determinants. In general, an effective amount of the composition provides efficient protein production in the cell, preferably more efficient than a composition containing a corresponding unmodified nucleic acid. Increased efficiency may be demonstrated by increased cell transfection (i.e., the percentage of cells transfected with the nucleic acid), increased protein translation from the nucleic acid, decreased nucleic acid degradation (as demonstrated, e.g., by increased duration of protein translation from a modified nucleic acid), or reduced innate immune response of the host cell.

[0001179] Aspects of the invention are directed to methods of inducing *in vivo* translation of a recombinant polypeptide in a mammalian subject in need thereof. Therein, an effective amount of a composition containing a nucleic acid that has at least one structural or chemical

modification and a translatable region encoding the recombinant polypeptide is administered to the subject using the delivery methods described herein. The nucleic acid is provided in an amount and under other conditions such that the nucleic acid is localized into a cell of the subject and the recombinant polypeptide is translated in the cell from the nucleic acid. The cell in which the nucleic acid is localized, or the tissue in which the cell is present, may be targeted with one or more than one rounds of nucleic acid administration.

[0001180] In certain embodiments, the administered polynucleotides directs production of one or more recombinant polypeptides that provide a functional activity which is substantially absent in the cell, tissue or organism in which the recombinant polypeptide is translated. For example, the missing functional activity may be enzymatic, structural, or gene regulatory in nature. In related embodiments, the administered polynucleotides directs production of one or more recombinant polypeptides that increases (e.g., synergistically) a functional activity which is present but substantially deficient in the cell in which the recombinant polypeptide is translated.

[0001181] In other embodiments, the administered polynucleotides directs production of one or more recombinant polypeptides that replace a polypeptide (or multiple polypeptides) that is substantially absent in the cell in which the recombinant polypeptide is translated. Such absence may be due to genetic mutation of the encoding gene or regulatory pathway thereof. In some embodiments, the recombinant polypeptide increases the level of an endogenous protein in the cell to a desirable level; such an increase may bring the level of the endogenous protein from a subnormal level to a normal level or from a normal level to a super-normal level.

[0001182] Alternatively, the recombinant polypeptide functions to antagonize the activity of an endogenous protein present in, on the surface of, or secreted from the cell. Usually, the activity of the endogenous protein is deleterious to the subject; for example, due to mutation of the endogenous protein resulting in altered activity or localization. Additionally, the recombinant polypeptide antagonizes, directly or indirectly, the activity of a biological moiety present in, on the surface of, or secreted from the cell. Examples of antagonized biological moieties include lipids (e.g., cholesterol), a lipoprotein (e.g., low density lipoprotein), a nucleic acid, a carbohydrate, a protein toxin such as shiga and tetanus toxins, or a small molecule toxin such as botulinum, cholera, and diphtheria toxins. Additionally, the antagonized biological molecule may be an endogenous protein that exhibits an undesirable activity, such as a cytotoxic or cytostatic activity.

[0001183] The recombinant proteins described herein may be engineered for localization within the cell, potentially within a specific compartment such as the nucleus, or are engineered for secretion from the cell or translocation to the plasma membrane of the cell.

[0001184] In some embodiments, modified mRNAs and their encoded polypeptides in accordance with the present invention may be used for treatment of any of a variety of diseases, disorders, and/or conditions, including but not limited to one or more of the following: autoimmune disorders *(e.g.* diabetes, lupus, multiple sclerosis, psoriasis, rheumatoid arthritis); inflammatory disorders *(e.g.* arthritis, pelvic inflammatory disease); infectious diseases *(e.g.* viral infections *(e.g.*, HIV, HCV, RSV), bacterial infections, fungal infections, sepsis); neurological disorders *(e.g.* Alzheimer's disease, Huntington's disease; autism; Duchenne muscular dystrophy); cardiovascular disorders *(e.g.* atherosclerosis, hypercholesterolemia, thrombosis, clotting disorders, angiogenic disorders such as macular degeneration); proliferative disorders *(e.g.* cancer, benign neoplasms); respiratory disorders *(e.g.* chronic obstructive pulmonary disease); digestive disorders *(e.g.* inflammatory bowel disease, ulcers); musculoskeletal disorders *(e.g.* fibromyalgia, arthritis); endocrine, metabolic, and nutritional disorders *(e.g.* diabetes, osteoporosis); urological disorders *(e.g.* renal disease); psychological disorders *(e.g.* depression, schizophrenia); skin disorders *(e.g.* wounds, eczema); blood and lymphatic disorders *(e.g.* anemia, hemophilia); *etc.*

[0001185] Diseases characterized by dysfunctional or aberrant protein activity include cystic fibrosis, sickle cell anemia, epidermolysis bullosa, amyotrophic lateral sclerosis, and glucoses-phosphate dehydrogenase deficiency. The present invention provides a method for treating such conditions or diseases in a subject by introducing nucleic acid or cell-based therapeutics containing the polynucleotides provided herein, wherein the polynucleotides encode for a protein that antagonizes or otherwise overcomes the aberrant protein activity present in the cell of the subject. Specific examples of a dysfunctional protein are the missense mutation variants of the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which produce a dysfunctional protein variant of CFTR protein, which causes cystic fibrosis.

[0001186] Diseases characterized by missing (or substantially diminished such that proper (normal or physiological protein function does not occur) protein activity include cystic fibrosis, Niemann-Pick type C, β thalassemia major, Duchenne muscular dystrophy, Hurler Syndrome, Hunter Syndrome, and Hemophilia A. Such proteins may not be present, or are essentially non-functional. The present invention provides a method for treating such conditions or diseases in a subject by introducing nucleic acid or cell-based therapeutics containing the polynucleotides provided herein, wherein the polynucleotides encode for a protein that replaces the protein activity missing from the target cells of the subject. Specific examples of a dysfunctional protein are the nonsense mutation variants of the cystic fibrosis transmembrane conductance

regulator (CFTR) gene, which produce a nonfunctional protein variant of CFTR protein, which causes cystic fibrosis.

[0001187] Thus, provided are methods of treating cystic fibrosis in a mammalian subject by contacting a cell of the subject with a polynucleotide having a translatable region that encodes a functional CFTR polypeptide, under conditions such that an effective amount of the CTFR polypeptide is present in the cell. Preferred target cells are epithelial, endothelial and mesothelial cells, such as the lung, and methods of administration are determined in view of the target tissue; i.e., for lung delivery, the RNA molecules are formulated for administration by inhalation, aerosolization (e.g., intratrachael aerosolixation), nebulization or instillation. As a non-limiting example, polynucleotides may be administered to the lung by the methods and compositions described in International Patent Publication Nos. WO2013 185069 and WO20 13 182683, the contents of each of which are herein incorporated by reference in their entirety. As another non-limiting example, the polynucleotides may be administered by aerosol delivery the methods and devices described in International Patent Publication No. WO2013 155513, the contents of which are herein incorporated by reference in its entirety.

[0001188] In another embodiment, the present invention provides a method for treating hyperlipidemia in a subject, by introducing into a cell population of the subject with a modified mRNA molecule encoding Sortilin, a protein recently characterized by genomic studies, thereby ameliorating the hyperlipidemia in a subject. The *SORT1* gene encodes a trans-Golgi network (TGN) transmembrane protein called Sortilin. Genetic studies have shown that one of five individuals has a single nucleotide polymorphism, rsl2740374, in the lpl3 locus of the SORT1 gene that predisposes them to having low levels of low-density lipoprotein (LDL) and very-low-density lipoprotein (VLDL). Each copy of the minor allele, present in about 30% of people, alters LDL cholesterol by 8 mg/dL, while two copies of the minor allele, present in about 5% of the population, lowers LDL cholesterol 16 mg/dL. Carriers of the minor allele have also been shown to have a 40% decreased risk of myocardial infarction. Functional *in vivo* studies in mice describes that overexpression of *SORT1* in mouse liver tissue led to significantly lower LDL-cholesterol levels, as much as 80% lower, and that silencing SORT1 increased LDL cholesterol approximately 200% (Musunuru K et al. From noncoding variant to phenotype via *SORT1* at the lpl3 cholesterol locus. *Nature* 2010; 466: 714-721).

[0001189] In another embodiment, the present invention provides a method for treating hematopoietic disorders, cardiovascular disease, oncology, diabetes, cystic fibrosis, neurological diseases, inborn errors of metabolism, skin and systemic disorders, and blindness. The identity of molecular targets to treat these specific diseases has been described (Templeton ed., Gene and

Cell Therapy: Therapeutic Mechanisms and Strategies, 3rd Edition, Bota Raton, FL:CRC Press; herein incorporated by reference in its entirety).

[0001190] Provided herein, are methods to prevent infection and/or sepsis in a subject at risk of developing infection and/or sepsis, the method comprising administering to a subject in need of such prevention a composition comprising a polynucleotide precursor encoding an anti-microbial polypeptide *(e.g.,* an anti-bacterial polypeptide), or a partially or fully processed form thereof in an amount sufficient to prevent infection and/or sepsis. In certain embodiments, the subject at risk of developing infection and/or sepsis may be a cancer patient. In certain embodiments, the cancer patient may have undergone a conditioning regimen. In some embodiments, the conditioning regiment may include, but is not limited to, chemotherapy, radiation therapy, or both. As a non-limiting example, a polynucleotide can encode Protein C, its zymogen or prepro-protein, the activated form of Protein C (APC) or variants of Protein C which are known in the art. The polynucleotides may be chemically modified and delivered to cells. Non-limiting examples of polypeptides which may be encoded within the chemically modified mRNAs of the present invention include those taught in US Patents 7,226,999; 7,498,305; 6,630,138 each of which is incorporated herein by reference in its entirety. These patents teach Protein C like molecules, variants and derivatives, any of which may be encoded within the chemically modified molecules of the present invention.

[0001191] Further provided herein, are methods to treat infection and/or sepsis in a subject, the method comprising administering to a subject in need of such treatment a composition comprising a polynucleotide precursor encoding an anti-microbial polypeptide *(e.g.,* an anti-bacterial polypeptide), *e.g.,* an anti-microbial polypeptide described herein, or a partially or fully processed form thereof in an amount sufficient to treat an infection and/or sepsis. In certain embodiments, the subject in need of treatment is a cancer patient. In certain embodiments, the cancer patient has undergone a conditioning regimen. In some embodiments, the conditioning regiment may include, but is not limited to, chemotherapy, radiation therapy, or both.

[0001192] In certain embodiments, the subject may exhibits acute or chronic microbial infections *(e.g.,* bacterial infections). In certain embodiments, the subject may have received or may be receiving a therapy. In certain embodiments, the therapy may include, but is not limited to, radiotherapy, chemotherapy, steroids, ultraviolet radiation, or a combination thereof. In certain embodiments, the patient may suffer from a microvascular disorder. In some embodiments, the microvascular disorder may be diabetes. In certain embodiments, the patient may have a wound. In some embodiments, the wound may be an ulcer. In a specific embodiment, the wound may be a diabetic foot ulcer. In certain embodiments, the subject may

have one or more burn wounds. In certain embodiments, the administration may be local or systemic. In certain embodiments, the administration may be subcutaneous. In certain embodiments, the administration may be intravenous. In certain embodiments, the administration may be oral. In certain embodiments, the administration may be topical. In certain embodiments, the administration may be by inhalation. In certain embodiments, the administration may be rectal. In certain embodiments, the administration may be vaginal.

[0001193] Other aspects of the present disclosure relate to transplantation of cells containing polynucleotides to a mammalian subject. Administration of cells to mammalian subjects is known to those of ordinary skill in the art, and include, but is not limited to, local implantation (e.g., topical or subcutaneous administration), organ delivery or systemic injection (e.g., intravenous injection or inhalation), and the formulation of cells in pharmaceutically acceptable carrier. Such compositions containing polynucleotides can be formulated for administration intramuscularly, transarterially, intraperitoneally, intravenously, intranasally, subcutaneously, endoscopically, transdermally, or intrathecally. In some embodiments, the composition may be formulated for extended release.

[0001194] The subject to whom the therapeutic agent may be administered suffers from or may be at risk of developing a disease, disorder, or deleterious condition. Provided are methods of identifying, diagnosing, and classifying subjects on these bases, which may include clinical diagnosis, biomarker levels, genome-wide association studies (GWAS), and other methods known in the art.

Wound Management

[0001195] The polynucleotides of the present invention may be used for wound treatment, *e.g.* of wounds exhibiting delayed healing. Provided herein are methods comprising the administration of polynucleotides in order to manage the treatment of wounds. Methods comprising the administration of polynucleotides in order to manage the treatment of wounds are described in co-pending International Patent Publication No. WO2015038892, the contents of which is incorporated by reference in its entirety, such as, but not limited to, in paragraphs [0001089] - [0001092].

Production of Antibodies

[0001196] In one embodiment of the invention, the polynucleotides may encode antibodies and fragments of such antibodies such as those described in co-pending International Patent Publication No. WO2015038892, the contents of which is incorporated by reference in its entirety, such as, but not limited to, in paragraphs [0001093] - [0001095].

Managing Infection

[0001197] In one embodiment, provided are methods for treating or preventing a microbial infection *(e.g.,* a bacterial infection) and/or a disease, disorder, or condition associated with a microbial or viral infection, or a symptom thereof, in a subject, by administering a polynucleotide encoding an anti-microbial polypeptide. The administration may be in combination with an anti-microbial agent *(e.g.,* an anti-bacterial agent), *e.g.,* an anti-microbial polypeptide or a small molecule anti-microbial compound described herein. The anti-microbial agents include, but are not limited to, anti-bacterial agents, anti-viral agents, anti-fungal agents, anti-protozoal agents, anti-parasitic agents, and anti-prion agents as well as compositions, delivery and methods of use of the polynucleotides herein are described in co-pending International Patent Publication No. WO20 1503 8892, the contents of which is incorporated by reference in its entirety, such as, but not limited to, in paragraphs [0001096] - [0001 116].

Modulation of the Immune Response

Avoidance of the immune response

[0001198] As described herein, a useful feature of the polynucleotides of the invention is the capacity to reduce, evade or avoid the innate immune response of a cell. In one aspect, provided herein are polynucleotides encoding a polypeptide of interest which when delivered to cells, results in a reduced immune response from the host as compared to the response triggered by a reference compound, e.g. an unmodified polynucleotide corresponding to a polynucleotide of the invention, or a different polynucleotides of the invention. As used herein, a "reference compound" is any molecule or substance which when administered to a mammal, results in an innate immune response having a known degree, level or amount of immune stimmulation. A reference compound need not be a nucleic acid molecule and it need not be any of the polynucleotides of the invention. Hence, the measure of polynucleotides avoidance, evasion or failure to trigger an immune response can be expressed in terms relative to any compound or substance which is known to trigger such a response.

[0001199] The term "innate immune response" includes a cellular response to exogenous single stranded nucleic acids, generally of viral or bacterial origin, which involves the induction of cytokine expression and release, particularly the interferons, and cell death. As used herein, the innate immune response or interferon response operates at the single cell level causing cytokine expression, cytokine release, global inhibition of protein synthesis, global destruction of cellular RNA, upregulation of major histocompatibility molecules, and/or induction of apoptotic death, induction of gene transcription of genes involved in apoptosis, anti-growth, and innate and adaptive immune cell activation. Some of the genes induced by type I IFNs include PKR, ADAR (adenosine deaminase acting on RNA), OAS (2',5'-oligoadenylate synthetase), RNase L, and Mx

proteins. PKR and ADAR lead to inhibition of translation initiation and RNA editing, respectively. OAS is a dsRNA-dependent synthetase that activates the endoribonuclease RNase L to degrade ssRNA.

[0001200] In some embodiments, the innate immune response comprises expression of a Type I or Type II interferon, and the expression of the Type I or Type II interferon is not increased more than two-fold compared to a reference from a cell which has not been contacted with a polynucleotide of the invention.

[0001201] In some embodiments, the innate immune response comprises expression of one or more IFN signature genes and where the expression of the one of more IFN signature genes is not increased more than three-fold compared to a reference from a cell which has not been contacted with the polynucleotides of the invention.

[0001202] While in some circumstances, it might be advantageous to eliminate the innate immune response in a cell, the invention provides polynucleotides that upon administration result in a substantially reduced (significantly less) the immune response, including interferon signaling, without entirely eliminating such a response.

[0001203] In some embodiments, the immune response is lower by 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 99%, 99.9%, or greater than 99.9% as compared to the immune response induced by a reference compound. The immune response itself may be measured by determining the expression or activity level of Type 1 interferons or the expression of interferon-regulated genes such as the toll-like receptors (e.g., TLR7 and TLR8). Reduction of innate immune response can also be measured by measuring the level of decreased cell death following one or more administrations to a cell population; e.g., cell death is 10%, 25%, 50%, 75%, 85%, 90%, 95%, or over 95% less than the cell death frequency observed with a reference compound. Moreover, cell death may affect fewer than 50%, 40%, 30%, 20%, 10%, 5%, 1%, 0.1%, 0.01% or fewer than 0.01% of cells contacted with the polynucleotides.

[0001204] In another embodiment, the polynucleotides of the present invention is significantly less immunogenic than an unmodified in vitro-synthesized polynucleotide with the same sequence or a reference compound. As used herein, "significantly less immunogenic" refers to a detectable decrease in immunogenicity. In another embodiment, the term refers to a fold decrease in immunogenicity. In another embodiment, the term refers to a decrease such that an effective amount of the polynucleotides can be administered without triggering a detectable immune response. In another embodiment, the term refers to a decrease such that the polynucleotides can be repeatedly administered without eliciting an immune response sufficient to detectably reduce expression of the recombinant protein. In another embodiment, the decrease

is such that the polynucleotides can be repeatedly administered without eliciting an immune response sufficient to eliminate detectable expression of the recombinant protein.

[0001205] In another embodiment, the polynucleotides is 2-fold less immunogenic than its unmodified counterpart or reference compound. In another embodiment, immunogenicity is reduced by a 3-fold factor. In another embodiment, immunogenicity is reduced by a 5-fold factor. In another embodiment, immunogenicity is reduced by a 7-fold factor. In another embodiment, immunogenicity is reduced by a 10-fold factor. In another embodiment, immunogenicity is reduced by a 15-fold factor. In another embodiment, immunogenicity is reduced by a fold factor. In another embodiment, immunogenicity is reduced by a 50-fold factor. In another embodiment, immunogenicity is reduced by a 100-fold factor. In another embodiment, immunogenicity is reduced by a 200-fold factor. In another embodiment, immunogenicity is reduced by a 500-fold factor. In another embodiment, immunogenicity is reduced by a 1000-fold factor. In another embodiment, immunogenicity is reduced by a 2000-fold factor. In another embodiment, immunogenicity is reduced by another fold difference.

[0001206] Methods of determining immunogenicity are well known in the art, and include, e.g. measuring secretion of cytokines (e.g. IL-12, IFNalpha, TNF-alpha, RANTES, MIP-1 alpha or beta, IL-6, IFN-beta, or IL-8), measuring expression of DC activation markers (e.g. CD83, HLA-DR, CD80 and CD86), or measuring ability to act as an adjuvant for an adaptive immune response.

[0001207] The polynucleotides of the invention, including the combination of modifications taught herein may have superior properties making them more suitable as therapeutic modalities.

[0001208] It has been determined that the "all or none" model in the art is sorely insufficient to describe the biological phenomena associated with the therapeutic utility of modified mRNA. The present inventors have determined that to improve protein production, one may consider the nature of the modification, or combination of modifications, the percent modification and survey more than one cytokine or metric to determine the efficacy and risk profile of a particular modified mRNA.

[0001209] In one aspect of the invention, methods of determining the effectiveness of a modified mRNA as compared to unmodified involves the measure and analysis of one or more cytokines whose expression is triggered by the administration of the exogenous nucleic acid of the invention. These values are compared to administration of an umodified nucleic acid or to a standard metric such as cytokine response, PolylC, R-848 or other standard known in the art.

[0001210] One example of a standard metric developed herein is the measure of the ratio of the level or amount of encoded polypeptide (protein) produced in the cell, tissue or organism to the

level or amount of one or more (or a panel) of cytokines whose expression is triggered in the cell, tissue or organism as a result of administration or contact with the modified nucleic acid. Such ratios are referred to herein as the Protein: Cytokine Ratio or "PC" Ratio. The higher the PC ratio, the more efficacioius the modified nucleic acid (polynucleotide encoding the protein measured). Preferred PC Ratios, by cytokine, of the present invention may be greater than 1, greater than 10, greater than 100, greater than 1000, greater than 10,000 or more. Modified nucleic acids having higher PC Ratios than a modified nucleic acid of a different or unmodified construct are preferred.

[0001211] The PC ratio may be further qualified by the percent modification present in the polynucleotide. For example, normalized to a 100% modified nucleic acid, the protein production as a function of cytokine (or risk) or cytokine profile can be determined.

[0001212] In one embodiment, the present invention provides a method for determining, across chemistries, cytokines or percent modification, the relative efficacy of any particular modified the polynucleotides by comparing the PC Ratio of the modified nucleic acid (polynucleotides).

[0001213] Polynucleotides containing varying levels of nucleobase subsitutions could be produced that maintain increased protein production and decreased immunostimulatory potential. The relative percentage of any modified nucleotide to its naturally occurring nucleotide counterpart can be varied during the IVT reaction (for instance, 100, 50, 25, 10, 5, 2.5, 1, 0.1, 0.01% 5 methyl cytidine usage versus cytidine; 100, 50, 25, 10, 5, 2.5, 1, 0.1, 0.01% pseudouridine or Nl-methyl-pseudouridine usage versus uridine). Polynucleotides can also be made that utilize different ratios using 2 or more different nucleotides to the same base (for instance, different ratios of pseudouridine and Nl-methyl-pseudouridine). Polynucleotides can also be made with mixed ratios at more than 1 "base" position, such as ratios of 5 methyl cytidine/cytidine and pseudouridine/Nl-methyl-pseudouridine/uridine at the same time. Use of modified mRNA with altered ratios of modified nucleotides can be beneficial in reducing potential exposure to chemically modified nucleotides. Lastly, positional introduction of modified nucleotides into the polynucleotides which modulate either protein production or immunostimulatory potential or both is also possible. The ability of such polynucleotides to demonstrate these improved properties can be assessed in vitro (using assays such as the PBMC assay described herein), and can also be assessed in vivo through measurement of both polynucleotides-encoded protein production and mediators of innate immune recognition such as cytokines.

[0001214] In another embodiment, the relative immunogenicity of the polynucleotides and its unmodified counterpart are determined by determining the quantity of the polynucleotides

required to elicit one of the above responses to the same degree as a given quantity of the unmodified nucleotide or reference compound. For example, if twice as much polynucleotides is required to elicit the same response, than the polynucleotides is two-fold less immunogenic than the unmodified nucleotide or the reference compound.

[0001215] In another embodiment, the relative immunogenicity of the polynucleotides and its unmodified counterpart are determined by determining the quantity of cytokine (e.g. IL-12, IFNalpha, TNF-alpha, RANTES, MIP-1 alpha or beta, IL-6, IFN-beta, or IL-8) secreted in response to administration of the polynucleotides, relative to the same quantity of the unmodified nucleotide or reference compound. For example, if one-half as much cytokine is secreted, than the polynucleotides is two-fold less immunogenic than the unmodified nucleotide. In another embodiment, background levels of stimulation are subtracted before calculating the immunogenicity in the above methods.

[0001216] Provided herein are also methods for performing the titration, reduction or elimination of the immune response in a cell or a population of cells. In some embodiments, the cell is contacted with varied doses of the same polynucleotides and dose response is evaluated. In some embodiments, a cell is contacted with a number of different polynucleotides at the same or different doses to determine the optimal composition for producing the desired effect. Regarding the immune response, the desired effect may be to avoid, evade or reduce the immune response of the cell. The desired effect may also be to alter the efficiency of protein production.

[0001217] The polynucleotides of the present invention may be used to reduce the immune response using the method described in International Publication No. WO2013003475, herein incorporated by reference in its entirety.

Activation of the immune response: Vaccines

[0001218] According to the present invention, the polynucleotides disclosed herein, may encode one or more vaccines. As used herein, a "vaccine" is a biological preparation that improves immunity to a particular disease or infectious agent. A vaccine introduces an antigen into the tissues or cells of a subject and elicits an immune response, thereby protecting the subject from a particular disease or pathogen infection. The polynucleotides of the present invention may encode an antigen and when the polynucleotides are expressed in cells, a desired immune reponse is achieved.

[0001219] The use of RNA as a vaccine overcomes the disadvantages of conventional genetic vaccination involving incorporating DNA into cells in terms of safeness, feasibility, applicability, and effectiveness to generate immune responses. RNA molecules are considered to be significantly safer than DNA vaccines, as RNAs are more easily degraded. They are

cleared quickly out of the organism and cannot integrate into the genome and influence the cell's gene expression in an uncontrollable manner. It is also less likely for RNA vaccines to cause severe side effects like the generation of autoimmune disease or anti-DNA antibodies (Bringmann A. et al., Journal of Biomedicine and Biotechnology (2010), vol. 2010, article ID623687). Transfetion with RNA requires only insertion into the cell's cytoplasm, which is easier to achieve than into the nucleus. Howerver, RNA is susceptible to RNase degradation and other natural decomposition in the cytoplasm of cells. Various attempts to increase the stability and shelf life of RNA vaccines. US 2005/0032730 to Von Der Mulbe et al. discloses improving the stability of mRNA vaccine compositions by increasing G(guanosine)/C(cytosine) content of the mRNA molecules. US 5580859 to Feigner et al. teaches incorporating polynucleotide sequences coding for regulatory proteins that binds to and regulates the stabilities of mRNA. While not wishing to be bound by theory, it is believed that the polynucleotides vaccines of the invention will result in improved stability and therapeutic efficacy due at least in part to the specificity, purity and selectivity of the construct designs.

[0001220] Additionally, certain modified nucleosides, or combinations thereof, when introduced into the polynucleotides of the invention will activate the innate immune response. Such activating molecules are useful as adjuvants when combined with polypeptides and/or other vaccines. In certain embodiments, the activating molecules contain a translatable region which encodes for a polypeptide sequence useful as a vaccine, thus providing the ability to be a self-adjuvant.

[0001221] In one embodiment, the polynucleotides of the present invention may be used in the prevention, treatment and diagnosis of diseases and physical disturbances caused by antigens or infectious agents. The polynucleotide of the present invention may encode at least one polypeptide of interest (e.g. antibody or antigen) and may be provided to an individual in order to stimulate the immune system to protect against the disease-causing agents. As a non-limiting example, the biological activity and/or effect from an antigen or infectious agent may be inhibited and/or abolished by providing one or more polynucleotides which have the ability to bind and neutralize the antigen and/or infectious agent.

[0001222] In one embodiment, the polynucleotides of the invention may encode an immunogen. The delivery of the polynucleotides encoding an immunogen may activate the immune response. As a non-limiting example, the polynucleotides encoding an immunogen may be delivered to cells to trigger multiple innate response pathways (see International Pub. No. WO2012006377 and US Patent Publication No. US20 130177639; herein incorporated by reference in its entirety). As another non-limiting example, the polynucleotides of the present invention encoding an

immunogen may be delivered to a vertebrate in a dose amount large enough to be immunogenic to the vertebrate (see International Pub. No. WO2012006372 and WO2012006369 and US Publication No. US20130149375 and US20130177640; the contents of each of which are herein incorporated by reference in their entirety). A non-limiting list of infectious disease that the polynucleotide vaccines may treat includes, viral infectious diseases such as AIDS (HIV), hepatitis A, B or C, herpes, herpes zoster (chicken pox), German measles (rubella virus), yellow fever, dengue fever etc. (flavi viruses), flu (influenza viruses), haemorrhagic infectious diseases (Marburg or Ebola viruses), bacterial infectious diseases such as Legionnaires' disease (Legionella), gastric ulcer (Helicobacter), cholera (Vibrio), E. coli infections, staphylococcal infections, salmonella infections or streptococcal infections, tetanus (Clostridium tetani), or protozoan infectious diseases (malaria, sleeping sickness, leishmaniasis, toxoplasmosis, i.e. infections caused by Plasmodium, trypanosomes, leishmania and toxoplasma).

[0001223] In one embodiment, the polynucleotides of the invention may encode a tumor antigen to treat cancer. A non-limiting list of tumor antigens includes, 707-AP, AFP, ART-4, BAGE, .beta.-catenin/m, Bcr-abl, CAMEL, CAP-1, CASP-8, CDC27/m, CDK4/m, CEA, CT, Cyp-B, DAM, ELF2M, ETV6-AML1, G250, GAGE, GnT-V, GplOO, HAGE, HER-2/neu, HLA-A*0201-R170I, HPV-E7, HSP70-2M, HAST-2, hTERT (or hTRT), iCE, KIAA0205, LAGE, LDLR/FUT, MAGE, MART-l/melan-A, MC1R, myosin/m, MUC1, MUM-1, -2, -3, NA88-A, NY-ESO-1, pl90 minor bcr-abl, Pml/RAR. alpha., PRAME, PSA, PSM, RAGE, RU1 or RU2, SAGE, SART-1 or SART-3, TEUAML1, TPI/m, TRP-1, TRP-2, TRP-2/INT2 and WT1.

[0001224] The polynucleotides of invention may encode a polypeptide sequence for a vaccine and may further comprise an inhibitor. The inhibitor may impair antigen presentation and/or inhibit various pathways known in the art. As a non-limiting example, the polynucleotides of the invention may be used for a vaccine in combination with an inhibitor which can impair antigen presentation (see International Pub. No. WO2012089225 and WO2012089338; each of which is herein incorporated by reference in their entirety).

[0001225] In one embodiment, the polynucleotides of the invention may be self-replicating RNA. Self-replicating RNA molecules can enhance efficiency of RNA delivery and expression of the enclosed gene product. In one embodiment, the polynucleotides may comprise at least one modification described herein and/or known in the art. In one embodiment, the self-replicating RNA can be designed so that the self-replicating RNA does not induce production of infectious viral particles. As a non-limiting example the self-replicating RNA may be designed by the methods described in US Pub. No. US201 10300205 and International Pub. No. WO201 1005799

and WO2013055905, the contents of each of which are herein incorporated by reference in their entirety.

[0001226] In one embodiment, the self-replicating polynucleotides of the invention may encode a protein which may raise the immune response. As a non-limiting example, the polynucleotides may be self-replicating mRNA may encode at least one antigen (see US Pub. No. US20 110300205, US20130171241, US201 301 77640 and US20 1301 77639 and International Pub. Nos. WO201 1005799, WO2012006372, WO2012006377, WO2013006838, WO2013006842, WO2012006369 and WO2013055905; the contents of each of which is herein incorporated by reference in their entirety). In one aspect, the self-replicating RNA may be administered to mammals at a large enough dose to raise the immune response in a large mammal (see e.g., International Publication No. WO2012006369, herein incorporated by reference in its entirety).

[0001227] In one embodiment, the self-replicating polynucleotides of the invention may be formulated using methods described herein or known in the art. As a non-limiting example, the self-replicating RNA may be formulated for delivery by the methods described in Geall et al (Nonviral delivery of self-amplifying RNA vaccines, PNAS 2012; PMID: 22908294; the contents of which is herein incorporated by reference in its entirety).

[0001228] As another non-limiting example, the polynucleotides of the present invention (e.g., nucleic acid molecules encoding an immunogen such as self-replicating RNA) may be substantially encapsulated within a PEGylated liposome (see International Patent Application No. WO2013033563; herein incorporated by reference in its entirety). In yet another non-limiting example, the self-replicating RNA may be formulated as described in International Application No. WO2013055905, herein incorporated by reference in its entirety. In one non-limiting example, the self-replicating RNA may be formulated using biodegradable polymer particles as described in International Publication No WO2012006359 or US Patent Publication No. US20130183355, the contents of each of which are herein incorporated by reference in its entirety.

[0001229] In one embodiment, the self-replicating RNA may be formulated in virion-like particles. As a non-limiting example, the self-replicating RNA is formulated in virion-like particles as described in International Publication No WO2012006376, herein incorporated by reference in its entirety.

[0001230] In another embodiment, the self-replicating RNA may be formulated in a liposome. As a non-limiting example, the self-replicating RNA may be formulated in liposomes as described in International Publication No. WO20 120067378, herein incorporated by reference in

its entirety. In one aspect, the liposomes may comprise lipids which have a pKa value which may be advantageous for delivery of polynucleotides such as, but not limited to, mRNA. In another aspect, the liposomes may have an essentially neutral surface charge at physiological pH and may therefore be effective for immunization (see e.g., the liposomes described in International Publication No. WO20 120067378, herein incorporated by reference in its entirety).

[0001231] In yet another embodiment, the self-replicating RNA may be formulated in a cationic oil-in-water emulsion. As a non-limiting example, the self-replicating RNA may be formulated in the cationic oil-in-water emulsion described in International Publication No. WO2012006380, herein incorporated by reference in its entirety. The cationic oil-in-water emulsions which may be used with the self replicating RNA described herein (e.g., polynucleotides) may be made by the methods described in International Publication No. WO20 120063 80, herein incorporated by reference in its entirety.

[0001232] In one embodiment, the polynucleotides of the present invention may encode amphipathic and/or immunogenic amphipathic peptides.

[0001233] In on embodiment, a formulation of the polynucleotides of the present invention may further comprise an amphipathic and/or immunogenic amphipathic peptide. As a non-limiting example, the polynucleotides comprising an amphipathic and/or immunogenic amphipathic peptide may be formulated as described in US. Pub. No. US201 10250237 and International Pub. Nos. WO20 10009277 and WO20 10009065; each of which is herein incorporated by reference in their entirety.

[0001234] In one embodiment, the polynucleotides of the present invention may be immunostimultory. As a non-limiting example, the polynucleotides may encode all or a part of a positive-sense or a negative-sense stranded RNA virus genome (see International Pub No. WO2012092569 and US Pub No. US20120177701, each of which is herein incorporated by reference in their entirety). In another non-limiting example, the immunostimultory polynucleotides of the present invention may be formulated with an excipient for administration as described herein and/or known in the art (see International Pub No. WO2012068295 and US Pub No. US20120213812, each of which is herein incorporated by reference in their entirety). The polynucleotides may further comprise a sequence region encoding a cytokine that promotes the immune response, such as a monokine, lymphokine, interleukin or chemokine, such as IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, INF-a, INF-γ, GM-CFS, LT-a, or growth factors such as hGH.

[0001235] In one embodiment, the response of the vaccine formulated by the methods described herein may be enhanced by the addition of various compounds to induce the therapeutic effect.

As a non-limiting example, the vaccine formulation may include a MHC II binding peptide or a peptide having a similar sequence to a MHC II binding peptide (see International Pub Nos. WO2012027365, WO201 103 1298 and US Pub No. US20120070493, US201 101 10965, each of which is herein incorporated by reference in their entirety). As another example, the vaccine formulations may comprise modified nicotinic compounds which may generate an antibody response to nicotine residue in a subject (see International Pub No. WO2012061717 and US Pub No. US201201 14677, each of which is herein incorporated by reference in their entirety).

[0001236] In one embodiment, the polynucleotides may encode at least one antibody or a fragment or portion thereof. The antibodies may be broadly neutralizing antibodies which may inhibit and protect against a broad range of infectious agents. As a non-limiting example, the polynucleotides encoding at least one antibody or fragment or portion thereof are provided to protect a subject against an infection disease and/or treat the disease. As another non-limiting example, the polynucleotides encoding two or more antibodies or fragments or portions thereof which are able to neutralize a wide spectrum of infectious agents are provided to protect a subject against an infection disease and/or treat the disease.

[0001237] In one embodiment, the polynucleotide may encode an antibody heavy chain or an antibody light chain. The optimal ratio of polynucleotide encoding antibody heavy chain and antibody light chain may be evaluated to determine the ratio that produces the maximal amount of a functional antibody and/or desired response. The polynucleotide may also encode a single svFv chain of an antibody.

[0001238] According to the present invention, the polynucleotides which encode one or more broadly neutralizing antibodies may be administrated to a subject prior to exposure to infectious viruses.

[0001239] In one embodiment, the effective amount of the polynucleotides provided to a cell, a tissue or a subject may be enough for immune prophylaxis.

[0001240] In some embodiment, the polynucleotide encoding cancer cell specific proteins may be formulated as a cancer vaccine. As a non-limiting example, the cancer vaccines comprising at least one polynucleotide of the present invention may be used prophylactically to prevent cancer. The vaccine may comprise an adjuvant and/or a preservative. As a non-limiting example, the adjuvant may be squalene. As another non-limiting example, the preservative may be thimerosal.

[0001241] In one embodiment, the present invention provides immunogenic compositions containing polynucleotides which encode one or more antibodies, and/or other anti-infection

reagents. These immunogenic compositions may comprise an adjuvant and/or a preservative. As a non-limiting example, the antibodies may be broadly neutralizing antibodies.

[0001242] In another instance, the present invention provides antibody therapeutics containing the polynucleotides which encode one or more antibodies, and/or other anti-infectous reagents.

[0001243] In one embodiment, the polynucleotide compostions of the present invention may be administrated with other prophylactic or therapeutic compounds. As a non-limiting example, the prophylactic or therapeutic compound may be an adjuvant or a booster. As used herein, when referring to a prophylactic composition, such as a vaccine, the term "booster" refers to an extra administration of the pr prophylactic ophalytic composition. A booster (or booster vaccine) may be given after an earlier administration of the prophylactic composition. The time of administration between the intial administration of the prophylactic composition and the booster may be, but is not limited to, 1 minute, 2 minutes, 3 minutes, 4 minutes, 5 minutes, 6 minutes, 7 minutes, 8 minutes, 9 minutes, 10 minutes, 15 minutes, 20 minutes 35 minutes, 40 minutes, 45 minutes, 50 minutes, 55 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 11 hours, 12 hours, 13 hours, 14 hours, 15 hours, 16 hours, 17 hours, 18 hours, 19 hours, 20 hours, 21 hours, 22 hours, 23 hours, 1 day, 36 hours, 2 days, 3 days, 4 days, 5 days, 6 days, 1 week, 10 days, 2 weeks, 3 weeks, 1 month, 2 months, 3 months, 4 months, 5 months, 6 months, 7 months, 8 months, 9 months, 10 months, 11 months, 1 year, 18 months, 2 years, 3 years, 4 years, 5 years, 6 years, 7 years, 8 years, 9 years, 10 years, 11 years, 12 years, 13 years, 14 years, 15 years, 16 years, 17 years, 18 years, 19 years, 20 years, 25 years, 30 years, 35 years, 40 years, 45 years, 50 years, 55 years, 60 years, 65 years, 70 years, 75 years, 80 years, 85 years, 90 years, 95 years or more than 99 years.

[0001244] In one embodiment, the polynucleotide may be administered intranasally similar to the administration of live vaccines. In another aspect the polynucleotide may be administered intramuscularly or intradermally similarly to the administration of inactivated vaccines known in the art.

[0001245] In one embodiment, the polynucleotides may be used to protect against and/or prevent the transmission of an emerging or engineered threat which may be known or unknown.

[0001246] In another embodiment, the polynucleotides may be formulated by the methods described herein. The formulations may comprise polynucleotides for more than one antibody or vaccine. In one aspect, the formulation may comprise polynucleotide which can can have a therapetutic and/or prophylactic effect on more than one disease, disorder or condition. As a non-limiting example, the formulation may comprise polynucleotides encoding an antigen, antibody or viral protein.

[0001247] In addition, the antibodies of the present invention may be used for research in many applications, such as, but not limited to, identifying and locating intracellular and extracellular proteins, protein interaction, signal pathways and cell biology.

[0001248] In another embodiment, the polynucleotide may be used in a vaccine such as, but not limited to, the modular vaccines described in International Publication No. WO2013093629, the contents of which are herein incorporated by reference in its entirety. As a non-limiting example, the polynucleotide encode at least one antigen, at least one subcellular localization element and at least one CD4 helper element. In one aspect, the subcellular localization element may be a signal peptide of protein sequence that results in the exportation of the antigen from the cytosol. In another aspect the CD4 helper element may be, but is not limited to, P30, NEF, P23TT, P32TT, P21TT, PfT3, P2TT, HBVnc, HA, HBsAg and MT (International Publication No. WO2013093629, the contents of which are herein incorporated by reference in its entirety).

[0001249] In one embodiment, the polynucleotide may be used in the prevention or treatment of RSV infection or reducing the risk of RSV infection. Vaishnaw et al. in US Patent Publication No. US201 31065499, the contents of which are herein incorporated by reference in its entirety, describe using a composition comprising a siRNA to treat and/or prevent a RSV infection. As a non-limiting example, the polynucleotide may be formulated for intranasal administration for the prevention and/or treatment of RSV (see e.g., US Patent Publication No. US20130165499, the contents of which are herein incorporated by reference in its entirety).

[0001250] In another embodiment, the polynucleotide may be used in to reduce the risk or inhibit the infection of influenza viruses such as, but not limited to, the highly pathogenic avian influenza virus (such as, but not limited to, H5N1 subtype) infection and human influenza virs (such as, but not limited to, H1N1 subtype and H3N2 subtype) infection. The polynucleotide described herein which may encode any of the protein sequences described in US Patent No. 8470771, the contents of which are herein incorporated by reference in its entirety, may be used in the treatment or to reduce the risk of an influenza infection.

[0001251] In one embodiment, the polynucleotide may be used to as a vaccine or modulating the immune response against a protein produced by a parasite. Bergmann-Leitner et al. in US Patent No. 8470560, the contents of which are herein incorporated by reference in its entirety, describe a DNA vaccine against the circumsporozoite protein (CSP) of malaria parasites. As a non-limiting example, the polynucleotide may encode the CR2 binding motif of C3d and may be used a vaccine or therapeutic to modulate the immune system against the CSP of malaria parasites.

[0001252] In one embodiment, the polynucleotide may be used to produce a virus which may be labeled with alkyne-modified biomolecules such as, but not limited to, those described in International Patent Publication No. WO20131 12778 and WO20131 12780, the contents of each of which are herein incorporated by reference in its entirety. The labeled viruses may increase the infectivity of the virus and thus may be beneficial in making vaccines. The labeled viruses may be produced by various methods including those described in International Patent Publication No. WO2013112778 and WO2013112780, the contents of each of which are herein incorporated by reference in its entirety.

[0001253] In one embodiment, the polynucleotide may be used as a vaccine and may further comprise an adjuvant which may enable the vaccine to elicit a higher immune response. As a non-limiting example, the adjuvant could be a sub-micron oil-in-water emulsion which can elicit a higher immune response in human pediatric populations (see e.g., the adjuvanted vaccines described in US Patent Publication No. US20120027813 and US Patent No. US8506966, the contents of each of which are herein incorporated by reference in its entirety).

[0001254] In another embodiment, the polynucleotide may be used to as a vaccine and may also comprise 5' cap analogs to improve the stability and increase the expression of the vaccine. Non-limiting examples of 5'cap analogs are described in US Patent Publication No. US20120195917, the contents of which are herein incorporated by reference in its entirety.

Naturally Occuring Mutants

[0001255] In another embodiment, the polynucleotides can be utilized to express variants of naturally occurring proteins that have an improved disease modifying activity, including increased biological activity, improved patient outcomes, or a protective function, etc. as described in co-pending International Patent Publication No. WO2015038892, the contents of which is incorporated by reference in its entirety, such as, but not limited to, in paragraphs [0001 174] - [0001 175].

Targeting of pathogenic organisms or diseased cells

[0001256] Provided herein are methods for targeting pathogenic microorganisms, such as bacteria, yeast, protozoa, helminthes and the like, or diseased cells such as cancer cells using polynucleotides that encode cytostatic or cytotoxic polypeptides. Preferably the mRNA introduced contains modified nucleosides or other nucleic acid sequence modifications that are translated exclusively, or preferentially, in the target pathogenic organism, to reduce possible off-target effects of the therapeutic. Such methods are useful for removing pathogenic organisms or killing diseased cells found in any biological material, including blood, semen, eggs, and transplant materials including embryos, tissues, and organs.

Bioprocessing

[0001257] The methods provided herein may be useful for enhancing protein product yield in a cell culture process as described in co-pending International Patent Publication No. WO2015038892, the contents of which is incorporated by reference in its entirety, such as, but not limited to, in paragraphs [0001 176] - [0001 187].

Cells

[0001258] In one embodiment, the cells are selected from the group consisting of mammalian cells, bacterial cells, plant, microbial, algal and fungal cells. In some embodiments, the cells are mammalian cells, such as, but not limited to, human, mouse, rat, goat, horse, rabbit, hamster or cow cells. In a further embodiment, the cells may be from an established cell line, including, but not limited to, HeLa, NS0, SP2/0, KEK 293T, Vero, Caco, Caco-2, MDCK, COS-1, COS-7, K562, Jurkat, CHO-K1, DG44, CHOK1SV, CHO-S, Huvec, CV-1, Huh-7, NIH3T3, HEK293, 293, A549, HepG2, IMR-90, MCF-7, U-20S, Per.C6, SF9, SF21 or Chinese Hamster Ovary (CHO) cells.

[0001259] In certain embodiments, the cells are fungal cells, such as, but not limited to, Chrysosporium cells, Aspergillus cells, Trichoderma cells, Dictyostelium cells, Candida cells, Saccharomyces cells, Schizosaccharomyces cells, and Penicillium cells.

[0001260] In certain embodiments, the cells are bacterial cells such as, but not limited to, E. coli, B. subtilis, or BL21 cells. Primary and secondary cells to be transfected by the methods of the invention can be obtained from a variety of tissues and include, but are not limited to, all cell types which can be maintained in culture. For examples, primary and secondary cells which can be transfected by the methods of the invention include, but are not limited to, fibroblasts, keratinocytes, epithelial cells (e.g., mammary epithelial cells, intestinal epithelial cells), endothelial cells, glial cells, neural cells, formed elements of the blood (e.g., lymphocytes, bone marrow cells), muscle cells and precursors of these somatic cell types. Primary cells may also be obtained from a donor of the same species or from another species (e.g., mouse, rat, rabbit, cat, dog, pig, cow, bird, sheep, goat, horse).

Purification and Isolation

[0001261] Those of ordinary skill in the art should be able to make a determination of the methods to use to purify or isolate of a protein of interest from cultured cells. Generally, this is done through a capture method using affinity binding or non-affinity purification. If the protein of interest is not secreted by the cultured cells, then a lysis of the cultured cells should be performed prior to purification or isolation. One may use unclarified cell culture fluid containing the protein of interest along with cell culture media components as well as cell culture additives,

such as anti-foam compounds and other nutrients and supplements, cells, cellular debris, host cell proteins, DNA, viruses and the like in the present invention. The process may be conducted in the bioreactor itself. The fluid may either be preconditioned to a desired stimulus such as pH, temperature or other stimulus characteristic or the fluid can be conditioned upon the addition of polymer(s) or the polymer(s) can be added to a carrier liquid that is properly conditioned to the required parameter for the stimulus condition required for that polymer to be solubilized in the fluid. The polymer may be allowed to circulate thoroughly with the fluid and then the stimulus may be applied (change in pH, temperature, salt concentration, etc) and the desired protein and polymer(s) precipitate can out of the solution. The polymer and the desired protein(s) can be separated from the rest of the fluid and optionally washed one or more times to remove any trapped or loosely bound contaminants. The desired protein may then be recovered from the polymer(s) by, for example, elution and the like. Preferably, the elution may be done under a set of conditions such that the polymer remains in its precipitated form and retains any impurities to it during the selected elution of the desired protein. The polymer and protein as well as any impurities may be solubilized in a new fluid such as water or a buffered solution and the protein may be recovered by a means such as affinity, ion exchanged, hydrophobic, or some other type of chromatography that has a preference and selectivity for the protein over that of the polymer or impurities. The eluted protein may then be recovered and may be subjected to additional processing steps, either batch like steps or continuous flow through steps if appropriate.

[0001262] In another embodiment, it may be useful to optimize the expression of a specific polypeptide in a cell line or collection of cell lines of potential interest, particularly a polypeptide of interest such as a protein variant of a reference protein having a known activity. In one embodiment, provided is a method of optimizing expression of a polypeptide of interest in a target cell, by providing a plurality of target cell types, and independently contacting with each of the plurality of target cell types a modified mRNA encoding a polypeptide. Additionally, culture conditions may be altered to increase protein production efficiency. Subsequently, the presence and/or level of the polypeptide of interest in the plurality of target cell types is detected and/or quantitated, allowing for the optimization of a polypeptide of interest's expression by selection of an efficient target cell and cell culture conditions relating thereto. Such methods may be useful when the polypeptide of interest contains one or more post-translational modifications or has substantial tertiary structure, which often complicate efficient protein production.

Protein recovery

[0001263] The protein of interest may be preferably recovered from the culture medium as a secreted polypeptide, or it can be recovered from host cell lysates if expressed without a secretory signal. It may be necessary to purify the protein of interest from other recombinant proteins and host cell proteins in a way that substantially homogenous preparations of the protein of interest are obtained. The cells and/or particulate cell debris may be removed from the culture medium or lysate. The product of interest may then be purified from contaminant soluble proteins, polypeptides and nucleic acids by, for example, fractionation on immunoaffinity or ion-exchange columns, ethanol precipitation, reverse phase HPLC (RP-HPLC), SEPHADEX® chromatography, chromatography on silica or on a cation exchange resin such as DEAE. Methods of purifying a protein heterologous expressed by a host cell are well known in the art.

[0001264] Methods and compositions described herein may be used to produce proteins which are capable of attenuating or blocking the endogenous agonist biological response and/or antagonizing a receptor or signaling molecule in a mammalian subject. For example, IL-12 and IL-23 receptor signaling may be enhanced in chronic autoimmune disorders such as multiple sclerosis and inflammatory diseases such as rheumatoid arthritis, psoriasis, lupus erythematosus, ankylosing spondylitis and Chron's disease (Kikly K, Liu L, Na S, Sedgwich JD (2006) Cur. Opin. Immunol. 18(6): 670-5). In another embodiment, a nucleic acid encodes an antagonist for chemokine receptors. Chemokine receptors CXCR-4 and CCR-5 are required for HIV enry into host cells (Arenzana-Seisdedos F et al, (1996) Nature. Oct 3; 383 (6599):400).

Gene Silencing

[0001265] The polynucleotides described herein are useful to silence (i.e., prevent or substantially reduce) expression of one or more target genes in a cell population. A polynucleotide encoding a polypeptide of interest capable of directing sequence-specific histone H3 methylation is introduced into the cells in the population under conditions such that the polypeptide is translated and reduces gene transcription of a target gene via histone H3 methylation and subsequent heterochromatin formation. In some embodiments, the silencing mechanism is performed on a cell population present in a mammalian subject. By way of non-limiting example, a useful target gene is a mutated Janus Kinase-2 family member, wherein the mammalian subject expresses the mutant target gene suffers from a myeloproliferative disease resulting from aberrant kinase activity.

[0001266] Co-administration of polynucleotides and RNAi agents are also provided herein.

Modulation of Biological Pathways

[0001267] The rapid translation polynucleotides introduced into cells provides a desirable mechanism of modulating target biological pathways. Such modulation includes antagonism or

agonism of a given pathway. In one embodiment, a method is provided for antagonizing a biological pathway in a cell by contacting the cell with an effective amount of a composition comprising a polynucleotide encoding a polypeptide of interest, under conditions such that the polynucleotides is localized into the cell and the polypeptide is capable of being translated in the cell from the polynucleotides, wherein the polypeptide inhibits the activity of a polypeptide functional in the biological pathway. Exemplary biological pathways are those defective in an autoimmune or inflammatory disorder such as multiple sclerosis, rheumatoid arthritis, psoriasis, lupus erythematosus, ankylosing spondylitis colitis, or Crohn's disease; in particular, antagonism of the IL-12 and IL-23 signaling pathways are of particular utility. (See Kikly K, Liu L, Na S, Sedgwick **JD** (2006) Curr. Opin. Immunol. 18 (6): 670-5).

[0001268] Further, provided are polynucleotides encoding an antagonist for chemokine receptors; chemokine receptors CXCR-4 and CCR-5 are required for, e.g., HIV entry into host cells (Arenzana-Seisdedos F et al, (1996) Nature. Oct 3;383(6599):400).

[0001269] Alternatively, provided are methods of agonizing a biological pathway in a cell by contacting the cell with an effective amount of a polynucleotide encoding a recombinant polypeptide under conditions such that the nucleic acid is localized into the cell and the recombinant polypeptide is capable of being translated in the cell from the nucleic acid, and the recombinant polypeptide induces the activity of a polypeptide functional in the biological pathway. Exemplary agonized biological pathways include pathways that modulate cell fate determination. Such agonization is reversible or, alternatively, irreversible.

Expression of Ligand or Receptor on Cell Surface

[0001270] In some aspects and embodiments of the aspects described herein, the polynucleotides described herein can be used to express a ligand or ligand receptor on the surface of a cell *(e.g.,* a homing moiety). A ligand or ligand receptor moiety attached to a cell surface can permit the cell to have a desired biological interaction with a tissue or an agent *in vivo.* A ligand can be an antibody, an antibody fragment, an aptamer, a peptide, a vitamin, a carbohydrate, a protein or polypeptide, a receptor, *e.g.,* cell-surface receptor, an adhesion molecule, a glycoprotein, a sugar residue, a therapeutic agent, a drug, a glycosaminoglycan, or any combination thereof. For example, a ligand can be an antibody that recognizes a cancer-cell specific antigen, rendering the cell capable of preferentially interacting with tumor cells to permit tumor-specific localization of a modified cell. A ligand can confer the ability of a cell composition to accumulate in a tissue to be treated, since a preferred ligand may be capable of interacting with a target molecule on the external face of a tissue to be treated. Ligands having limited cross-reactivity to other tissues are generally preferred.

[0001271] In some cases, a ligand can act as a homing moiety which permits the cell to target to a specific tissue or interact with a specific ligand. Such homing moieties can include, but are not limited to, any member of a specific binding pair, antibodies, monoclonal antibodies, or derivatives or analogs thereof, including without limitation: Fv fragments, single chain Fv (scFv) fragments, Fab' fragments, F(ab')2 fragments, single domain antibodies, camelized antibodies and antibody fragments, humanized antibodies and antibody fragments, and multivalent versions of the foregoing; multivalent binding reagents including without limitation: monospecific or bispecific antibodies, such as disulfide stabilized Fv fragments, scFv tandems ((SCFV)2 fragments), diabodies, tribodies or tetrabodies, which typically are covalently linked or otherwise stabilized *(i.e.,* leucine zipper or helix stabilized) scFv fragments; and other homing moieties include for example, aptamers, receptors, and fusion proteins.

[0001272] In some embodiments, the homing moiety may be a surface-bound antibody, which can permit tuning of cell targeting specificity. This is especially useful since highly specific antibodies can be raised against an epitope of interest for the desired targeting site. In one embodiment, multiple antibodies are expressed on the surface of a cell, and each antibody can have a different specificity for a desired target. Such approaches can increase the avidity and specificity of homing interactions.

[0001273] A skilled artisan can select any homing moiety based on the desired localization or function of the cell, for example an estrogen receptor ligand, such as tamoxifen, can target cells to estrogen-dependent breast cancer cells that have an increased number of estrogen receptors on the cell surface. Other non-limiting examples of ligand/receptor interactions include CCRI *(e.g.,* for treatment of inflamed joint tissues or brain in rheumatoid arthritis, and/or multiple sclerosis), CCR7, CCR8 *(e.g.,* targeting to lymph node tissue), CCR6, CCR9,CCR10 *(e.g.,* to target to intestinal tissue), CCR4, CCR10 *(e.g.,* for targeting to skin), CXCR4 *(e.g.,* for general enhanced transmigration), HCELL *(e.g.,* for treatment of inflammation and inflammatory disorders, bone marrow), Alpha4beta7 *(e.g.,* for intestinal mucosa targeting), VLA-4/VCAM- 1 *(e.g.,* targeting to endothelium). In general, any receptor involved in targeting *(e.g.,* cancer metastasis) can be harnessed for use in the methods and compositions described herein.

Modulation of Cell Lineage

[0001274] Provided are methods of inducing an alteration in cell fate in a target mammalian cell. The target mammalian cell may be a precursor cell and the alteration may involve driving differentiation into a lineage, or blocking such differentiation. Alternatively, the target mammalian cell may be a differentiated cell, and the cell fate alteration includes driving de-differentiation into a pluripotent precursor cell, or blocking such de-differentiation, such as the

dedifferentiation of cancer cells into cancer stem cells. In situations where a change in cell fate is desired, effective amounts of mRNAs encoding a cell fate inductive polypeptide is introduced into a target cell under conditions such that an alteration in cell fate is induced. In some embodiments, the modified mRNAs are useful to reprogram a subpopulation of cells from a first phenotype to a second phenotype. Such a reprogramming may be temporary or permanent. Optionally, the reprogramming induces a target cell to adopt an intermediate phenotype.

[0001275] Additionally, the methods of the present invention are particularly useful to generate induced pluripotent stem cells (iPS cells) because of the high efficiency of transfection, the ability to re-transfect cells, and the tenability of the amount of recombinant polypeptides produced in the target cells. Further, the use of iPS cells generated using the methods described herein is expected to have a reduced incidence of teratoma formation.

[0001276] Also provided are methods of reducing cellular differentiation in a target cell population. For example, a target cell population containing one or more precursor cell types is contacted with a composition having an effective amount of polynucleotides encoding a polypeptide, under conditions such that the polypeptide is translated and reduces the differentiation of the precursor cell. In non-limiting embodiments, the target cell population contains injured tissue in a mammalian subject or tissue affected by a surgical procedure. The precursor cell is, e.g., a stromal precursor cell, a neural precursor cell, or a mesenchymal precursor cell.

[0001277] In a specific embodiment, provided are polynucleotides that encode one or more differentiation factors Gata4, Mef2c and Tbx4. These mRNA-generated factors are introduced into fibroblasts and drive the reprogramming into cardiomyocytes. Such a reprogramming can be performed *in vivo*, by contacting an mRNA-containing patch or other material to damaged cardiac tissue to facilitate cardiac regeneration. Such a process promotes cardiomyocyte genesis as opposed to fibrosis.

Mediation of cell death

[0001278] In one embodiment, polynucleotides compositions can be used to induce apoptosis in a cell *(e.g.,* a cancer cell) by increasing the expression of a death receptor, a death receptor ligand or a combination thereof. This method can be used to induce cell death in any desired cell and has particular usefulness in the treatment of cancer where cells escape natural apoptotic signals.

[0001279] Apoptosis can be induced by multiple independent signaling pathways that converge upon a final effector mechanism consisting of multiple interactions between several "death receptors" and their ligands, which belong to the tumor necrosis factor (TNF) receptor/ligand

superfamily. The best-characterized death receptors are CD95 ("Fas"), TNFRI (p55), death receptor 3 (DR3 or Apo3/TRAMO), DR4 and DR5 (apo2-TRAIL-R2). The final effector mechanism of apoptosis may be the activation of a series of proteinases designated as caspases. The activation of these caspases results in the cleavage of a series of vital cellular proteins and cell death. The molecular mechanism of death receptors/ligands-induced apoptosis is well known in the art. For example, Fas/FasL-mediated apoptosis is induced by binding of three FasL molecules which induces trimerization of Fas receptor via C-terminus death domains (DDs), which in turn recruits an adapter protein FADD (Fas-associated protein with death domain) and Caspase-8. The oligomerization of this trimolecular complex, Fas/FAIDD/caspase-8, results in proteolytic cleavage of proenzyme caspase-8 into active caspase-8 that, in turn, initiates the apoptosis process by activating other downstream caspases through proteolysis, including caspase-3. Death ligands in general are apoptotic when formed into trimers or higher order of structures. As monomers, they may serve as antiapoptotic agents by competing with the trimers for binding to the death receptors.

[0001280] In one embodiment, the polynucleotides composition encodes for a death receptor *(e.g.,* Fas, TRAIL, TRAMO, TNFR, TLR etc). Cells made to express a death receptor by transfection of polynucleotides become susceptible to death induced by the ligand that activates that receptor. Similarly, cells made to express a death ligand, *e.g.,* on their surface, will induce death of cells with the receptor when the transfected cell contacts the target cell. In another embodiment, the polynucleotides composition encodes for a death receptor ligand *(e.g.,* FasL, TNF, etc). In another embodiment, the polynucleotides composition encodes a caspase *(e.g.,* caspase 3, caspase 8, caspase 9 etc). Where cancer cells often exhibit a failure to properly differentiate to a non-proliferative or controlled proliferative form, in another embodiment, the synthetic, polynucleotides composition encodes for both a death receptor and its appropriate activating ligand. In another embodiment, the synthetic, polynucleotides composition encodes for a differentiation factor that when expressed in the cancer cell, such as a cancer stem cell, will induce the cell to differentiate to a non-pathogenic or nonself-renewing phenotype *(e.g.,* reduced cell growth rate, reduced cell division etc) or to induce the cell to enter a dormant cell phase *(e.g.,* **Go** resting phase).

[0001281] One of skill in the art will appreciate that the use of apoptosis-inducing techniques may require that the polynucleotides are appropriately targeted to *e.g.,* tumor cells to prevent unwanted wide-spread cell death. Thus, one can use a delivery mechanism *(e.g.,* attached ligand or antibody, targeted liposome etc) that recognizes a cancer antigen such that the polynucleotides are expressed only in cancer cells.

Cosmetic Applications

[0001282] In one embodiment, the polynucleotides may be used in the treatment, amelioration or prophylaxis of cosmetic conditions. Such conditions include acne, rosacea, scarring, wrinkles, eczema, shingles, psoriasis, age spots, birth marks, dry skin, calluses, rash (e.g., diaper, heat), scabies, hives, warts, insect bites, vitiligo, dandruff, freckles, and general signs of aging.

VI. Kits and Devices

Kits

[0001283] The invention provides a variety of kits for conveniently and/or effectively carrying out methods of the present invention. Typically kits will comprise sufficient amounts and/or numbers of components to allow a user to perform multiple treatments of a subject(s) and/or to perform multiple experiments.

[0001284] In one aspect, the present invention provides kits comprising the molecules (polynucleotides) of the invention. In one embodiment, the kit comprises one or more functional antibodies or function fragments thereof.

[0001285] The kits can be for protein production, comprising polynucleotides comprising a translatable region. The kit may further comprise packaging and instructions and/or a delivery agent to form a formulation composition. The delivery agent may comprise a saline, a buffered solution, a lipidoid or any delivery agent disclosed herein.

[0001286] In one embodiment, the buffer solution may include sodium chloride, calcium chloride, phosphate and/or EDTA. In another embodiment, the buffer solution may include, but is not limited to, saline, saline with 2mM calcium, 5% sucrose, 5% sucrose with 2mM calcium, 5% Mannitol, 5% Mannitol with 2mM calcium, Ringer's lactate, sodium chloride, sodium chloride with 2mM calcium and mannose (See e.g., U.S. Pub. No. 20120258046; herein incorporated by reference in its entirety). In a futher embodiment, the buffer solutions may be precipitated or it may be lyophilized. The amount of each component may be varied to enable consistent, reproducible higher concentration saline or simple buffer formulations. The components may also be varied in order to increase the stability of modified RNA in the buffer solution over a period of time and/or under a variety of conditions. In one aspect, the present invention provides kits for protein production, comprising: a polynucleotide comprising a translatable region, provided in an amount effective to produce a desired amount of a protein encoded by the translatable region when introduced into a target cell; a second polynucleotide comprising an inhibitory nucleic acid, provided in an amount effective to substantially inhibit the innate immune response of the cell; and packaging and instructions.

[0001287] In one aspect, the present invention provides kits for protein production, comprising a polynucleotide comprising a translatable region, wherein the polynucleotide exhibits reduced degradation by a cellular nuclease, and packaging and instructions.

[0001288] In one aspect, the present invention provides kits for protein production, comprising a polynucleotide comprising a translatable region, wherein the polynucleotide exhibits reduced degradation by a cellular nuclease, and a mammalian cell suitable for translation of the translatable region of the first nucleic acid.

[0001289] Kits using the polynucleotides described herein are described in International Publication No. WO2013 15 1666 filed March 9, 2013 (Attorney Docket Number M300.20), International Publication No. WO201415221 l(Attorney Docket Number M030.20), the contents of each of which are incorporated herein by reference in their entirety.

Devices

[0001290] The present invention provides for devices which may incorporate polynucleotides that encode polypeptides of interest. These devices contain in a stable formulation the reagents to synthesize a polynucleotide in a formulation available to be immediately delivered to a subject in need thereof, such as a human patient

[0001291] Devices for administration may be employed to deliver the polynucleotides of the present invention according to single, multi- or split-dosing regimens taught herein. Such devices are taught in, for example, International Publication No. WO2013 15 1666 filed March 9, 2013 (Attorney Docket Number M300.20), International Publication No. WO20 141 522 11(Attorney Docket Number M030.20), the contents of each of which are incorporated herein by reference in their entirety.

[0001292] Method and devices known in the art for multi-administration to cells, organs and tissues are contemplated for use in conjunction with the methods and compositions disclosed herein as embodiments of the present invention. These include, for example, those methods and devices having multiple needles, hybrid devices employing for example lumens or catheters as well as devices utilizing heat, electric current or radiation driven mechanisms.

[0001293] According to the present invention, these multi-administration devices may be utilized to deliver the single, multi- or split doses contemplated herein. Such devices are taught for example in, International Publication No. WO2013151666 filed March 9, 2013 (Attorney Docket Number M300.20), International Publication No. WO201415221 1(Attorney Docket Number M030.20), the contents of each of which are incorporated herein by reference in their entirety.

[0001294] In one embodiment, the polynucleotide is administered subcutaneously or intramuscularly via at least 3 needles to three different, optionally adjacent, sites simultaneously, or within a 60 minutes period (e.g., administration to 4 ,5, 6, 7, 8, 9, or 10 sites simultaneously or within a 60 minute period).

[0001295] Methods of delivering therapeutic agents using solid biodegradable microneedles are described by O'hagan et al. in US Patent Publication No. US20130287832, the contents of which are herein incorporated by reference in its entirety. The microneedles are fabricated from the therapeutic agent (e.g., influenza vaccine) in combination with at least one solid excipient. After penetrating the skin, the microneedles dissolve in situ and release the therapeutic agent to the subject. As a non-limiting example, the therapeutic agents used in the fabrication of the microneedles are the polynucleotides described herein.

[0001296] A microneedle assembly for transdermal drug delivery is described by Ross et al. in US Patent No. US8636696, the contents of which are herein incorporated by reference in its entirety. The assembly has a first surface and a second surface where the microneedles project outwardly from the second surface of the support. The assembly may include a channel and aperture to form a junction which allows fluids (e.g., therapeutic agents or drugs) to pass.

Methods and Devices utilizing catheters and/or lumens

[0001297] Methods and devices using catheters and lumens may be employed to administer the polynucleotides of the present invention on a single, multi- or split dosing schedule. Such methods and devices are described in International Publication No. WO2013151666 filed March 9, 2013 (Attorney Docket Number M300.20), International Publication No. WO2014152211(Attorney Docket Number M030.20), the contents of each of which are incorporated herein by reference in their entirety.

Methods and Devices utilizing electrical current

[0001298] Methods and devices utilizing electric current may be employed to deliver the polynucleotides of the present invention according to the single, multi- or split dosing regimens taught herein. Such methods and devices are described in International Publication No. WO2013151666 filed March 9, 2013 (Attorney Docket Number M300.20), International Publication No. WO20 141 522 11(Attorney Docket Number M030.20), the contents of each of which are incorporated herein by reference in their entirety.

VII. Definitions

[0001299] At various places in the present specification, substituents of compounds of the present disclosure are disclosed in groups or in ranges. It is specifically intended that the present disclosure include each and every individual subcombination of the members of such groups and

ranges. For example, the term "C_{1-6} alkyl" is specifically intended to individually disclose methyl, ethyl, C_3 alkyl, C_4 alkyl, C_5 alkyl, and C_6 alkyl. Herein a phrase of the form "optionally substituted X" *(e.g.,* optionally substituted alkyl) is intended to be equivalent to "X, wherein X is optionally substituted" *(e.g.,* "alkyl, wherein said alkyl is optionally substituted"). It is not intended to mean that the feature "X" *(e.g.* alkyl) *per se* is optional.

[0001300] *About:* As used herein, the term "about" means +/- 10% of the recited value.

[0001301] *Administered in combination:* As used herein, the term "administered in combination" or "combined administration" means that two or more agents are administered to a subject at the same time or within an interval such that there may be an overlap of an effect of each agent on the patient. In some embodiments, they are administered within about 60, 30, 15, 10, 5, or 1 minute of one another. In some embodiments, the administrations of the agents are spaced sufficiently closely together such that a combinatorial *(e.g.,* a synergistic) effect is achieved.

[0001302] *Adjuvant:* As used herein, the term "adjuvant" means a substance that enhances a subject's immune response to an antigen.

[0001303] *Animal:* As used herein, the term "animal" refers to any member of the animal kingdom. In some embodiments, "animal" refers to humans at any stage of development. In some embodiments, "animal" refers to non-human animals at any stage of development. In certain embodiments, the non-human animal is a mammal *(e.g.,* a rodent, a mouse, a rat, a rabbit, a monkey, a dog, a cat, a sheep, cattle, a primate, or a pig). In some embodiments, animals include, but are not limited to, mammals, birds, reptiles, amphibians, fish, and worms. In some embodiments, the animal is a transgenic animal, genetically-engineered animal, or a clone.

[0001304] *Antigen:* As used herein, the term "antigen" refers to the substance that binds specifically to the respective antibody. An antigen may originate either from the body, such as cancer antigen used herein, or from the external environment, for instance, from infectious agents.

[0001305] *Antigens of interest or desired antigens:* As used herein, the terms "antigens of interest" or "desired antigens" include those proteins and other biomolecules provided herein that are immunospecifically bound by the antibodies and fragments, mutants, variants, and alterations thereof described herein. Examples of antigens of interest include, but are not limited to, insulin, insulin-like growth factor, hGH, tPA, cytokines, such as interleukins (IL), e.g., IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, interferon (IFN) alpha, IFN beta, IFN gamma, IFN omega or IFN tau, tumor necrosis

factor (TNF), such as TNF alpha and TNF beta, TNF gamma, TRAIL; G-CSF, GM-CSF, M-CSF, MCP-1 and VEGF.

[0001306] *Approximately:* As used herein, the term "approximately" or "about," as applied to one or more values of interest, refers to a value that is similar to a stated reference value. In certain embodiments, the term "approximately" or "about" refers to a range of values that fall within 25%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, or less in either direction (greater than or less than) of the stated reference value unless otherwise stated or otherwise evident from the context (except where such number would exceed 100% of a possible value).

[0001307] *Associated with:* As used herein, the terms "associated with," "conjugated," "linked," "attached," and "tethered," when used with respect to two or more moieties, means that the moieties are physically associated or connected with one another, either directly or via one or more additional moieties that serves as a linking agent, to form a structure that is sufficiently stable so that the moieties remain physically associated under the conditions in which the structure is used, *e.g.*, physiological conditions. An "association" need not be strictly through direct covalent chemical bonding. It may also suggest ionic or hydrogen bonding or a hybridization based connectivity sufficiently stable such that the "associated" entities remain physically associated.

[0001308] *Bifunctional:* As used herein, the term "Afunctional" refers to any substance, molecule or moiety which is capable of or maintains at least two functions. The functions may effect the same outcome or a different outcome. The structure that produces the function may be the same or different. For example, bifunctional modified RNAs of the present invention may encode a cytotoxic peptide (a first function) while those nucleosides which comprise the encoding RNA are, in and of themselves, cytotoxic (second function). In this example, delivery of the bifunctional modified RNA to a cancer cell would produce not only a peptide or protein molecule which may ameliorate or treat the cancer but would also deliver a cytotoxic payload of nucleosides to the cell should degradation, instead of translation of the modified RNA, occur.

[0001309] *Biocompatible:* As used herein, the term "biocompatible" means compatible with living cells, tissues, organs or systems posing little to no risk of injury, toxicity or rejection by the immune system.

[0001310] *Biodegradable:* As used herein, the term "biodegradable" means capable of being broken down into innocuous products by the action of living things.

[0001311] *Biologically active:* As used herein, the phrase "biologically active" refers to a characteristic of any substance that has activity in a biological system and/or organism. For

instance, a substance that, when administered to an organism, has a biological effect on that organism, is considered to be biologically active. In particular embodiments, a polynucleotide of the present invention may be considered biologically active if even a portion of the polynucleotides is biologically active or mimics an activity considered biologically relevant.

[0001312] *Cancer stem cells:* As used herein, "cancer stem cells" are cells that can undergo self-renewal and/or abnormal proliferation and differentiation to form a tumor.

[0001313] *Chemical terms:* The following provides the definition of various chemical terms from "acyl" to "thiol."

[0001314] The term "acyl," as used herein, represents a hydrogen or an alkyl group (e.g., a haloalkyl group), as defined herein, that is attached to the parent molecular group through a carbonyl group, as defined herein, and is exemplified by formyl (i.e., a carboxyaldehyde group), acetyl, trifluoroacetyl, propionyl, butanoyl and the like. Exemplary unsubstituted acyl groups include from 1 to 7, from 1 to 11, or from 1 to 21 carbons. In some embodiments, the alkyl group is further substituted with 1, 2, 3, or 4 substituents as described herein.

[0001315] Non-limiting examples of optionally substituted acyl groups include, alkoxycarbonyl, alkoxycarbonylacyl, arylalkoxycarbonyl, aryloyl, carbamoyl, carboxyaldehyde, (heterocyclyl) imino, and (heterocyclyl)oyl:

[0001316] The "alkoxycarbonyl" group, which as used herein, represents an alkoxy, as defined herein, attached to the parent molecular group through a carbonyl atom (e.g., -C(0)-OR, where R is H or an optionally substituted Ci_6, Ci_io, or Ci-20 alkyl group). Exemplary unsubstituted alkoxycarbonyl include from 1 to 21 carbons (e.g., from 1 to 11 or from 1 to 7 carbons). In some embodiments, the alkoxy group is further substituted with 1, 2, 3, or 4 substituents as described herein.

[0001317] The "alkoxycarbonylacyl" group, which as used herein, represents an acyl group, as defined herein, that is substituted with an alkoxycarbonyl group, as defined herein (e.g., -C(O) -alkyl-C(0)-OR, where R is an optionally substituted Ci_6, Ci_io, or Ci-20 alkyl group). Exemplary unsubstituted alkoxycarbonylacyl include from 3 to 41 carbons (e.g., from 3 to 10, from 3 to 13, from 3 to 17, from 3 to 21, or from 3 to 31 carbons, such as Ci_6 alkoxycarbonyl-Ci-6 acyl, Ci_io alkoxycarbonyl-Ci-10 acyl, or Ci-20 alkoxycarbonyl-Ci-20 acyl). In some embodiments, each alkoxy and alkyl group is further independently substituted with 1, 2, 3, or 4 substituents, as described herein (e.g., a hydroxy group) for each group.

[0001318] The "arylalkoxycarbonyl" group, which as used herein, represents an arylalkoxy group, as defined herein, attached to the parent molecular group through a carbonyl (e.g., -C(O)-O-alkyl-aryl). Exemplary unsubstituted arylalkoxy groups include from 8 to 31 carbons (e.g.,

from 8 to 17 or from 8 to 21 carbons, such as *Ce-w* aryl-Ci-6 alkoxy-carbonyl, *Ce-w* aryl-Ci-io alkoxy-carbonyl, or *Ce-w* aryl-Ci-2_0 alkoxy-carbonyl). In some embodiments, the arylalkoxycarbonyl group can be substituted with 1, 2, 3, or 4 substituents as defined herein.

[0001319] The "aryloyl" group, which as used herein, represents an aryl group, as defined herein, that is attached to the parent molecular group through a carbonyl group. Exemplary unsubstituted aryloyl groups are of 7 to 11 carbons. In some embodiments, the aryl group can be substituted with 1, 2, 3, or 4 substituents as defined herein.

[0001320] The "carbamoyl" group, which as used herein, represents -C(0)-N(R^{N1})2, where the meaning of each R^{N1} is found in the definition of "amino" provided herein.

[0001321] The "carboxyaldehyde" group, which as used herein, represents an acyl group having the structure -CHO.

[0001322] The "(heterocyclyl) imino" group, which as used herein, represents a heterocyclyl group, as defined herein, attached to the parent molecular group through an imino group. In some embodiments, the heterocyclyl group can be substituted with 1, 2, 3, or 4 substituent groups as defined herein.

[0001323] The "(heterocyclyl)oyl" group, which as used herein, represents a heterocyclyl group, as defined herein, attached to the parent molecular group through a carbonyl group. In some embodiments, the heterocyclyl group can be substituted with 1, 2, 3, or 4 substituent groups as defined herein.

[0001324] The term "alkyl," as used herein, is inclusive of both straight chain and branched chain saturated groups from 1 to 20 carbons (e.g., from 1 to 10 or from 1 to 6), unless otherwise specified. Alkyl groups are exemplified by methyl, ethyl, n- and iso-propyl, n-, sec-, iso- and tert-butyl, neopentyl, and the like, and may be optionally substituted with one, two, three, or, in the case of alkyl groups of two carbons or more, four substituents independently selected from the group consisting of: (1) Ci-6 alkoxy; (2) C_{1-6} alkylsulfinyl; (3) amino, as defined herein (e.g., unsubstituted amino (i.e., -NH_2) or a substituted amino (i.e., -N(R^{N1})$_2$, where R^{N1} is as defined for amino); (4) *Ce-w* aryl-Ci-6 alkoxy; (5) azido; (6) halo; (7) (C2-9 heterocyclyl)oxy; (8) hydroxy, optionally substituted with an O-protecting group; (9) nitro; (10) oxo (e.g., carboxyaldehyde or acyl); (11) C_{1-7} spirocyclyl; (12) thioalkoxy; (13) thiol; (14) -C02$R^{A'}$, optionally substituted with an O-protecting group and where $R^{A'}$ is selected from the group consisting of (a) C_{1-20} alkyl (e.g., Ci_6 alkyl), (b) C2-20 alkenyl (e.g., C_{2-6} alkenyl), (c) C_6-io aryl, (d) hydrogen, (e) Ci_6 alk-C_{6-io} aryl, (f) amino-Ci-2_0 alkyl, (g) polyethylene glycol of -(CH2) $_{s2}$(OCH2CH2) $_{si}$(CH2) $_{s3}$0R', wherein si is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to

10), and R' is H or C_{1-20} alkyl, and (h) amino-polyethylene glycol of -$NR^{N1}(CH_2)_{S}2(CH2CH_20)_si(CH2)_{s3}NR^{N1}$, wherein si is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and each R^{N1} is, independently, hydrogen or optionally substituted Ci_{-6} alkyl; (15) -C(0)$NR^{B'}R^{C'}$, where each of $R^{B'}$ and $R^{C'}$ is, independently, selected from the group consisting of (a) hydrogen, (b) Ci_6 alkyl, (c) *Ce-w* aryl, and (d) Ci_6 alk-C6-io aryl; (16) -SC^$R^{D'}$, where $R^{D'}$ is selected from the group consisting of (a) Ci-6 alkyl, (b) *Ce-w* aryl, (c) Ci_6 alk-C6-io aryl, and (d) hydroxy; (17) -S0 2$NR^{E'}R^{F'}$, where each of $R^{E'}$ and $R^{F'}$ is, independently, selected from the group consisting of (a) hydrogen, (b) C_{1-6} alkyl, (c) *Ce-w* aryl and (d) Ci_6 alk-C6-io aryl; (18) -C(0)$R^{G'}$, where $R^{G'}$ is selected from the group consisting of (a) $Ci_{-2}o$ alkyl (e.g., Ci_{-6} alkyl), (b) C2-20 alkenyl (e.g., C_{2-6} alkenyl), (c) C_6-io aryl, (d) hydrogen, (e) Ci_6 alk-C6-io aryl, (f) amino-Ci-20 alkyl, (g) polyethylene glycol of -$(CH2)_{S}2(OCH2CH2)_si(CH2)_{s3}0R'$, wherein si is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and R' is H or C_{1-20} alkyl, and (h) amino-polyethylene glycol of -$NR^{N1}(CH2)_{S}2(CH_2CH_20)_si(CH2)_{s3}NR^{N1}$, wherein si is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and each R^{N1} is, independently, hydrogen or optionally substituted Ci_6 alkyl; (19) -$NR^{H'}$C(0)$R^{I'}$, wherein $R^{H'}$ is selected from the group consisting of (al) hydrogen and (bl) Ci_6 alkyl, and $R^{1'}$ is selected from the group consisting of (a2) Ci-2_0 alkyl (e.g., Ci_6 alkyl), (b2) C2-2_0 alkenyl (e.g., C2-6 alkenyl), (c2) C_{6-10} aryl, (d2) hydrogen, (e2) Ci_6 alk-C6-io aryl, (f2) amino-Ci-20 alkyl, (g2) polyethylene glycol of -$(CH2)_{S}2(OCH2CH2)_si(CH2)_{s3}0R'$, wherein si is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and R' is H or Ci-20 alkyl, and (h2) amino-polyethylene glycol of -$NR^{N1}(CH2)_{S}2(CH_2CH_20)_si(CH2)_{s3}NR^{N1}$, wherein si is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and each R^{N1} is, independently, hydrogen or optionally substituted Ci_6 alkyl; (20) -$NR^{J'}$C(0)$OR^{K'}$, wherein $R^{J'}$ is selected from the group consisting of (al) hydrogen and (bl) Ci_6 alkyl, and $R^{K'}$ is selected from the group consisting of (a2) Ci-2_0 alkyl (e.g., Ci_6 alkyl), (b2) C2-2_0 alkenyl (e.g., C2-6 alkenyl), (c2) C6-10 aryl, (d2) hydrogen, (e2) Ci_6 alk-C6-io aryl, (f2) amino-Ci-20 alkyl, (g2) polyethylene glycol of -$(CH2)_{S}2(OCH2CH2)_si(CH2)_{s3}0R'$, wherein si is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4,

from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and R' is H or C_{1-20} alkyl, and (h2) amino-polyethylene glycol of $-NR^{N1}(CH_2)_{s2}(CH_2CH_20)_si(CH_2)_{s3}NR^{N1}$, wherein si is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and each R^{N1} is, independently, hydrogen or optionally substituted C_{1-6} alkyl; and (21) amidine. In some embodiments, each of these groups can be further substituted as described herein. For example, the alkylene group of a Ci-alkaryl can be further substituted with an oxo group to afford the respective aryloyl substituent.

[0001325] The term "alkylene," as used herein, represent a saturated divalent hydrocarbon group derived from a straight or branched chain saturated hydrocarbon by the removal of two hydrogen atoms, and is exemplified by methylene, ethylene, isopropylene, and the like. The term "C_{x-y} alkylene" and the prefix "C_{x-y} alk-" represent alkylene groups having between x and y carbons. Exemplary values for x are 1, 2, 3, 4, 5, and 6, and exemplary values for y are 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 18, or 20 (e.g., C_{1-6}, C_{1-10}, C_{2-20}, C_{2-6}, C_{2-10}, or C_{2-20} alkylene). In some embodiments, the alkylene can be further substituted with 1, 2, 3, or 4 substituent groups as defined herein for an alkyl group. Similarly, the suffix "-ene" appended to any group indicates that the group is a divalent group.

[0001326] Non-limiting examples of optionally substituted alkyl and alkylene groups include acylaminoalkyl, acyloxyalkyl, alkoxyalkyl, alkoxycarbonylalkyl, alkylsulfinyl, alkylsulfinylalkyl, aminoalkyl, carbamoylalkyl, carboxyalkyl, carboxyaminoalkyl, haloalkyl, hydroxyalkyl, perfluoroalkyl, and sulfoalkyl:

[0001327] The "acylaminoalkyl" group, which as used herein, represents an acyl group, as defined herein, attached to an amino group that is in turn attached to the parent molecular group through an alkylene group, as defined herein (i.e., -alkyl-N(R^{N1})-C(0)-R, where R is H or an optionally substituted **Ci-6,** Ci-io, or $Ci_{-2}o$ alkyl group (e.g., haloalkyl) and R^{N1} is as defined herein). Exemplary unsubstituted acylaminoalkyl groups include from 1 to 41 carbons (e.g., from 1 to 7, from 1 to 13, from 1 to 21, from 2 to 7, from 2 to 13, from 2 to 21, or from 2 to 41 carbons). In some embodiments, the alkylene group is further substituted with 1, 2, 3, or 4 substituents as described herein, and/or the amino group is $-NH_2$ or $-NHR^{N1}$, wherein R^{N1} is, independently, OH, $N0_2$, NH_2, NR^{N2}_2, $S0_2OR^{N2}$, $S0_2R^{N2}$, SOR^{N2}, alkyl, aryl, acyl (e.g., acetyl, trifluoroacetyl, or others described herein), or alkoxycarbonylalkyl, and each R^{N2} can be H, alkyl, or aryl.

[0001328] The "acyloxyalkyl" group, which as used herein, represents an acyl group, as defined herein, attached to an oxygen atom that in turn is attached to the parent molecular group though

an alkylene group (i.e., -alkyl-0-C(0)-R, where R is H or an optionally substituted Ci_6, Ci_io, or Ci-20 alkyl group). Exemplary unsubstituted acyloxyalkyl groups include from 1 to 2 1 carbons (e.g., from 1 to 7 or from 1 to 11 carbons). In some embodiments, the alkylene group is, independently, further substituted with 1, 2, 3, or 4 substituents as described herein.

[0001329] The "alkoxyalkyl" group, which as used herein, represents an alkyl group that is substituted with an alkoxy group. Exemplary unsubstituted alkoxyalkyl groups include between 2 to 40 carbons (e.g., from 2 to 12 or from 2 to 20 carbons, such as Ci_6 alkoxy-Ci-6 alkyl, Ci_io alkoxy-Ci-io alkyl, or Ci-20 alkoxy-Ci-20 alkyl). In some embodiments, the alkyl and the alkoxy each can be further substituted with 1, 2, 3, or 4 substituent groups as defined herein for the respective group.

[0001330] The "alkoxycarbonylalkyl" group, which as used herein, represents an alkyl group, as defined herein, that is substituted with an alkoxycarbonyl group, as defined herein (e.g., -alkyl-C(0)-OR, where R is an optionally substituted Ci-20, Ci_io, or Ci_6 alkyl group). Exemplary unsubstituted alkoxycarbonylalkyl include from 3 to 4 1 carbons (e.g., from 3 to 10, from 3 to 13, from 3 to 17, from 3 to 21, or from 3 to 31 carbons, such as Ci_6 alkoxycarbonyl-Ci-6 alkyl, Ci_io alkoxycarbonyl-Ci-10 alkyl, or Ci-20 alkoxycarbonyl-Ci-20 alkyl). In some embodiments, each alkyl and alkoxy group is further independently substituted with 1, 2, 3, or 4 substituents as described herein (e.g., a hydroxy group).

[0001331] The "alkylsulfinylalkyl" group, which as used herein, represents an alkyl group, as defined herein, substituted with an alkylsulfinyl group. Exemplary unsubstituted alkylsulfinylalkyl groups are from 2 to 12, from 2 to 20, or from 2 to 40 carbons. In some embodiments, each alkyl group can be further substituted with 1, 2, 3, or 4 substituent groups as defined herein.

[0001332] The "aminoalkyl" group, which as used herein, represents an alkyl group, as defined herein, substituted with an amino group, as defined herein. The alkyl and amino each can be further substituted with 1, 2, 3, or 4 substituent groups as described herein for the respective group (e.g., $C0_2R^{A'}$, where $R^{A'}$ is selected from the group consisting of (a) Ci_6 alkyl, (b) C6-10 aryl, (c) hydrogen, and (d) Ci_6 alk-C6-io aryl, e.g., carboxy, and/or an *N*-protecting group).

[0001333] The "carbamoylalkyl" group, which as used herein, represents an alkyl group, as defined herein, substituted with a carbamoyl group, as defined herein. The alkyl group can be further substituted with 1, 2, 3, or 4 substituent groups as described herein.

[0001334] The "carboxyalkyl" group, which as used herein, represents an alkyl group, as defined herein, substituted with a carboxy group, as defined herein. The alkyl group can be

further substituted with 1, 2, 3, or 4 substituent groups as described herein, and the carboxy group can be optionally substituted with one or more O-protecting groups.

[0001335] The "carboxyaminoalkyl" group, which as used herein, represents an aminoalkyl group, as defined herein, substituted with a carboxy, as defined herein. The carboxy, alkyl, and amino each can be further substituted with 1, 2, 3, or 4 substituent groups as described herein for the respective group (e.g., $C0_2R^{A'}$, where $R^{A'}$ is selected from the group consisting of (a) Ci_6 alkyl, (b) *Ce-w* aryl, (c) hydrogen, and (d) C_{1-6} alk-C6-io aryl, e.g., carboxy, and/or an *N*-protecting group, and/or an O-protecting group).

[0001336] The "haloalkyl" group, which as used herein, represents an alkyl group, as defined herein, substituted with a halogen group (i.e., F, CI, Br, or I). A haloalkyl may be substituted with one, two, three, or, in the case of alkyl groups of two carbons or more, four halogens. Haloalkyl groups include perfluoroalkyls (e.g., $-CF_3$), $-CHF_2$, $-CH_2F$, $-CC1_3$, $-CH_2CH_2Br$, $-CH_2CH(CH_2CH_2Br)CH_3$, and $-CHICH_3$. In some embodiments, the haloalkyl group can be further substituted with 1, 2, 3, or 4 substituent groups as described herein for alkyl groups.

[0001337] The "hydroxyalkyl" group, which as used herein, represents an alkyl group, as defined herein, substituted with one to three hydroxy groups, with the proviso that no more than one hydroxy group may be attached to a single carbon atom of the alkyl group, and is exemplified by hydroxymethyl, dihydroxypropyl, and the like. In some embodiments, the hydroxyalkyl group can be substituted with 1, 2, 3, or 4 substituent groups (e.g., O-protecting groups) as defined herein for an alkyl.

[0001338] The "perfluoroalkyl" group, which as used herein, represents an alkyl group, as defined herein, where each hydrogen radical bound to the alkyl group has been replaced by a fluoride radical. Perfluoroalkyl groups are exemplified by trifluoromethyl, pentafluoroethyl, and the like.

[0001339] The "sulfoalkyl" group, which as used herein, represents an alkyl group, as defined herein, substituted with a sulfo group of $-SO_3H$. In some embodiments, the alkyl group can be further substituted with 1, 2, 3, or 4 substituent groups as described herein, and the sulfo group can be further substituted with one or more O-protecting groups (e.g., as described herein).

[0001340] The term "alkenyl," as used herein, represents monovalent straight or branched chain groups of, unless otherwise specified, from 2 to 20 carbons (e.g., from 2 to 6 or from 2 to 10 carbons) containing one or more carbon-carbon double bonds and is exemplified by ethenyl, 1-propenyl, 2-propenyl, 2-methyl-l-propenyl, 1-butenyl, 2-butenyl, and the like. Alkenyls include both cis and trans isomers. Alkenyl groups may be optionally substituted with 1, 2, 3, or 4 substituent groups that are selected, independently, from amino, aryl, cycloalkyl, or heterocyclyl

(e.g., heteroaryl), as defined herein, or any of the exemplary alkyl substituent groups described herein.

[0001341] Non-limiting examples of optionally substituted alkenyl groups include, alkoxycarbonylalkenyl, aminoalkenyl, and hydroxy alkenyl:

[0001342] The "alkoxycarbonylalkenyl" group, which as used herein, represents an alkenyl group, as defined herein, that is substituted with an alkoxycarbonyl group, as defined herein (e.g., -alkenyl-C(0)-OR, where R is an optionally substituted C_{1-20}, C_{1-10}, or Ci_6 alkyl group). Exemplary unsubstituted alkoxycarbonylalkenyl include from 4 to 41 carbons (e.g., from 4 to 10, from 4 to 13, from 4 to 17, from 4 to 21, or from 4 to 31 carbons, such as C_{1-6} alkoxycarbonyl-C2-6 alkenyl, C_{1-10} alkoxycarbonyl-C2-io alkenyl, or C_{1-20} alkoxycarbonyl-C2-2o alkenyl). In some embodiments, each alkyl, alkenyl, and alkoxy group is further independently substituted with 1, 2, 3, or 4 substituents as described herein (e.g., a hydroxy group).

[0001343] The "aminoalkenyl" group, which as used herein, represents an alkenyl group, as defined herein, substituted with an amino group, as defined herein. The alkenyl and amino each can be further substituted with 1, 2, 3, or 4 substituent groups as described herein for the respective group (e.g., C0 2$R^{A'}$, where $R^{A'}$ is selected from the group consisting of (a) Ci_6 alkyl, (b) C_{6-10} aryl, (c) hydrogen, and (d) Ci_6 alk-C6-io aryl, e.g., carboxy, and/or an *N*-protecting group).

[0001344] The "hydroxyalkenyl" group, which as used herein, represents an alkenyl group, as defined herein, substituted with one to three hydroxy groups, with the proviso that no more than one hydroxy group may be attached to a single carbon atom of the alkyl group, and is exemplified by dihydroxypropenyl, hydroxyisopentenyl, and the like. In some embodiments, the hydroxyalkenyl group can be substituted with 1, 2, 3, or 4 substituent groups (e.g., O-protecting groups) as defined herein for an alkyl.

[0001345] The term "alkynyl," as used herein, represents monovalent straight or branched chain groups from 2 to 20 carbon atoms (e.g., from 2 to 4, from 2 to 6, or from 2 to 10 carbons) containing a carbon-carbon triple bond and is exemplified by ethynyl, 1-propynyl, and the like. Alkynyl groups may be optionally substituted with 1, 2, 3, or 4 substituent groups that are selected, independently, from aryl, cycloalkyl, or heterocyclyl (e.g., heteroaryl), as defined herein, or any of the exemplary alkyl substituent groups described herein.

[0001346] Non-limiting examples of optionally substituted alkynyl groups include alkoxycarbonylalkynyl, aminoalkynyl, and hydroxy alkynyl:

[0001347] The "alkoxycarbonylalkynyl" group, which as used herein, represents an alkynyl group, as defined herein, that is substituted with an alkoxycarbonyl group, as defined herein

(e.g., -alkynyl-C(0)-OR, where R is an optionally substituted C_{1-20}, Ci_io, or Ci_6 alkyl group). Exemplary unsubstituted alkoxycarbonylalkynyl include from 4 to 41 carbons (e.g., from 4 to 10, from 4 to 13, from 4 to 17, from 4 to 21, or from 4 to 31 carbons, such as C_{1-6} alkoxycarbonyl-C2-6 alkynyl, C_{1-10} alkoxycarbonyl-C2-io alkynyl, or C_{1-20} alkoxycarbonyl-C2-2o alkynyl). In some embodiments, each alkyl, alkynyl, and alkoxy group is further independently substituted with 1, 2, 3, or 4 substituents as described herein (e.g., a hydroxy group).

[0001348] The "aminoalkynyl" group, which as used herein, represents an alkynyl group, as defined herein, substituted with an amino group, as defined herein. The alkynyl and amino each can be further substituted with 1, 2, 3, or 4 substituent groups as described herein for the respective group (e.g., $C0_2R^{A'}$, where $R^{A'}$ is selected from the group consisting of (a) Ci_6 alkyl, (b) C_{6-10} aryl, (c) hydrogen, and (d) Ci_6 alk-C6-io aryl, e.g., carboxy, and/or an *N*-protecting group).

[0001349] The "hydroxyalkynyl" group, which as used herein, represents an alkynyl group, as defined herein, substituted with one to three hydroxy groups, with the proviso that no more than one hydroxy group may be attached to a single carbon atom of the alkyl group. In some embodiments, the hydroxyalkynyl group can be substituted with 1, 2, 3, or 4 substituent groups (e.g., O-protecting groups) as defined herein for an alkyl.

[0001350] The term "amino," as used herein, represents -N(R^{N1})2, wherein each R^{N1} is, independently, H, OH, $N0_2$, $N(R^{N2})_2$, $S0_2OR^{N2}$, $S0_2R^{N2}$, SOR^{N2}, an *N*-protecting group, alkyl, alkenyl, alkynyl, alkoxy, aryl, alkaryl, cycloalkyl, alkcycloalkyl, carboxyalkyl (e.g., optionally substituted with an O-protecting group, such as optionally substituted arylalkoxycarbonyl groups or any described herein), sulfoalkyl, acyl (e.g., acetyl, trifluoroacetyl, or others described herein), alkoxycarbonylalkyl (e.g., optionally substituted with an O-protecting group, such as optionally substituted arylalkoxycarbonyl groups or any described herein), heterocyclyl (e.g., heteroaryl), or alkheterocyclyl (e.g., alkheteroaryl), wherein each of these recited R^{N1} groups can be optionally substituted, as defined herein for each group; or two R^{N1} combine to form a heterocyclyl or an *N*-protecting group, and wherein each R^{N2} is, independently, H, alkyl, or aryl. The amino groups of the invention can be an unsubstituted amino (i.e., $-NH_2$) or a substituted amino (i.e., $-N(R^{N1})_2$). In a preferred embodiment, amino is $-NH_2$ or $-NHR^{N1}$, wherein R^{N1} is, independently, OH, $N0_2$, NH_2, NR^{N2}_2, $S0_2OR^{N2}$, $S0_2R^{N2}$, SOR^{N2}, alkyl, carboxyalkyl, sulfoalkyl, acyl (e.g., acetyl, trifluoroacetyl, or others described herein), alkoxycarbonylalkyl (e.g., t-butoxycarbonylalkyl) or aryl, and each R^{N2} can be H, Ci-20 alkyl (e.g., Ci_6 alkyl), or C_{6-10} aryl.

[0001351] Non-limiting examples of optionally substituted amino groups include acylamino and carbamyl:

[0001352] The "acylamino" group, which as used herein, represents an acyl group, as defined herein, attached to the parent molecular group though an amino group, as defined herein (i.e., -N(R^{N1})-C(0)-R, where R is H or an optionally substituted C_{1-6}, C_{1-i0}, or C_{1-20} alkyl group (e.g., haloalkyl) and R^{N1} is as defined herein). Exemplary unsubstituted acylamino groups include from 1 to 41 carbons (e.g., from 1 to 7, from 1 to 13, from 1 to 21, from 2 to 7, from 2 to 13, from 2 to 21, or from 2 to 41 carbons). In some embodiments, the alkyl group is further substituted with 1, 2, 3, or 4 substituents as described herein, and/or the amino group is -NH_2 or -NHR^{N1}, wherein R^{N1} is, independently, OH, NO_2, NH_2, NR^{N2}_2, SO_2OR^{N2}, SO_2R^{N2}, SOR^{N2}, alkyl, aryl, acyl (e.g., acetyl, trifluoroacetyl, or others described herein), or alkoxycarbonylalkyl, and each R^{N2} can be H, alkyl, or aryl.

[0001353] The "carbamyl" group, which as used herein, refers to a carbamate group having the structure -$NR^{N1}C(=O)OR$ or -$OC(=O)N(R^{N1})_2$, where the meaning of each R^{N1} is found in the definition of "amino" provided herein, and R is alkyl, cycloalkyl, alkcycloalkyl, aryl, alkaryl, heterocyclyl (e.g., heteroaryl), or alkheterocyclyl (e.g., alkheteroaryl), as defined herein.

[0001354] The term "amino acid," as described herein, refers to a molecule having a side chain, an amino group, and an acid group (e.g., a carboxy group of -CO_2H or a sulfo group of -SO_3H), wherein the amino acid is attached to the parent molecular group by the side chain, amino group, or acid group (e.g., the side chain). In some embodiments, the amino acid is attached to the parent molecular group by a carbonyl group, where the side chain or amino group is attached to the carbonyl group. Exemplary side chains include an optionally substituted alkyl, aryl, heterocyclyl, alkaryl, alkheterocyclyl, aminoalkyl, carbamoylalkyl, and carboxyalkyl. Exemplary amino acids include alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, hydroxynorvaline, isoleucine, leucine, lysine, methionine, norvaline, ornithine, phenylalanine, proline, pyrrolysine, selenocysteine, serine, taurine, threonine, tryptophan, tyrosine, and valine. Amino acid groups may be optionally substituted with one, two, three, or, in the case of amino acid groups of two carbons or more, four substituents independently selected from the group consisting of: (1) C_{i_6} alkoxy; (2) C_{i_6} alkylsulfinyl; (3) amino, as defined herein (e.g., unsubstituted amino (i.e., -NH_2) or a substituted amíno (i.e., -$N(R^{N1})_2$, where R^{N1} is as defined for amino); (4) *Ce-w* aryl-Ci-6 alkoxy; (5) azido; (6) halo; (7) (C_{2-9} heterocyclyl)oxy; (8) hydroxy; (9) nitro; (10) oxo (e.g., carboxyaldehyde or acyl); (11) C_{i_7} spirocyclyl; (12) thioalkoxy; (13) thiol; (14) -$CO_2R^{A'}$, where $R^{A'}$ is selected from the group consisting of (a) C_{1-20} alkyl (e.g., C_{1-6} alkyl), (b) C_{2-20} alkenyl (e.g., C_{2_6} alkenyl), (c) C_{6-10} aryl, (d) hydrogen, (e) C_{1-6} alk-Ce-io aryl, (f) amino-$Ci_{\_20}$ alkyl, (g) polyethylene glycol of -$(CH_2)_{s2}(OCH_2CH_2)si(CH_2)s30R'$, wherein si is an integer from 1 to 10 (e.g., from 1 to 6 or from

1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and R' is H or C_{1-20} alkyl, and (h) amino-polyethylene glycol of -NR N1(CH 2)S2(CH2CH 20)si(CH2) s3NRN1, wherein si is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and each R^{N1} is, independently, hydrogen or optionally substituted Ci_6 alkyl; (15) -C(0)NR B'R C', where each of $R^{B'}$ and $R^{C'}$ is, independently, selected from the group consisting of (a) hydrogen, (b) Ci_6 alkyl, (c) C 6-io aryl, and (d) Ci_6 alk-C6-io aryl; (16) -SC^R D', where $R^{D'}$ is selected from the group consisting of (a) Ci_6 alkyl, (b) C6-10 aryl, (c) Ci_6 alk-C6-io aryl, and (d) hydroxy; (17) -S0 2NR E'R F', where each of $R^{E'}$ and $R^{F'}$ is, independently, selected from the group consisting of (a) hydrogen, (b) Ci_6 alkyl, (c) C6-io aryl and (d) Ci_6 alk-C6-io aryl; (18) -C(0)R G', where R G' is selected from the group consisting of (a) Ci-20 alkyl (e.g., Ci_6 alkyl), (b) C2-20 alkenyl (e.g., C2-6 alkenyl), (c) C6-io aryl, (d) hydrogen, (e) Ci_6 alk-C6-io aryl, (f) amino-Ci_20 alkyl, (g) polyethylene glycol of -(CH2) S2(OCH2CH2) si(CH2) S30R', wherein si is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and R' is H or Ci-20 alkyl, and (h) amino-polyethylene glycol of -NR N1(CH 2)S2(CH2CH 20)si(CH2)S3NRN1, wherein si is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and each R^{N1} is, independently, hydrogen or optionally substituted Ci_6 alkyl; (19) -NR H'C(0)R I', wherein $R^{H'}$ is selected from the group consisting of (al) hydrogen and (bl) Ci_6 alkyl, and $R^{I'}$ is selected from the group consisting of (a2) Ci-20 alkyl (e.g., Ci_6 alkyl), (b2) C2-20 alkenyl (e.g., C2-6 alkenyl), (c2) C6-10 aryl, (d2) hydrogen, (e2) Ci_6 alk-C6-io aryl, (f2) amino-Ci-20 alkyl, (g2) polyethylene glycol of -(CH2) S2(OCH2CH2) si(CH2)S30R', wherein si is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and R' is H or Ci-20 alkyl, and (h2) amino-polyethylene glycol of -NR N1(CH2) S2(CH 2CH 20)si(CH2)S3NRN1, wherein si is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and each R^{N1} is, independently, hydrogen or optionally substituted Ci_6 alkyl; (20) -NR J'C(0)OR K', wherein $R^{J'}$ is selected from the group consisting of (al) hydrogen and (bl) Ci_6 alkyl, and $R^{K'}$ is selected from the group consisting of (a2) Ci-20 alkyl (e.g., Ci_6 alkyl), (b2) C2-20 alkenyl (e.g., C2-6 alkenyl), (c2) C6-10 aryl, (d2) hydrogen, (e2) Ci_6 alk-C6-io aryl, (f2) amino-Ci-20 alkyl, (g2) polyethylene glycol of -(CH2) S2(OCH2CH2) si(CH2)S30R', wherein si is an integer

from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and R' is H or Ci_2o alkyl, and (h2) amino-polyethylene glycol of -NR^{N1}(CH$_2$)$_S$2(CH2CH$_2$0)$_s$i(CH2)$_{S3}$NR^{N1}, wherein si is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and each R^{N1} is, independently, hydrogen or optionally substituted Ci_6 alkyl; and (21) amidine. In some embodiments, each of these groups can be further substituted as described herein.

[0001355] The term "aryl," as used herein, represents a mono-, bicyclic, or multicyclic carbocyclic ring system having one or two aromatic rings and is exemplified by phenyl, naphthyl, 1,2-dihydronaphthyl, 1,2,3,4-tetrahydronaphthyl, anthracenyl, phenanthrenyl, fluorenyl, indanyl, indenyl, and the like, and may be optionally substituted with 1, 2, 3, 4, or 5 substituents independently selected from the group consisting of: (1) C1-7 acyl (e.g., carboxy aldehyde); (2) Ci-20 alkyl (e.g., Ci_6 alkyl, Ci_6 alkoxy-Ci-6 alkyl, Ci_6 alkylsulfinyl-Ci-6 alkyl, amino-Ci-6 alkyl, azido-Ci_6 alkyl, (carboxyaldehyde)-Ci_6 alkyl, halo-Ci_6 alkyl (e.g., perfluoroalkyl), hydroxy-Ci-6 alkyl, nitro-Ci-6 alkyl, or Ci_6 thioalkoxy-Ci-6 alkyl); (3) Ci-20 alkoxy (e.g., Ci_6 alkoxy, such as perfluoroalkoxy); (4) Ci_6 alkylsulfinyl; (5) C 6-1$_0$ aryl; (6) amino; (7) Ci-6 alk-C6-io aryl; (8) azido; (9) C3-8 cycloalkyl; (10) Ci-6 alk-C3-8 cycloalkyl; (11) halo; (12) Ci-12 heterocyclyl (e.g., Ci-12 heteroaryl); (13) (Ci-12 heterocyclyl)oxy; (14) hydroxy; (15) nitro; (16) Ci-20 thioalkoxy (e.g., Ci_6 thioalkoxy); (17) -(CH$_2$)$_q$C02R$^{A'}$, where q is an integer from zero to four, and R$^{A'}$ is selected from the group consisting of (a) Ci_6 alkyl, (b) C 6-1$_0$ aryl, (c) hydrogen, and (d) Ci_6 alk-C6-io aryl; (18) -(CH$_2$)$_q$CONR$^{B'}$R$^{C'}$, where q is an integer from zero to four and where R$^{B'}$ and R$^{C'}$ are independently selected from the group consisting of (a) hydrogen, (b) Ci$_{-6}$ alkyl, (c) C$_6$-io aryl, and (d) Ci$_{-6}$ alk-C$_6$-io aryl; (19) -(CH$_2$)$_q$S0$_2$R$^{D'}$, where q is an integer from zero to four and where R$^{D'}$ is selected from the group consisting of (a) alkyl, (b) C 6-10 aryl, and (c) alk-C6-io aryl; (20) -(CH$_2$)$_q$S02NR$^{E'}$R$^{F'}$, where q is an integer from zero to four and where each of R$^{E'}$ and R$^{F'}$ is, independently, selected from the group consisting of (a) hydrogen, (b) Ci_6 alkyl, (c) C 6-10 aryl, and (d) Ci_6 alk-C6-io aryl; (21) thiol; (22) C 6-10 aryloxy; (23) C3-8 cycloalkoxy; (24) C 6-10 aryl-Ci-6 alkoxy; (25) Ci_6 alk-Ci-12 heterocyclyl (e.g., Ci_6 alk-Ci-12 heteroaryl); (26) C2-2$_0$ alkenyl; and (27) C2-2$_0$ alkynyl. In some embodiments, each of these groups can be further substituted as described herein. For example, the alkylene group of a Ci-alkaryl or a Ci-alkheterocyclyl can be further substituted with an oxo group to afford the respective aryloyl and (heterocyclyl)oyl substituent group.

[0001356] The "arylalkyl" group, which as used herein, represents an aryl group, as defined herein, attached to the parent molecular group through an alkylene group, as defined herein. Exemplary unsubstituted arylalkyl groups are from 7 to 30 carbons (e.g., from 7 to 16 or from 7 to 20 carbons, such as C_{1-6} alk-C6-io aryl, C_{1-10} alk-C6-io aryl, or C_{1-20} alk-C6-io aryl). In some embodiments, the alkylene and the aryl each can be further substituted with 1, 2, 3, or 4 substituent groups as defined herein for the respective groups. Other groups preceded by the prefix "alk-" are defined in the same manner, where "alk" refers to a Ci_6 alkylene, unless otherwise noted, and the attached chemical structure is as defined herein.

[0001357] The term "azido" represents an $-N_3$ group, which can also be represented as -N=N=N.

[0001358] The term "bicyclic," as used herein, refer to a structure having two rings, which may be aromatic or non-aromatic. Bicyclic structures include spirocyclyl groups, as defined herein, and two rings that share one or more bridges, where such bridges can include one atom or a chain including two, three, or more atoms. Exemplary bicyclic groups include a bicyclic carbocyclyl group, where the first and second rings are carbocyclyl groups, as defined herein; a bicyclic aryl groups, where the first and second rings are aryl groups, as defined herein; bicyclic heterocyclyl groups, where the first ring is a heterocyclyl group and the second ring is a carbocyclyl (e.g., aryl) or heterocyclyl (e.g., heteroaryl) group; and bicyclic heteroaryl groups, where the first ring is a heteroaryl group and the second ring is a carbocyclyl (e.g., aryl) or heterocyclyl (e.g., heteroaryl) group. In some embodiments, the bicyclic group can be substituted with 1, 2, 3, or 4 substituents as defined herein for cycloalkyl, heterocyclyl, and aryl groups.

[0001359] The term "boranyl," as used herein, represents $-B(R^{B1})_3$, where each R^{B1} is, independently, selected from the group consisting of H and optionally substituted alkyl. In some embodiments, the boranyl group can be substituted with 1, 2, 3, or 4 substituents as defined herein for alkyl.

[0001360] The terms "carbocyclic" and "carbocyclyl," as used herein, refer to an optionally substituted C_{3-12} monocyclic, bicyclic, or tricyclic structure in which the rings, which may be aromatic or non-aromatic, are formed by carbon atoms. Carbocyclic structures include cycloalkyl, cycloalkenyl, cycloalkynyl, and aryl groups.

[0001361] The term "carbonyl," as used herein, represents a C(O) group, which can also be represented as C=0.

[0001362] The term "carboxy," as used herein, means -CO2H.

[0001363] The term "cyano," as used herein, represents an -CN group.

[0001364] The term "cycloalkyl," as used herein represents a monovalent saturated or unsaturated non-aromatic cyclic hydrocarbon group from three to eight carbons, unless otherwise specified, and is exemplified by cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl, bicycle heptyl, and the like. When what would otherwise be a cycloalkyl group includes one or more carbon-carbon double bonds, the group is referred to as a "cycloalkenyl" group. For the purposes of this invention, cycloalkenyl excludes aryl groups. When what would otherwise be a cycloalkyl group includes one or more carbon-carbon triple bonds, the group is referred to as a "cycloalkynyl" group. Exemplary cycloalkenyl groups include cyclopentenyl, cyclohexenyl, and the like. The cycloalkyl groups of this invention can be optionally substituted with: (1) C_{1-7} acyl (e.g., carboxyaldehyde); (2) C_{1-20} alkyl (e.g., C_{1-6} alkyl, Ci_6 alkoxy-Ci-6 alkyl, Ci_6 alkylsulfinyl-Ci-6 alkyl, amino-Ci-6 alkyl, azido-Ci_6 alkyl, (carboxyaldehyde)-Ci_6 alkyl, halo-Ci_6 alkyl (e.g., perfluoroalkyl), hydroxy-Ci-6 alkyl, nitro-Ci-6 alkyl, or Ci_6 thioalkoxy-Ci_6 alkyl); (3) C_{1-20} alkoxy (e.g., C_{1-6} alkoxy, such as perfluoroalkoxy); (4) Ci_6 alkylsulfinyl; (5) *Ce-w* aryl; (6) amino; (7) C_{1-6} alk-C6-io aryl; (8) azido; (9) $_{C3-8}$ cycloalkyl; (10) Ci_6 alk-C3_8 cycloalkyl; (11) halo; (12) C_{1-12} heterocyclyl (e.g., C_{1-12} heteroaryl); (13) (C_{1-12} heterocyclyl)oxy; (14) hydroxy; (15) nitro; (16) C_{1-20} thioalkoxy (e.g., Ci_6 thioalkoxy); (17) -$(CH_2)_q C0_2 R^{A'}$, where q is an integer from zero to four, and $R^{A'}$ is selected from the group consisting of (a) C_{1-6} alkyl, (b) *Ce-w* aryl, (c) hydrogen, and (d) Ci-6 alk-C6-io aryl; (18) -$(CH2)_q CONR^{B'}R^{C'}$, where q is an integer from zero to four and where $R^{B'}$ and $R^{C'}$ are independently selected from the group consisting of (a) hydrogen, (b) C_{6-10} alkyl, (c) C_{6-10} aryl, and (d) d_{-6} alk-C_{6-10} aryl; (19) -$(CH_2)_q S0_2 R^{D'}$, where q is an integer from zero to four and where $R^{D'}$ is selected from the group consisting of (a) C_6-io alkyl, (b) C_6-io aryl, and (c) Ci_{-6} alk-C_6-io aryl; (20) -$(CH_2)_q S0_2 NR^{E'}R^{F'}$, where q is an integer from zero to four and where each of $R^{E'}$ and $R^{F'}$ is, independently, selected from the group consisting of (a) hydrogen, (b) *Ce-w* alkyl, (c) *Ce-w* aryl, and (d) Ci_6 alk-C6-io aryl; (21) thiol; (22) *Ce-w* aryloxy; (23) $_{C3-8}$ cycloalkoxy; (24) Ce-ıo aryl-Ci-6 alkoxy; (25) Ci-6 alk-Ci-i_2 heterocyclyl (e.g., Ci_6 alk-C_{1-12} heteroaryl); (26) oxo; (27) C_{2-2}o alkenyl; and (28) C_{2-2}o alkynyl. In some embodiments, each of these groups can be further substituted as described herein. For example, the alkylene group of a Ci-alkaryl or a Ci-alkheterocyclyl can be further substituted with an oxo group to afford the respective aryloyl and (heterocyclyl) oyl substituent group.

[0001365] The "cycloalkylalkyl" group, which as used herein, represents a cycloalkyl group, as defined herein, attached to the parent molecular group through an alkylene group, as defined herein (e.g., an alkylene group of from 1 to 4, from 1 to 6, from 1 to 10, or form 1 to 20 carbons). In some embodiments, the alkylene and the cycloalkyl each can be further substituted with 1, 2, 3, or 4 substituent groups as defined herein for the respective group.

[0001366] The term "diastereomer," as used herein means stereoisomers that are not mirror images of one another and are non-superimposable on one another.

[0001367] The term "enantiomer," as used herein, means each individual optically active form of a compound of the invention, having an optical purity or enantiomeric excess (as determined by methods standard in the art) of at least 80% (i.e., at least 90% of one enantiomer and at most 10% of the other enantiomer), preferably at least 90% and more preferably at least 98%.

[0001368] The term "halo," as used herein, represents a halogen selected from bromine, chlorine, iodine, or fluorine.

[0001369] The term "heteroalkyl," as used herein, refers to an alkyl group, as defined herein, in which one or two of the constituent carbon atoms have each been replaced by nitrogen, oxygen, or sulfur. In some embodiments, the heteroalkyl group can be further substituted with 1, 2, 3, or 4 substituent groups as described herein for alkyl groups. The terms "heteroalkenyl" and heteroalkynyl," as used herein refer to alkenyl and alkynyl groups, as defined herein, respectively, in which one or two of the constituent carbon atoms have each been replaced by nitrogen, oxygen, or sulfur. In some embodiments, the heteroalkenyl and heteroalkynyl groups can be further substituted with 1, 2, 3, or 4 substituent groups as described herein for alkyl groups.

[0001370] Non-limiting examples of optionally substituted heteroalkyl, heteroalkenyl, and heteroalkynyl groups include acyloxy, alkenyloxy, alkoxy, alkoxyalkoxy, alkoxycarbonylalkoxy, alkynyloxy, aminoalkoxy, arylalkoxy, carboxyalkoxy, cycloalkoxy, haloalkoxy, (heterocyclyl)oxy, perfluoroalkoxy, thioalkoxy, and thioheterocyclylalkyl:

[0001371] The "acyloxy" group, which as used herein, represents an acyl group, as defined herein, attached to the parent molecular group though an oxygen atom (i.e., -0-C(0)-R, where R is H or an optionally substituted C_{1-6}, $C_{1-}w$, or C_{1-20} alkyl group). Exemplary unsubstituted acyloxy groups include from 1 to 21 carbons (e.g., from 1 to 7 or from 1 to 11 carbons). In some embodiments, the alkyl group is further substituted with 1, 2, 3, or 4 substituents as described herein.

[0001372] The "alkenyloxy" group, which as used here, represents a chemical substituent of formula -OR, where R is a $C2\text{-}2_0$ alkenyl group (e.g., C2-6 or C_{2-10} alkenyl), unless otherwise specified. Exemplary alkenyloxy groups include ethenyloxy, propenyloxy, and the like. In some embodiments, the alkenyl group can be further substituted with 1, 2, 3, or 4 substituent groups as defined herein (e.g., a hydroxy group).

[0001373] The "alkoxy" group, which as used herein, represents a chemical substituent of formula -OR, where R is a C_{1-20} alkyl group (e.g., Ci-6 or C_{1-10} alkyl), unless otherwise specified.

Exemplary alkoxy groups include methoxy, ethoxy, propoxy (e.g., n-propoxy and isopropoxy), t-butoxy, and the like. In some embodiments, the alkyl group can be further substituted with 1, 2, 3, or 4 substituent groups as defined herein (e.g., hydroxy or alkoxy).

[0001374] The "alkoxyalkoxy" group, which as used herein, represents an alkoxy group that is substituted with an alkoxy group. Exemplary unsubstituted alkoxyalkoxy groups include between 2 to 40 carbons (e.g., from 2 to 12 or from 2 to 20 carbons, such as C_{1-6} alkoxy-Ci_6 alkoxy, C_{1-10} alkoxy-Ci_io alkoxy, or C_{1-20} alkoxy-Ci-20 alkoxy). In some embodiments, the each alkoxy group can be further substituted with 1, 2, 3, or 4 substituent groups as defined herein.

[0001375] The "alkoxycarbonylalkoxy" group, which as used herein, represents an alkoxy group, as defined herein, that is substituted with an alkoxycarbonyl group, as defined herein (e.g., -0-alkyl-C(0)-OR, where R is an optionally substituted C_{1-6}, Ci_io, or C_{1-20} alkyl group). Exemplary unsubstituted alkoxycarbonylalkoxy include from 3 to 41 carbons (e.g., from 3 to 10, from 3 to 13, from 3 to 17, from 3 to 21, or from 3 to 31 carbons, such as C_{1-6} alkoxycarbonyl-Ci-6 alkoxy, C_{1-10} alkoxycarbonyl-Ci-io alkoxy, or C_{1-20} alkoxycarbonyl-Ci-20 alkoxy). In some embodiments, each alkoxy group is further independently substituted with 1, 2, 3, or 4 substituents, as described herein (e.g., a hydroxy group).

[0001376] The "alkynyloxy" group, which as used herein, represents a chemical substituent of formula -OR, where R is a C2-20 alkynyl group (e.g., C2-6 or C2-10 alkynyl), unless otherwise specified. Exemplary alkynyloxy groups include ethynyloxy, propynyloxy, and the like. In some embodiments, the alkynyl group can be further substituted with 1, 2, 3, or 4 substituent groups as defined herein (e.g., a hydroxy group).

[0001377] The "aminoalkoxy" group, which as used herein, represents an alkoxy group, as defined herein, substituted with an amino group, as defined herein. The alkyl and amino each can be further substituted with 1, 2, 3, or 4 substituent groups as described herein for the respective group (e.g., C02R$^{A'}$, where R$^{A'}$ is selected from the group consisting of (a) Ci-6 alkyl, (b) C_{6-10} aryl, (c) hydrogen, and (d) Ci_6 alk-C6-io aryl, e.g., carboxy).

[0001378] The "arylalkoxy" group, which as used herein, represents an alkaryl group, as defined herein, attached to the parent molecular group through an oxygen atom. Exemplary unsubstituted arylalkoxy groups include from 7 to 30 carbons (e.g., from 7 to 16 or from 7 to 20 carbons, such as *Ce-w* aryl-Ci-6 alkoxy, *Ce-w* aryl-Ci-10 alkoxy, or *Ce-w* aryl-Ci-20 alkoxy). In some embodiments, the arylalkoxy group can be substituted with 1, 2, 3, or 4 substituents as defined herein.

[0001379] The "aryloxy" group, which as used herein, represents a chemical substituent of formula -OR', where R' is an aryl group of 6 to 18 carbons, unless otherwise specified. In some embodiments, the aryl group can be substituted with 1, 2, 3, or 4 substituents as defined herein.

[0001380] The "carboxyalkoxy" group, which as used herein, represents an alkoxy group, as defined herein, substituted with a carboxy group, as defined herein. The alkoxy group can be further substituted with 1, 2, 3, or 4 substituent groups as described herein for the alkyl group, and the carboxy group can be optionally substituted with one or more O-protecting groups.

[0001381] The "cycloalkoxy" group, which as used herein, represents a chemical substituent of formula -OR, where R is a C_{3-8} cycloalkyl group, as defined herein, unless otherwise specified. The cycloalkyl group can be further substituted with 1, 2, 3, or 4 substituent groups as described herein. Exemplary unsubstituted cycloalkoxy groups are from 3 to 8 carbons. In some embodiment, the cycloalkyl group can be further substituted with 1, 2, 3, or 4 substituent groups as described herein.

[0001382] The "haloalkoxy" group, which as used herein, represents an alkoxy group, as defined herein, substituted with a halogen group (i.e., F, CI, Br, or I). A haloalkoxy may be substituted with one, two, three, or, in the case of alkyl groups of two carbons or more, four halogens. Haloalkoxy groups include perfluoroalkoxys (e.g., $-OCF_3$), $-OCHF_2$, $-OCH_2F$, $-OCCI_3$, $-OCH_2CH_2Br$, $-OCH_2CH(CH_2CH_2Br)CH_3$, and -OCHICH3. In some embodiments, the haloalkoxy group can be further substituted with 1, 2, 3, or 4 substituent groups as described herein for alkyl groups.

[0001383] The "(heterocyclyl)oxy" group, which as used herein, represents a heterocyclyl group, as defined herein, attached to the parent molecular group through an oxygen atom. In some embodiments, the heterocyclyl group can be substituted with 1, 2, 3, or 4 substituent groups as defined herein.

[0001384] The "perfluoroalkoxy" group, which as used herein, represents an alkoxy group, as defined herein, where each hydrogen radical bound to the alkoxy group has been replaced by a fluoride radical. Perfluoroalkoxy groups are exemplified by trifluoromethoxy, pentafluoroethoxy, and the like.

[0001385] The "alkylsulfinyl" group, which as used herein, represents an alkyl group attached to the parent molecular group through an -S(O)- group. Exemplary unsubstituted alkylsulfinyl groups are from 1 to 6, from 1 to 10, or from 1 to 20 carbons. In some embodiments, the alkyl group can be further substituted with 1, 2, 3, or 4 substituent groups as defined herein.

[0001386] The "thioarylalkyl" group, which as used herein, represents a chemical substituent of formula -SR, where R is an arylalkyl group. In some embodiments, the arylalkyl group can be further substituted with 1, 2, 3, or 4 substituent groups as described herein.

[0001387] The "thioalkoxy" group as used herein, represents a chemical substituent of formula -SR, where R is an alkyl group, as defined herein. In some embodiments, the alkyl group can be further substituted with 1, 2, 3, or 4 substituent groups as described herein.

[0001388] The "thioheterocyclylalkyl" group, which as used herein, represents a chemical substituent of formula -SR, where R is an heterocyclylalkyl group. In some embodiments, the heterocyclylalkyl group can be further substituted with 1, 2, 3, or 4 substituent groups as described herein.

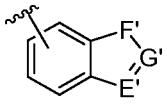
[0001389] The term "heteroaryl," as used herein, represents that subset of heterocyclyls, as defined herein, which are aromatic: i.e., they contain *4n+2* pi electrons within the mono- or multicyclic ring system. Exemplary unsubstituted heteroaryl groups are of 1 to 12 (e.g., 1 to 11, 1 to 10, 1 to 9, 2 to 12, 2 to 11, 2 to 10, or 2 to 9) carbons. In some embodiment, the heteroaryl is substituted with 1, 2, 3, or 4 substituents groups as defined for a heterocyclyl group.

[0001390] The term "heteroarylalkyl" refers to a heteroaryl group, as defined herein, attached to the parent molecular group through an alkylene group, as defined herein. Exemplary unsubstituted heteroarylalkyl groups are from 2 to 32 carbons (e.g., from 2 to 22, from 2 to 18, from 2 to 17, from 2 to 16, from 3 to 15, from 2 to 14, from 2 to 13, or from 2 to 12 carbons, such as Ci-6 alk-Ci-12 heteroaryl, Ci_io alk-Ci-12 heteroaryl, or Ci-20 alk-Ci-12 heteroaryl). In some embodiments, the alkylene and the heteroaryl each can be further substituted with 1, 2, 3, or 4 substituent groups as defined herein for the respective group. Heteroarylalkyl groups are a subset of heterocyclylalkyl groups.

[0001391] The term "heterocyclyl," as used herein represents a 5-, 6- or 7-membered ring, unless otherwise specified, containing one, two, three, or four heteroatoms independently selected from the group consisting of nitrogen, oxygen, and sulfur. The 5-membered ring has zero to two double bonds, and the 6- and 7-membered rings have zero to three double bonds. Exemplary unsubstituted heterocyclyl groups are of 1 to 12 (e.g., 1 to 11, 1 to 10, 1 to 9, 2 to 12, 2 to 11, 2 to 10, or 2 to 9) carbons. The term "heterocyclyl" also represents a heterocyclic compound having a bridged multicyclic structure in which one or more carbons and/or heteroatoms bridges two non-adjacent members of a monocyclic ring, e.g., a quinuclidinyl group. The term "heterocyclyl" includes bicyclic, tricyclic, and tetracyclic groups in which any of the above heterocyclic rings is fused to one, two, or three carbocyclic rings, e.g., an aryl ring, a cyclohexane ring, a cyclohexene ring, a cyclopentane ring, a cyclopentene ring, or another

monocyclic heterocyclic ring, such as indolyl, quinolyl, isoquinolyl, tetrahydroquinolyl, benzofuryl, benzothienyl and the like. Examples of fused heterocyclyls include tropanes and l,2,3,5,8,8a-hexahydroindolizine. Heterocyclics include pyrrolyl, pyrrolinyl, pyrrolidinyl, pyrazolyl, pyrazolinyl, pyrazolidinyl, imidazolyl, imidazolinyl, imidazolidinyl, pyridyl, piperidinyl, homopiperidinyl, pyrazinyl, piperazinyl, pyrimidinyl, pyridazinyl, oxazolyl, oxazolidinyl, isoxazolyl, isoxazolidiniyl, morpholinyl, thiomorpholinyl, thiazolyl, thiazolidinyl, isothiazolyl, isothiazolidinyl, indolyl, indazolyl, quinolyl, isoquinolyl, quinoxalinyl, dihydroquinoxalinyl, quinazolinyl, cinnolinyl, phthalazinyl, benzimidazolyl, benzothiazolyl, benzoxazolyl, benzothiadiazolyl, furyl, thienyl, thiazolidinyl, isothiazolyl, triazolyl, tetrazolyl, oxadiazolyl (e.g., 1,2,3-oxadiazolyl), purinyl, thiadiazolyl (e.g., 1,2,3-thiadiazolyl), tetrahydrofuranyl, dihydrofuranyl, tetrahydrothienyl, dihydrothienyl, dihydroindolyl, dihydroquinolyl, tetrahydroquinolyl, tetrahydroisoquinolyl, dihydroisoquinolyl, pyranyl, dihydropyranyl, dithiazolyl, benzofuranyl, isobenzoiuranyl, benzothienyl, and the like, including dihydro and tetrahydro forms thereof, where one or more double bonds are reduced and replaced with hydrogens. Still other exemplary heterocyclyls include: 2,3,4,5-tetrahydro-2-oxo-oxazolyl; 2,3-dihydro-2-oxo-lH-imidazolyl; 2,3,4,5-tetrahydro-5-oxo-lH-pyrazolyl (e.g., 2,3,4,5-tetrahydro-2-phenyl-5-oxo-lH-pyrazolyl); 2,3,4,5-tetrahydro-2,4-dioxo-lH- imidazolyl (e.g., 2,3,4,5-tetrahydro-2,4-dioxo-5-methyl-5-phenyl-lH-imidazolyl); 2,3-dihydro-2-thioxo- 1,3,4-oxadiazolyl (e.g., 2,3-dihydro-2-thioxo-5-phenyl-l,3,4-oxadiazolyl); 4,5-dihydro-5-oxo-l *H*-triazolyl (e.g., 4,5-dihydro-3-methyl-4-amino 5-oxo-l *H*-triazolyl); l,2,3,4-tetrahydro-2,4-dioxopyridinyl (e.g., l,2,3,4-tetrahydro-2,4-dioxo-3,3-diethylpyridinyl); 2,6-dioxo-piperidinyl (e.g., 2,6-dioxo-3-ethyl-3-phenylpiperidinyl); 1,6-dihydro-6-oxopyridiminyl; l,6-dihydro-4-oxopyrimidinyl (e.g., 2-(methylthio)-l,6-dihydro-4-oxo-5-methylpyrimidin-l-yl); 1,2,3,4-tetrahydro-2,4-dioxopyrimidinyl (e.g., l,2,3,4-tetrahydro-2,4-dioxo-3-ethylpyrimidinyl); 1,6-dihydro-6-oxo-pyridazinyl (e.g., l,6-dihydro-6-oxo-3-ethylpyridazinyl); l,6-dihydro-6-oxo-1,2,4-triazinyl (e.g., l,6-dihydro-5-isopropyl-6-oxo-l,2,4-triazinyl); 2,3-dihydro-2-oxo-l *H*-indolyl (e.g., 3,3-dimethyl-2,3-dihydro-2-oxo-l *H*-indolyl and 2,3-dihydro-2-oxo-3,3'-spiropropane- l*H*-indol- 1-yl); 1,3-dihydro- 1-oxo-2*H*-iso-indolyl; 1,3-dihydro- 1,3-dioxo-2*H*-iso-indolyl; l*H*-benzopyrazolyl (e.g., l-(ethoxycarbonyl)- l*H*-benzopyrazolyl); 2,3-dihydro-2-oxo-l*H*-benzimidazolyl (e.g., 3-ethyl-2,3-dihydro-2-oxo-l *H*-benzimidazolyl); 2,3-dihydro-2-oxo-benzoxazolyl (e.g., 5-chloro-2,3-dihydro-2-oxo-benzoxazolyl); 2,3-dihydro-2-oxo-benzoxazolyl; 2-oxo-2H-benzopyranyl; 1,4-benzodioxanyl; 1,3-benzodioxanyl; 2,3-dihydro-3-oxo,4*H*-l,3-benzothiazinyl; 3,4-dihydro-4-oxo-3*H*-quinazolinyl (e.g., 2-methyl-3,4-dihydro-4-oxo-3 *H*-quinazolinyl); 1,2,3,4-tetrahydro-2,4-dioxo-3 *H*-quinazolyl (e.g., 1-ethyl-1 ,2,3,4-tetrahydro-2,4-

dioxo-3*H*-quinazolyl); l,2,3,6-tetrahydro-2,6-dioxo-7 *H*-purinyl (e.g., l,2,3,6-tetrahydro-l,3-dimethyl-2,6-dioxo-7 *H* -purinyl); l,2,3,6-tetrahydro-2,6-dioxo-l *H* -purinyl (e.g., 1,2,3,6-tetrahydro-3,7-dimethyl-2,6-dioxo-l *H* -purinyl); 2-oxobenz[c,<i]indolyl; l,l-dioxo-2H-naphth[l,8-c,i/]isothiazolyl; and 1,8-naphthylenedicarboxamido. Additional heterocyclics include 3,3a,4,5,6,6a-hexahydro-pyrrolo[3,4-b]pyrrol-(2H)-yl, and 2,5-diazabicyclo[2.2.1]heptan-2-yl, homopiperazinyl (or diazepanyl), tetrahydropyranyl, dithiazolyl, benzofuranyl, benzothienyl, oxepanyl, thiepanyl, azocanyl, oxecanyl, and thiocanyl. Heterocyclic groups also include groups of the formula



, where

[0001392] E' is selected from the group consisting of -N- and -CH-; F' is selected from the group consisting of -N=CH-, -NH-CH_2-, -NH-C(O)-, -NH-, -CH=N-, -CH_2-NH-, -C(0)-NH-, -CH=CH-, -CH_2-, -CH2CH2-, -CH2O-, -OCH2-, -0-, and -S-; and G' is selected from the group consisting of -CH- and -N-. Any of the heterocyclyl groups mentioned herein may be optionally substituted with one, two, three, four or five substituents independently selected from the group consisting of: (1) C1-7 acyl (e.g., carboxyaldehyde); (2) Ci-2_0 alkyl (e.g., Ci_6 alkyl, Ci_6 alkoxy-C1-6 alkyl, Ci_6 alkylsulfinyl-Ci-6 alkyl, amino-Ci-6 alkyl, azido-Ci_6 alkyl, (carboxyaldehyde)-Ci-$_6$ alkyl, halo-Ci_6 alkyl (e.g., perfluoroalkyl), hydroxy-Ci_6 alkyl, nitro-Ci-6 alkyl, or Ci_6 thioalkoxy-Ci-6 alkyl); (3) Ci-20 alkoxy (e.g., Ci_6 alkoxy, such as perfluoroalkoxy); (4) Ci_6 alkylsulfinyl; (5) C 6-10 aryl; (6) amino; (7) Ci_6 alk-C6-io aryl; (8) azido; (9) C3-8 cycloalkyl; (10) Ci-6 alk-C3_8 cycloalkyl; (11) halo; (12) Ci-12 heterocyclyl (e.g., C2-12 heteroaryl); (13) (Ci-12 heterocyclyl)oxy; (14) hydroxy; (15) nitro; (16) Ci-20 thioalkoxy (e.g., Ci-6 thioalkoxy); (17) -$(CH_2)_q$C0 2R$^{A'}$, where q is an integer from zero to four, and R$^{A'}$ is selected from the group consisting of (a) Ci_6 alkyl, (b) C 6-10 aryl, (c) hydrogen, and (d) Ci_6 alk-C6-io aryl; (18) -(CH2) $_q$CONR$^{B'}$R$^{C'}$, where q is an integer from zero to four and where R$^{B'}$ and R$^{C'}$ are independently selected from the group consisting of (a) hydrogen, (b) Ci_6 alkyl, (c) C 6-1_0 aryl, and (d) Ci_6 alk-C6-io aryl; (19) -$(CH_2)_q$S02R $^{D'}$, where q is an integer from zero to four and where R$^{D'}$ is selected from the group consisting of (a) Ci_6 alkyl, (b) C 6-10 aryl, and (c) Ci_6 alk-C6-io aryl; (20) -$(CH_2)_q$S02NR $^{E'}$R$^{F'}$, where q is an integer from zero to four and where each of R$^{E'}$ and R$^{F'}$ is, independently, selected from the group consisting of (a) hydrogen, (b) Ci_6 alkyl, (c) C 6-1_0 aryl, and (d) Ci_6 alk-C6-io aryl; (21) thiol; (22) C 6-10 aryloxy; (23) C3-8 cycloalkoxy; (24) arylalkoxy; (25) Ci_6 alk-Ci-12 heterocyclyl (e.g., Ci_6 alk-Ci-12 heteroaryl); (26) oxo; (27) (Ci-12 heterocyclyl)imino; (28) C2-2_0 alkenyl; and (29) C2-2_0 alkynyl. In some embodiments, each of

these groups can be further substituted as described herein. For example, the alkylene group of a Ci-alkaryl or a Ci-alkheterocyclyl can be further substituted with an oxo group to afford the respective aryloyl and (heterocyclyl)oyl substituent group.

[0001393] The "heterocyclylalkyl" group, which as used herein, represents a heterocyclyl group, as defined herein, attached to the parent molecular group through an alkylene group, as defined herein. Exemplary unsubstituted heterocyclylalkyl groups are from 2 to 32 carbons (e.g., from 2 to 22, from 2 to 18, from 2 to 17, from 2 to 16, from 3 to 15, from 2 to 14, from 2 to 13, or from 2 to 12 carbons, such as Ci_6 alk-Ci-12 heterocyclyl, C_{1-10} alk-C_{1-12} heterocyclyl, or C_{1-20} alk-C_{1-12} heterocyclyl). In some embodiments, the alkylene and the heterocyclyl each can be further substituted with 1, 2, 3, or 4 substituent groups as defined herein for the respective group.

[0001394] The term "hydrocarbon," as used herein, represents a group consisting only of carbon and hydrogen atoms.

[0001395] The term "hydroxy," as used herein, represents an -OH group.

[0001396] The term "isomer," as used herein, means any tautomer, stereoisomer, enantiomer, or diastereomer of any compound of the invention. It is recognized that the compounds of the invention can have one or more chiral centers and/or double bonds and, therefore, exist as stereoisomers, such as double-bond isomers (i.e., geometric E/Z isomers) or diastereomers (e.g., enantiomers (i.e., (+) or (-)) or cis/trans isomers). According to the invention, the chemical structures depicted herein, and therefore the compounds of the invention, encompass all of the corresponding stereoisomers, that is, both the stereomerically pure form (e.g., geometrically pure, enantiomerically pure, or diastereomerically pure) and enantiomeric and stereoisomeric mixtures, e.g., racemates. Enantiomeric and stereoisomeric mixtures of compounds of the invention can typically be resolved into their component enantiomers or stereoisomers by well-known methods, such as chiral-phase gas chromatography, chiral-phase high performance liquid chromatography, crystallizing the compound as a chiral salt complex, or crystallizing the compound in a chiral solvent. Enantiomers and stereoisomers can also be obtained from stereomerically or enantiomerically pure intermediates, reagents, and catalysts by well-known asymmetric synthetic methods.

[0001397] The term *"N*-protected amino," as used herein, refers to an amino group, as defined herein, to which is attached one or two *N*-protecting groups, as defined herein.

[0001398] The term *"N*-protecting group," as used herein, represents those groups intended to protect an amino group against undesirable reactions during synthetic procedures. Commonly used *N*-protecting groups are disclosed in Greene, "Protective Groups in Organic Synthesis," 3rd Edition (John Wiley & Sons, New York, 1999), which is incorporated herein by reference. *N*-

protecting groups include acyl, aryloyl, or carbamyl groups such as formyl, acetyl, propionyl, pivaloyl, t-butylacetyl, 2-chloroacetyl, 2-bromoacetyl, trifluoroacetyl, trichloroacetyl, phthalyl, o-nitrophenoxyacetyl, a-chlorobutyryl, benzoyl, 4-chlorobenzoyl, 4-bromobenzoyl, 4-nitrobenzoyl, and chiral auxiliaries such as protected or unprotected D, L or D, L-amino acids such as alanine, leucine, phenylalanine, and the like; sulfonyl-containing groups such as benzenesulfonyl, p-toluenesulfonyl, and the like; carbamate forming groups such as benzyloxycarbonyl, p-chlorobenzyloxycarbonyl, p-methoxybenzyloxycarbonyl, p-nitrobenzyloxycarbonyl, 2-nitrobenzyloxycarbonyl, p-bromobenzyloxycarbonyl, 3,4-dimethoxybenzyloxycarbonyl, 3,5-dimethoxybenzyloxycarbonyl, 2,4-dimethoxybenzyloxycarbonyl, 4-methoxybenzyloxycarbonyl, 2-nitro-4,5-dimethoxybenzyloxycarbonyl, 3,4,5-trimethoxybenzyloxycarbonyl, l-(p-biphenylyl)-l-methylethoxycarbonyl, a,a-dimethyl-3,5-dimethoxybenzyloxycarbonyl, benzhydryloxy carbonyl, t-butyloxycarbonyl, diisopropylmethoxycarbonyl, isopropyloxycarbonyl, ethoxycarbonyl, methoxycarbonyl, allyloxycarbonyl, 2,2,2,-trichloroethoxycarbonyl, phenoxycarbonyl, 4-nitrophenoxy carbonyl, fluorenyl-9-methoxycarbonyl, cyclopentyloxycarbonyl, adamantyloxycarbonyl, cyclohexyloxycarbonyl, phenylthiocarbonyl, and the like, alkaryl groups such as benzyl, triphenylmethyl, benzyloxymethyl, and the like and silyl groups, such as trimethylsilyl, and the like. Preferred *N*-protecting groups are formyl, acetyl, benzoyl, pivaloyl, t-butylacetyl, alanyl, phenylsulfonyl, benzyl, t-butyloxycarbonyl (Boc), and benzyloxycarbonyl (Cbz).

[0001399] The term "nitro," as used herein, represents an $-NO_2$ group.

[0001400] The term "O-protecting group," as used herein, represents those groups intended to protect an oxygen containing (e.g., phenol, hydroxyl, or carbonyl) group against undesirable reactions during synthetic procedures. Commonly used O-protecting groups are disclosed in Greene, "Protective Groups in Organic Synthesis," 3rd Edition (John Wiley & Sons, New York, 1999), which is incorporated herein by reference. Exemplary O-protecting groups include acyl, aryloyl, or carbamyl groups, such as formyl, acetyl, propionyl, pivaloyl, t-butylacetyl, 2-chloroacetyl, 2-bromoacetyl, trifluoroacetyl, trichloroacetyl, phthalyl, o-nitrophenoxyacetyl, a-chlorobutyryl, benzoyl, 4-chlorobenzoyl, 4-bromobenzoyl, i-butyldimethylsilyl, *tri-iso*-propylsilyloxymethyl, 4,4'-dimethoxytrityl, isobutyryl, phenoxyacetyl, 4-isopropylpehenoxyacetyl, dimethylformamidino, and 4-nitrobenzoyl; alkylcarbonyl groups, such as acyl, acetyl, propionyl, pivaloyl, and the like; optionally substituted arylcarbonyl groups, such as benzoyl; silyl groups, such as trimethylsilyl (TMS), tert-butyldimethylsilyl (TBDMS), tri-iso-propylsilyloxymethyl (TOM), triisopropylsilyl (TIPS), and the like; ether-forming groups with

the hydroxyl, such methyl, methoxymethyl, tetrahydropyranyl, benzyl, p-methoxybenzyl, trityl, and the like; alkoxycarbonyls, such as methoxycarbonyl, ethoxycarbonyl, isopropoxycarbonyl, n-isopropoxycarbonyl, n-butyloxycarbonyl, isobutyloxycarbonyl, sec-butyloxycarbonyl, t-butyloxycarbonyl, 2-ethylhexyloxycarbonyl, cyclohexyloxycarbonyl, methyloxycarbonyl, and the like; alkoxyalkoxycarbonyl groups, such as methoxymethoxycarbonyl, ethoxymethoxycarbonyl, 2-methoxyethoxycarbonyl, 2-ethoxyethoxycarbonyl, 2-butoxyethoxycarbonyl, 2-methoxyethoxymethoxycarbonyl, allyloxycarbonyl, propargyloxycarbonyl, 2-butenoxycarbonyl, 3-methyl-2-butenoxycarbonyl, and the like; haloalkoxycarbonyls, such as 2-chloroethoxycarbonyl, 2-chloroethoxycarbonyl, 2,2,2-trichloroethoxycarbonyl, and the like; optionally substituted arylalkoxycarbonyl groups, such as benzyloxycarbonyl, p-methylbenzyloxycarbonyl, p-methoxybenzyloxycarbonyl, p-nitrobenzyloxycarbonyl, 2,4-dinitrobenzyloxycarbonyl, 3,5-dimethylbenzyloxycarbonyl, p-chlorobenzyloxycarbonyl, p-bromobenzyloxy-carbonyl, fluorenylmethyloxycarbonyl, and the like; and optionally substituted aryloxycarbonyl groups, such as phenoxycarbonyl, p-nitrophenoxycarbonyl, o-nitrophenoxycarbonyl, 2,4-dinitrophenoxycarbonyl, p-methyl-phenoxycarbonyl, m-methylphenoxycarbonyl, o-bromophenoxycarbonyl, 3,5-dimethylphenoxycarbonyl, p-chlorophenoxycarbonyl, 2-chloro-4-nitrophenoxy-carbonyl, and the like); substituted alkyl, aryl, and alkaryl ethers (e.g., trityl; methylthiomethyl; methoxymethyl; benzyloxymethyl; siloxymethyl; 2,2,2,-trichloroethoxymethyl; tetrahydropyranyl; tetrahydrofuranyl; ethoxyethyl; l-[2-(trimethylsilyl)ethoxy] ethyl; 2-trimethylsilylethyl; t-butyl ether; p-chlorophenyl, p-methoxyphenyl, p-nitrophenyl, benzyl, p-methoxybenzyl, and nitrobenzyl); silyl ethers (e.g., trimethylsilyl; triethylsilyl; triisopropylsilyl; dimethylisopropylsilyl; t-butyldimethylsilyl; t-butyldiphenylsilyl; tribenzylsilyl; triphenylsilyl; and diphenymethylsilyl); carbonates (e.g., methyl, methoxymethyl, 9-fluorenylmethyl; ethyl; 2,2,2-trichloroethyl; 2-(trimethylsilyl)ethyl; vinyl, allyl, nitrophenyl; benzyl; methoxybenzyl; 3,4-dimethoxybenzyl; and nitrobenzyl); carbonyl-protecting groups (e.g., acetal and ketal groups, such as dimethyl acetal, 1,3-dioxolane, and the like; acylal groups; and dithiane groups, such as 1,3-dithianes, 1,3-dithiolane, and the like); carboxylic acid-protecting groups (e.g., ester groups, such as methyl ester, benzyl ester, t-butyl ester, orthoesters, and the like; and oxazoline groups.

[0001401] The term "oxo" as used herein, represents **=0.**

[0001402] The prefix "perfluoro," as used herein, represents anyl group, as defined herein, where each hydrogen radical bound to the alkyl group has been replaced by a fluoride radical.

For example, perfluoroalkyl groups are exemplified by trifluoromethyl, pentafluoroethyl, and the like.

[0001403] The term "protected hydroxyl," as used herein, refers to an oxygen atom bound to an O-protecting group.

[0001404] The term "spirocyclyl," as used herein, represents a C2-7 alkylene diradical, both ends of which are bonded to the same carbon atom of the parent group to form a spirocyclic group, and also a Ci_6 heteroalkylene diradical, both ends of which are bonded to the same atom. The heteroalkylene radical forming the spirocyclyl group can containing one, two, three, or four heteroatoms independently selected from the group consisting of nitrogen, oxygen, and sulfur. In some embodiments, the spirocyclyl group includes one to seven carbons, excluding the carbon atom to which the diradical is attached. The spirocyclyl groups of the invention may be optionally substituted with 1, 2, 3, or 4 substituents provided herein as optional substituents for cycloalkyl and/or heterocyclyl groups.

[0001405] The term "stereoisomer," as used herein, refers to all possible different isomeric as well as conformational forms which a compound may possess (e.g., a compound of any formula described herein), in particular all possible stereochemically and conformationally isomeric forms, all diastereomers, enantiomers and/or conformers of the basic molecular structure. Some compounds of the present invention may exist in different tautomeric forms, all of the latter being included within the scope of the present invention.

[0001406] The term "sulfonyl," as used herein, represents an -S**(0**)2- group.

[0001407] The term "thiol," as used herein represents an -SH group.

[0001408] *Chimera:* As used herein, "chimera" is an entity having two or more incongruous or heterogeneous parts or regions.

[0001409] *Chimeric polynucleotide:* As used herein, "chimeric polynucleotides" are those nucleic acid polymers having portions or regions which differ in size and/or chemical modification pattern, chemical modification position, chemical modification percent or chemical modification population and combinations of the foregoing.

[0001410] *Compound:* As used herein, the term "compound," is meant to include all stereoisomers, geometric isomers, tautomers, and isotopes of the structures depicted.

[0001411] The compounds described herein can be asymmetric *(e.g.,* having one or more stereocenters). All stereoisomers, such as enantiomers and diastereomers, are intended unless otherwise indicated. Compounds of the present disclosure that contain asymmetrically substituted carbon atoms can be isolated in optically active or racemic forms. Methods on how to prepare optically active forms from optically active starting materials are known in the art,

such as by resolution of racemic mixtures or by stereoselective synthesis. Many geometric isomers of olefins, C=N double bonds, and the like can also be present in the compounds described herein, and all such stable isomers are contemplated in the present disclosure. Cis and trans geometric isomers of the compounds of the present disclosure are described and may be isolated as a mixture of isomers or as separated isomeric forms.

[0001412] Compounds of the present disclosure also include tautomeric forms. Tautomeric forms result from the swapping of a single bond with an adjacent double bond and the concomitant migration of a proton. Tautomeric forms include prototropic tautomers which are isomeric protonation states having the same empirical formula and total charge. Examples prototropic tautomers include ketone - enol pairs, amide - imidic acid pairs, lactam - lactim pairs, amide - imidic acid pairs, enamine - imine pairs, and annular forms where a proton can occupy two or more positions of a heterocyclic system, such as, 1H- and 3H-imidazole, 1H-, 2H- and 4H- 1,2,4-triazole, 1H- and 2H- isoindole, and 1H- and 2H-pyrazole. Tautomeric forms can be in equilibrium or sterically locked into one form by appropriate substitution.

[0001413] Compounds of the present disclosure also include all of the isotopes of the atoms occurring in the intermediate or final compounds. "Isotopes" refers to atoms having the same atomic number but different mass numbers resulting from a different number of neutrons in the nuclei. For example, isotopes of hydrogen include tritium and deuterium.

[0001414] The compounds and salts of the present disclosure can be prepared in combination with solvent or water molecules to form solvates and hydrates by routine methods.

[0001415] *Committed:* As used herein, the term "committed" means, when referring to a cell, when the cell is far enough into the differentiation pathway where, under normal circumstances, it will continue to differentiate into a specific cell type or subset of cell type instead of into a different cell type or reverting to a lesser differentiated cell type.

[0001416] *Conserved:* As used herein, the term "conserved" refers to nucleotides or amino acid residues of a polynucleotide sequence or polypeptide sequence, respectively, that are those that occur unaltered in the same position of two or more sequences being compared. Nucleotides or amino acids that are relatively conserved are those that are conserved amongst more related sequences than nucleotides or amino acids appearing elsewhere in the sequences.

[0001417] In some embodiments, two or more sequences are said to be "completely conserved" if they are 100% identical to one another. In some embodiments, two or more sequences are said to be "highly conserved" if they are at least 70% identical, at least 80% identical, at least 90% identical, or at least 95% identical to one another. In some embodiments, two or more sequences are said to be "highly conserved" if they are about 70% identical, about 80% identical, about

90% identical, about 95%, about 98%, or about 99% identical to one another. In some embodiments, two or more sequences are said to be "conserved" if they are at least 30% identical, at least 40% identical, at least 50% identical, at least 60% identical, at least 70% identical, at least 80% identical, at least 90% identical, or at least 95% identical to one another. In some embodiments, two or more sequences are said to be "conserved" if they are about 30% identical, about 40% identical, about 50% identical, about 60% identical, about 70% identical, about 80% identical, about 90% identical, about 95% identical, about 98% identical, or about 99% identical to one another. Conservation of sequence may apply to the entire length of an polynucleotide or polypeptide or may apply to a portion, region or feature thereof.

[0001418] *Controlled Release:* As used herein, the term "controlled release" refers to a pharmaceutical composition or compound release profile that conforms to a particular pattern of release to effect a therapeutic outcome.

[0001419] *Cyclic or Cyclized:* As used herein, the term "cyclic" refers to the presence of a continuous loop. Cyclic molecules need not be circular, only joined to form an unbroken chain of subunits. Cyclic molecules such as the engineered RNA or mRNA of the present invention may be single units or multimers or comprise one or more components of a complex or higher order structure.

[0001420] *Cytostatic:* As used herein, "cytostatic" refers to inhibiting, reducing, suppressing the growth, division, or multiplication of a cell *(e.g.,* a mammalian cell *(e.g.,* a human cell)), bacterium, virus, fungus, protozoan, parasite, prion, or a combination thereof.

[0001421] *Cytotoxic:* As used herein, "cytotoxic" refers to killing or causing injurious, toxic, or deadly effect on a cell *(e.g.,* a mammalian cell *(e.g.,* a human cell)), bacterium, virus, fungus, protozoan, parasite, prion, or a combination thereof.

[0001422] *Delivery:* As used herein, "delivery" refers to the act or manner of delivering a compound, substance, entity, moiety, cargo or payload.

[0001423] *Delivery Agent:* As used herein, "delivery agent" refers to any substance which facilitates, at least in part, the *in vivo* delivery of a polynucleotide to targeted cells.

[0001424] *Destabilized:* As used herein, the term "destable," "destabilize," or "destabilizing region" means a region or molecule that is less stable than a starting, wild-type or native form of the same region or molecule.

[0001425] *Detectable label:* As used herein, "detectable label" refers to one or more markers, signals, or moieties which are attached, incorporated or associated with another entity that is readily detected by methods known in the art including radiography, fluorescence, chemiluminescence, enzymatic activity, absorbance and the like. Detectable labels include

radioisotopes, fluorophores, chromophores, enzymes, dyes, metal ions, ligands such as biotin, avidin, streptavidin and haptens, quantum dots, and the like. Detectable labels may be located at any position in the peptides or proteins disclosed herein. They may be within the amino acids, the peptides, or proteins, or located at the N- or C- termini.

[0001426] *Diastereomer:* As used herein, the term "diastereomer," means stereoisomers that are not mirror images of one another and are non-superimposable on one another.

[0001427] *Digest:* As used herein, the term "digest" means to break apart into smaller pieces or components. When referring to polypeptides or proteins, digestion results in the production of peptides.

[0001428] *Differentiated cell:* As used herein, the term "differentiated cell" refers to any somatic cell that is not, in its native form, pluripotent. Differentiated cell also encompasses cells that are partially differentiated.

[0001429] *Differentiation:* As used herein, the term "differentiation factor" refers to a developmental potential altering factor such as a protein, RNA or small molecule that can induce a cell to differentiate to a desired cell-type.

[0001430] *Differentiate:* As used herein, "differentiate" refers to the process where an uncommitted or less committed cell acquires the features of a committed cell.

[0001431] *Distal:* As used herein, the term "distal" means situated away from the center or away from a point or region of interest.

[0001432] *Dosing regimen:* As used herein, a "dosing regimen" is a schedule of administration or physician determined regimen of treatment, prophylaxis, or palliative care.

[0001433] *Dose splitting factor (DSF)-ratio* of PUD of dose split treatment divided by PUD of total daily dose or single unit dose. The value is derived from comparison of dosing regimens groups.

[0001434] *Enantiomer:* As used herein, the term "enantiomer" means each individual optically active form of a compound of the invention, having an optical purity or enantiomeric excess (as determined by methods standard in the art) of at least 80% (i.e., at least 90% of one enantiomer and at most 10% of the other enantiomer), preferably at least 90% and more preferably at least 98%.

[0001435] *Encapsulate:* As used herein, the term "encapsulate" means to enclose, surround or encase.

[0001436] *Encoded protein cleavage signal:* As used herein, "encoded protein cleavage signal" refers to the nucleotide sequence which encodes a protein cleavage signal.

[0001437] *Engineered:* As used herein, embodiments of the invention are "engineered" when they are designed to have a feature or property, whether structural or chemical, that varies from a starting point, wild type or native molecule.

[0001438] *Effective Amount:* As used herein, the term "effective amount" of an agentis that amount sufficient to effect beneficial or desired results, for example, clinical results, and, as such, an "effective amount" depends upon the context in which it is being applied. For example, in the context of administering an agent that treats cancer, an effective amount of an agent is, for example, an amount sufficient to achieve treatment, as defined herein, of cancer, as compared to the response obtained without administration of the agent.

[0001439] *Exosome:* As used herein, "exosome" is a vesicle secreted by mammalian cells or a complex involved in RNA degradation.

[0001440] *Expression:* As used herein, "expression" of a nucleic acid sequence refers to one or more of the following events: (1) production of an RNA template from a DNA sequence *(e.g.,* by transcription); (2) processing of an RNA transcript *(e.g.,* by splicing, editing, 5' cap formation, and/or 3' end processing); (3) translation of an RNA into a polypeptide or protein; and (4) post-translational modification of a polypeptide or protein.

[0001441] *Feature:* As used herein, a "feature" refers to a characteristic, a property, or a distinctive element.

[0001442] *Formulation:* As used herein, a "formulation" includes at least a polynucleotide and a delivery agent.

[0001443] *Fragment:* A "fragment," as used herein, refers to a portion. For example, fragments of proteins may comprise polypeptides obtained by digesting full-length protein isolated from cultured cells.

[0001444] *Functional:* As used herein, a "functional" biological molecule is a biological molecule in a form in which it exhibits a property and/or activity by which it is characterized.

[0001445] *Homology:* As used herein, the term "homology" refers to the overall relatedness between polymeric molecules, *e.g.* between nucleic acid molecules *(e.g.* DNA molecules and/or RNA molecules) and/or between polypeptide molecules. In some embodiments, polymeric molecules are considered to be "homologous" to one another if their sequences are at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% identical or similar. The term "homologous" necessarily refers to a comparison between at least two sequences (polynucleotide or polypeptide sequences). In accordance with the invention, two polynucleotide sequences are considered to be homologous if the polypeptides they encode are at least about 50%, 60%, 70%, 80%, 90%, 95%, or even 99% for at least one stretch of at least

about 20 amino acids. In some embodiments, homologous polynucleotide sequences are characterized by the ability to encode a stretch of at least 4-5 uniquely specified amino acids. For polynucleotide sequences less than 60 nucleotides in length, homology is determined by the ability to encode a stretch of at least 4-5 uniquely specified amino acids. In accordance with the invention, two protein sequences are considered to be homologous if the proteins are at least about 50%, 60%, 70%, 80%, or 90% identical for at least one stretch of at least about 20 amino acids.

[0001446] *Identity:* As used herein, the term "identity" refers to the overall relatedness between polymeric molecules, *e.g.,* between polynucleotide molecules *(e.g.* DNA molecules and/or RNA molecules) and/or between polypeptide molecules. Calculation of the percent identity of two polynucleotide sequences, for example, can be performed by aligning the two sequences for optimal comparison purposes *(e.g.,* gaps can be introduced in one or both of a first and a second nucleic acid sequences for optimal alignment and non-identical sequences can be disregarded for comparison purposes). In certain embodiments, the length of a sequence aligned for comparison purposes is at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or 100% of the length of the reference sequence. The nucleotides at corresponding nucleotide positions are then compared. When a position in the first sequence is occupied by the same nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which needs to be introduced for optimal alignment of the two sequences. The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm. For example, the percent identity between two nucleotide sequences can be determined using methods such as those described in Computational Molecular Biology, Lesk, A. M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D. W., ed., Academic Press, New York, 1993; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; Computer Analysis of Sequence Data, Part I, Griffin, A. M., and Griffin, H. G., eds., Humana Press, New Jersey, 1994; and Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991; each of which is incorporated herein by reference. For example, the percent identity between two nucleotide sequences can be determined using the algorithm of Meyers and Miller (CABIOS, 1989, 4:11-17), which has been incorporated into the ALIGN program (version 2.0) using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4. The percent identity between two nucleotide

sequences can, alternatively, be determined using the GAP program in the GCG software package using an NWSgapdna.CMP matrix. Methods commonly employed to determine percent identity between sequences include, but are not limited to those disclosed in Carillo, H., and Lipman, D., SIAM J Applied Math., 48:1073 (1988); incorporated herein by reference. Techniques for determining identity are codified in publicly available computer programs. Exemplary computer software to determine homology between two sequences include, but are not limited to, GCG program package, Devereux, J., *et al, Nucleic Acids Research,* 12(1), 387 (1984)), BLASTP, BLASTN, and FASTA Altschul, S. F. *etal, J. Molec. Biol,* 215, 403 (1990)).

[0001447] *Infectious Agent:* As used herein, the phrase "infectious agent" means an agent capable of producing an infection.

[0001448] *Inhibit expression of a gene:* As used herein, the phrase "inhibit expression of a gene" means to cause a reduction in the amount of an expression product of the gene. The expression product can be an RNA transcribed from the gene *(e.g.,* an mRNA) or a polypeptide translated from an mRNA transcribed from the gene. Typically a reduction in the level of an mRNA results in a reduction in the level of a polypeptide translated therefrom. The level of expression may be determined using standard techniques for measuring mRNA or protein.

[0001449] *Infectious agent:* As used herein, an "infectious agent" refers to any microorganism, virus, infectious substance, or biological product that may be engineered as a result of biotechnology, or any naturally occurring or bioengineered component of any such microorganism, virus, infectious substance, or biological product, can cause emerging and contagious disease, death or other biological malfunction in a human, an animal, a plant or another living organism.

[0001450] *Influenza:* As used herein, "influenza" or "flu" is an infectious disease of birds and mammals caused by RNA viruses of the family Orthomyxoviridae, the influenza viruses.

[0001451] *Isomer:* As used herein, the term "isomer" means any tautomer, stereoisomer, enantiomer, or diastereomer of any compound of the invention. It is recognized that the compounds of the invention can have one or more chiral centers and/or double bonds and, therefore, exist as stereoisomers, such as double-bond isomers (i.e., geometric E/Z isomers) or diastereomers (e.g., enantiomers (i.e., (+) or (-)) or cis/trans isomers). According to the invention, the chemical structures depicted herein, and therefore the compounds of the invention, encompass all of the corresponding stereoisomers, that is, both the stereomerically pure form (e.g., geometrically pure, enantiomerically pure, or diastereomerically pure) and enantiomeric and stereoisomeric mixtures, e.g., racemates. Enantiomeric and stereoisomeric mixtures of

compounds of the invention can typically be resolved into their component enantiomers or stereoisomers by well-known methods, such as chiral-phase gas chromatography, chiral-phase high performance liquid chromatography, crystallizing the compound as a chiral salt complex, or crystallizing the compound in a chiral solvent. Enantiomers and stereoisomers can also be obtained from stereomerically or enantiomerically pure intermediates, reagents, and catalysts by well-known asymmetric synthetic methods.

[0001452] *In vitro:* As used herein, the term "*i« vitro"* refers to events that occur in an artificial environment, *e.g.,* in a test tube or reaction vessel, in cell culture, in a Petri dish, *etc.,* rather than within an organism *(e.g.,* animal, plant, or microbe).

[0001453] *In vivo:* As used herein, the term *"in vivo"* refers to events that occur within an organism *(e.g.,* animal, plant, or microbe or cell or tissue thereof).

[0001454] *Isolated:* As used herein, the term "isolated" refers to a substance or entity that has been separated from at least some of the components with which it was associated (whether in nature or in an experimental setting). Isolated substances may have varying levels of purity in reference to the substances from which they have been associated. Isolated substances and/or entities may be separated from at least about 10%, about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, or more of the other components with which they were initially associated. In some embodiments, isolated agents are more than about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or more than about 99% pure. As used herein, a substance is "pure" if it is substantially free of other components. *Substantially isolated:* By "substantially isolated" is meant that the compound is substantially separated from the environment in which it was formed or detected. Partial separation can include, for example, a composition enriched in the compound of the present disclosure. Substantial separation can include compositions containing at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95%, at least about 97%, or at least about 99% by weight of the compound of the present disclosure, or salt thereof. Methods for isolating compounds and their salts are routine in the art.

[0001455] *IVT Polynucleotide:* As used herein, an "IVT polynucleotide" is a linear polynucleotide which may be made using only *in vitro* transcription (IVT) enzymatic synthesis methods.

[0001456] *Linker:* As used herein, a "linker" refers to a group of atoms, e.g., 10-1,000 atoms, and can be comprised of the atoms or groups such as, but not limited to, carbon, amino, alkylamino, oxygen, sulfur, sulfoxide, sulfonyl, carbonyl, and imine. The linker can be attached

to a modified nucleoside or nucleotide on the nucleobase or sugar moiety at a first end, and to a payload, e.g., a detectable or therapeutic agent, at a second end. The linker may be of sufficient length as to not interfere with incorporation into a nucleic acid sequence. The linker can be used for any useful purpose, such as to form polynucleotide multimers (e.g., through linkage of two or more chimeric polynucleotides molecules or IVT polynucleoties) or polynucleotides conjugates, as well as to administer a payload, as described herein. Examples of chemical groups that can be incorporated into the linker include, but are not limited to, alkyl, alkenyl, alkynyl, amido, amino, ether, thioether, ester, alkylene, heteroalkylene, aryl, or heterocyclyl, each of which can be optionally substituted, as described herein. Examples of linkers include, but are not limited to, unsaturated alkanes, polyethylene glycols (e.g., ethylene or propylene glycol monomeric units, e.g., diethylene glycol, dipropylene glycol, triethylene glycol, tripropylene glycol, tetraethylene glycol, or tetraethylene glycol), and dextran polymers and derivatives thereof, Other examples include, but are not limited to, cleavable moieties within the linker, such as, for example, a disulfide bond (-S-S-) or an azo bond (-N=N-), which can be cleaved using a reducing agent or photolysis. Non-limiting examples of a selectively cleavable bond include an amido bond can be cleaved for example by the use of tris(2-carboxyethyl)phosphine (TCEP), or other reducing agents, and/or photolysis, as well as an ester bond can be cleaved for example by acidic or basic hydrolysis.

[0001457] *MicroRNA (miRNA) binding site:* As used herein, a microRNA (miRNA) binding site represents a nucleotide location or region of a nucleic acid transcript to which at least the "seed" region of a miRNA binds.

[0001458] *Modified:* As used herein "modified" refers to a changed state or structure of a molecule of the invention. Molecules may be modified in many ways including chemically, structurally, and functionally. In one embodiment, the mRNA molecules of the present invention are modified by the introduction of non-natural nucleosides and/or nucleotides, e.g., as it relates to the natural ribonucleotides A, U, G, and C. Noncanonical nucleotides such as the cap structures are not considered "modified" although they differ from the chemical structure of the A, C, G, U ribonucleotides.

[0001459] *Mucus:* As used herein, "mucus" refers to the natural substance that is viscous and comprises mucin glycoproteins.

[0001460] *Naturally occurring:* As used herein, "naturally occurring" means existing in nature without artificial aid.

[0001461] *Neutralizing antibody:* As used herein, a "neutralizing antibody" refers to an antibody which binds to its antigen and defends a cell from an antigen or infectious agent by neutralizing or abolishing any biological activity it has.

[0001462] *Non-human vertebrate:* As used herein, a "non human vertebrate" includes all vertebrates except *Homo sapiens*, including wild and domesticated species. Examples of non-human vertebrates include, but are not limited to, mammals, such as alpaca, banteng, bison, camel, cat, cattle, deer, dog, donkey, gayal, goat, guinea pig, horse, llama, mule, pig, rabbit, reindeer, sheep water buffalo, and yak.

[0001463] *Off-target:* As used herein, "off target" refers to any unintended effect on any one or more target, gene, or cellular transcript.

[0001464] *Open reading frame:* As used herein, "open reading frame" or "ORF" refers to a sequence which does not contain a stop codon in a given reading frame.

[0001465] *Operably linked:* As used herein, the phrase "operably linked" refers to a functional connection between two or more molecules, constructs, transcripts, entities, moieties or the like.

[0001466] *Optionally substituted:* Herein a phrase of the form "optionally substituted X" *(e.g.,* optionally substituted alkyl) is intended to be equivalent to "X, wherein X is optionally substituted" *(e.g.,* "alkyl, wherein the alkyl is optionally substituted"). It is not intended to mean that the feature "X" *(e.g.* alkyl) *per se* is optional.

[0001467] *Part:* As used herein, a "part" or "region" of a polynucleotide is defined as any portion of the polynucleotide which is less than the entire length of the polynucleotide.

[0001468] *Peptide:* As used herein, "peptide" is less than or equal to 50 amino acids long, e.g., about 5, 10, 15, 20, 25, 30, 35, 40, 45, or 50 amino acids long.

[0001469] *Paratope:* As used herein, a "paratope" refers to the antigen-binding site of an antibody.

[0001470] *Patient:* As used herein, "patient" refers to a subject who may seek or be in need of treatment, requires treatment, is receiving treatment, will receive treatment, or a subject who is under care by a trained professional for a particular disease or condition.

[0001471] *Pharmaceutically acceptable:* The phrase "pharmaceutically acceptable" is employed herein to refer to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

[0001472] *Pharmaceutically acceptable excipients:* The phrase "pharmaceutically acceptable excipient," as used herein, refers any ingredient other than the compounds described herein (for

example, a vehicle capable of suspending or dissolving the active compound) and having the properties of being substantially nontoxic and non-inflammatory in a patient. Excipients may include, for example: antiadherents, antioxidants, binders, coatings, compression aids, disintegrants, dyes (colors), emollients, emulsifiers, fillers (diluents), film formers or coatings, flavors, fragrances, glidants (flow enhancers), lubricants, preservatives, printing inks, sorbents, suspensing or dispersing agents, sweeteners, and waters of hydration. Exemplary excipients include, but are not limited to: butylated hydroxytoluene (BHT), calcium carbonate, calcium phosphate (dibasic), calcium stearate, croscarmellose, crosslinked polyvinyl pyrrolidone, citric acid, crospovidone, cysteine, ethylcellulose, gelatin, hydroxypropyl cellulose, hydroxypropyl methylcellulose, lactose, magnesium stearate, maltitol, mannitol, methionine, methylcellulose, methyl paraben, microcrystalline cellulose, polyethylene glycol, polyvinyl pyrrolidone, povidone, pregelatinized starch, propyl paraben, retinyl palmitate, shellac, silicon dioxide, sodium carboxymethyl cellulose, sodium citrate, sodium starch glycolate, sorbitol, starch (corn), stearic acid, sucrose, talc, titanium dioxide, vitamin A, vitamin E, vitamin C, and xylitol.

[0001473] *Pharmaceutically acceptable salts:* The present disclosure also includes pharmaceutically acceptable salts of the compounds described herein. As used herein, "pharmaceutically acceptable salts" refers to derivatives of the disclosed compounds wherein the parent compound is modified by converting an existing acid or base moiety to its salt form (e.g., by reacting the free base group with a suitable organic acid). Examples of pharmaceutically acceptable salts include, but are not limited to, mineral or organic acid salts of basic residues such as amines; alkali or organic salts of acidic residues such as carboxylic acids; and the like. Representative acid addition salts include acetate, acetic acid, adipate, alginate, ascorbate, aspartate, benzenesulfonate, benzene sulfonic acid, benzoate, bisulfate, borate, butyrate, camphorate, camphorsulfonate, citrate, cyclopentanepropionate, digluconate, dodecylsulfate, ethanesulfonate, fumarate, glucoheptonate, glycerophosphate, hemisulfate, heptonate, hexanoate, hydrobromide, hydrochloride, hydroiodide, 2-hydroxy-ethanesulfonate, lactobionate, lactate, laurate, lauryl sulfate, malate, maleate, malonate, methanesulfonate, 2-naphthalenesulfonate, nicotinate, nitrate, oleate, oxalate, palmitate, pamoate, pectinate, persulfate, 3-phenylpropionate, phosphate, picrate, pivalate, propionate, stearate, succinate, sulfate, tartrate, thiocyanate, toluenesulfonate, undecanoate, valerate salts, and the like. Representative alkali or alkaline earth metal salts include sodium, lithium, potassium, calcium, magnesium, and the like, as well as nontoxic ammonium, quaternary ammonium, and amine cations, including, but not limited to ammonium, tetramethylammonium, tetraethylammonium, methylamine, dimethylamine, trimethylamine, triethylamine, ethylamine, and the like. The pharmaceutically acceptable salts

of the present disclosure include the conventional non-toxic salts of the parent compound formed, for example, from non-toxic inorganic or organic acids. The pharmaceutically acceptable salts of the present disclosure can be synthesized from the parent compound which contains a basic or acidic moiety by conventional chemical methods. Generally, such salts can be prepared by reacting the free acid or base forms of these compounds with a stoichiometric amount of the appropriate base or acid in water or in an organic solvent, or in a mixture of the two; generally, nonaqueous media like ether, ethyl acetate, ethanol, isopropanol, or acetonitrile are preferred. Lists of suitable salts are found in *Remington's Pharmaceutical Sciences,* 17th ed., Mack Publishing Company, Easton, Pa., 1985, p. 1418, *Pharmaceutical Salts: Properties, Selection, and Use,* P.H. Stahl and C.G. Wermuth (eds.), Wiley-VCH, 2008, and Berge et al, *Journal of Pharmaceutical Science,* 66, 1-19 (1977), each of which is incorporated herein by reference in its entirety.

[0001474] *Pharmaceutically acceptable solvate:* The term "pharmaceutically acceptable solvate," as used herein, means a compound of the invention wherein molecules of a suitable solvent are incorporated in the crystal lattice. A suitable solvent is physiologically tolerable at the dosage administered. For example, solvates may be prepared by crystallization, recrystallization, or precipitation from a solution that includes organic solvents, water, or a mixture thereof. Examples of suitable solvents are ethanol, water (for example, mono-, di-, and tri-hydrates), *N*-methylpyrrolidinone (NMP), dimethyl sulfoxide (DMSO), *N,N'*-dimethylformamide (DMF), *N,N'*-dimethylacetamide (DMAC), 1,3-dimethyl-2-imidazolidinone (DMEU), 1,3-dimethyl-3,4,5,6-tetrahydro-2-(1H)-pyrimidinone (DMPU), acetonitrile (ACN), propylene glycol, ethyl acetate, benzyl alcohol, 2-pyrrolidone, benzyl benzoate, and the like. When water is the solvent, the solvate is referred to as a "hydrate."

[0001475] *Pharmacokinetic:* As used herein, "pharmacokinetic" refers to any one or more properties of a molecule or compound as it relates to the determination of the fate of substances administered to a living organism. Pharmacokinetics is divided into several areas including the extent and rate of absorption, distribution, metabolism and excretion. This is commonly referred to as ADME where: (A) Absorption is the process of a substance entering the blood circulation; (D) Distribution is the dispersion or dissemination of substances throughout the fluids and tissues of the body; (M) Metabolism (or Biotransformation) is the irreversible transformation of parent compounds into daughter metabolites; and (E) Excretion (or Elimination) refers to the elimination of the substances from the body. In rare cases, some drugs irreversibly accumulate in body tissue.

[0001476] *Physicochemical:* As used herein, "physicochemical" means of or relating to a physical and/or chemical property.

[0001477] *Polypeptide per unit drug (PUD):* As used herein, a PUD or product per unit drug, is defined as a subdivided portion of total daily dose, usually 1 mg, pg, kg, etc., of a product (such as a polypeptide) as measured in body fluid or tissue, usually defined in concentration such as pmol/mL, mmol/mL, etc divided by the measure in the body fluid.

[0001478] *Preventing:* As used herein, the term "preventing" refers to partially or completely delaying onset of an infection, disease, disorder and/or condition; partially or completely delaying onset of one or more symptoms, features, or clinical manifestations of a particular infection, disease, disorder, and/or condition; partially or completely delaying onset of one or more symptoms, features, or manifestations of a particular infection, disease, disorder, and/or condition; partially or completely delaying progression from an infection, a particular disease, disorder and/or condition; and/or decreasing the risk of developing pathology associated with the infection, the disease, disorder, and/or condition.

[0001479] *Prodrug:* The present disclosure also includes prodrugs of the compounds described herein. As used herein, "prodrugs" refer to any substance, molecule or entity which is in a form predicate for that substance, molecule or entity to act as a therapeutic upon chemical or physical alteration. Prodrugs may by covalently bonded or sequestered in some way and which release or are converted into the active drug moiety prior to, upon or after administered to a mammalian subject. Prodrugs can be prepared by modifying functional groups present in the compounds in such a way that the modifications are cleaved, either in routine manipulation or *in vivo,* to the parent compounds. Prodrugs include compounds wherein hydroxyl, amino, sulfhydryl, or carboxyl groups are bonded to any group that, when administered to a mammalian subject, cleaves to form a free hydroxyl, amino, sulfhydryl, or carboxyl group respectively. Preparation and use of prodrugs is discussed in T. Higuchi and V. Stella, "Pro-drugs as Novel Delivery Systems," Vol. 14 of the A.C.S. Symposium Series, and in *Bioreversible Carriers in Drug Design,* ed. Edward B. Roche, American Pharmaceutical Association and Pergamon Press, 1987, both of which are hereby incorporated by reference in their entirety.

[0001480] *Proliferate:* As used herein, the term "proliferate" means to grow, expand or increase or cause to grow, expand or increase rapidly. "Proliferative" means having the ability to proliferate. "Anti-proliferative" means having properties counter to or inapposite to proliferative properties.

[0001481] *Progenitor cell:* As used herein, the term "progenitor cell" refers to cells that have greater developmental potential relative to a cell which it can give rise to by differentiation.

[0001482] *Prophylactic:* As used herein, "prophylactic" refers to a therapeutic or course of action used to prevent the spread of disease.

[0001483] *Prophylaxis:* As used herein, a "prophylaxis" refers to a measure taken to maintain health and prevent the spread of disease. An "immune phrophylaxis" refers to a measure to produce active or passive immunity to prevent the spread of disease.

[0001484] *Protein cleavage site:* As used herein, "protein cleavage site" refers to a site where controlled cleavage of the amino acid chain can be accomplished by chemical, enzymatic or photochemical means.

[0001485] *Protein cleavage signal:* As used herein "protein cleavage signal" refers to at least one amino acid that flags or marks a polypeptide for cleavage.

[0001486] *Protein of interest:* As used herein, the terms "proteins of interest" or "desired proteins" include those provided herein and fragments, mutants, variants, and alterations thereof.

[0001487] *Proximal:* As used herein, the term "proximal" means situated nearer to the center or to a point or region of interest.

[0001488] *Pseudouridine:* As used herein, pseudouridine refers to the C-glycoside isomer of the nucleoside uridine. A "pseudouridine analog" is any modification, variant, isoform or derivative of pseudouridine. For example, pseudouridine analogs include but are not limited to 1-carboxymethyl-pseudouridine, 1-propynyl-pseudouridine, 1-taurinomethyl-pseudouridine, 1-taurinomethyl-4-thio-pseudouridine, 1-methylpseudouridine **($m^1\psi$),** 1-methyl-4-thio-pseudouridine ($m^1s^4\psi$), 4-thio-1-methyl-pseudouridine, 3-methyl-pseudouridine ($m^3\psi$), 2-thio-l-methyl-pseudouridine, 1-methyl- 1-deaza-pseudouridine, 2-thio- 1-methyl- 1-deaza-pseudouridine, dihydropseudouridine, 2-thio-dihydropseudouridine, 2-methoxyuridine, 2-methoxy-4-thio-uridine, 4-methoxy-pseudouridine, 4-methoxy-2-thio-pseudouridine, N1-methyl-pseudouridine, l-methyl-3-(3-amino-3-carboxypropyl)pseudouridine ($acp^3\ \psi$), and 2'-0-methyl-pseudouridine (ψr^n).

[0001489] *Purified:* As used herein, "purify," "purified," "purification" means to make substantially pure or clear from unwanted components, material defilement, admixture or imperfection.

[0001490] *Repeated transfection:* As used herein, the term "repeated transfection" refers to transfection of the same cell culture with a polynucleotide a plurality of times. The cell culture can be transfected at least twice, at least 3 times, at least 4 times, at least 5 times, at least 6 times, at least 7 times, at least 8 times, at least 9 times, at least 10 times, at least 11 times, at least 12 times, at least 13 times, at least 14 times, at least 15 times, at least 16 times, at least 17 times at

least 18 times, at least 19 times, at least 20 times, at least 25 times, at least 30 times, at least 35 times, at least 40 times, at least 45 times, at least 50 times or more.

[0001491] *Sample:* As used herein, the term "sample" or "biological sample" refers to a subset of its tissues, cells or component parts (e.g. body fluids, including but not limited to blood, mucus, lymphatic fluid, synovial fluid, cerebrospinal fluid, saliva, amniotic fluid, amniotic cord blood, urine, vaginal fluid and semen). A sample further may include a homogenate, lysate or extract prepared from a whole organism or a subset of its tissues, cells or component parts, or a fraction or portion thereof, including but not limited to, for example, plasma, serum, spinal fluid, lymph fluid, the external sections of the skin, respiratory, intestinal, and genitourinary tracts, tears, saliva, milk, blood cells, tumors, organs. A sample further refers to a medium, such as a nutrient broth or gel, which may contain cellular components, such as proteins or nucleic acid molecule.

[0001492] *Signal Sequences:* As used herein, the phrase "signal sequences" refers to a sequence which can direct the transport or localization of a protein.

[0001493] *Single unit dose:* As used herein, a "single unit dose" is a dose of any therapeutic administered in one dose/at one time/single route/single point of contact, i.e., single administration event.

[0001494] *Similarity:* As used herein, the term "similarity" refers to the overall relatedness between polymeric molecules, *e.g.* between polynucleotide molecules *(e.g.* DNA molecules and/or RNA molecules) and/or between polypeptide molecules. Calculation of percent similarity of polymeric molecules to one another can be performed in the same manner as a calculation of percent identity, except that calculation of percent similarity takes into account conservative substitutions as is understood in the art.

[0001495] *Split dose:* As used herein, a "split dose" is the division of single unit dose or total daily dose into two or more doses.

[0001496] *Stable:* As used herein "stable" refers to a compound that is sufficiently robust to survive isolation to a useful degree of purity from a reaction mixture, and preferably capable of formulation into an efficacious therapeutic agent.

[0001497] *Stabilized:* As used herein, the term "stabilize", "stabilized," "stabilized region" means to make or become stable.

[0001498] *Stereoisomer:* As used herein, the term "stereoisomer" refers to all possible different isomeric as well as conformational forms which a compound may possess (e.g., a compound of any formula described herein), in particular all possible stereochemically and conformationally isomeric forms, all diastereomers, enantiomers and/or conformers of the basic molecular

structure. Some compounds of the present invention may exist in different tautomeric forms, all of the latter being included within the scope of the present invention.

[0001499] *Subject:* As used herein, the term "subject" or "patient" refers to any organism to which a composition in accordance with the invention may be administered, *e.g.,* for experimental, diagnostic, prophylactic, and/or therapeutic purposes. Typical subjects include animals *(e.g.,* mammals such as mice, rats, rabbits, non-human primates, and humans) and/or plants.

[0001500] *Substantially:* As used herein, the term "substantially" refers to the qualitative condition of exhibiting total or near-total extent or degree of a characteristic or property of interest. One of ordinary skill in the biological arts will understand that biological and chemical phenomena rarely, if ever, go to completion and/or proceed to completeness or achieve or avoid an absolute result. The term "substantially" is therefore used herein to capture the potential lack of completeness inherent in many biological and chemical phenomena.

[0001501] *Substantially equal:* As used herein as it relates to time differences between doses, the term means plus/minus 2%.

[0001502] *Substantially simultaneously:* As used herein and as it relates to plurality of doses, the term means within 2 seconds.

[0001503] *Suffering from:* An individual who is "suffering from" a disease, disorder, and/or condition has been diagnosed with or displays one or more symptoms of a disease, disorder, and/or condition.

[0001504] *Susceptible to:* An individual who is "susceptible to" a disease, disorder, and/or condition has not been diagnosed with and/or may not exhibit symptoms of the disease, disorder, and/or condition but harbors a propensity to develop a disease or its symptoms. In some embodiments, an individual who is susceptible to a disease, disorder, and/or condition (for example, cancer) may be characterized by one or more of the following: (1) a genetic mutation associated with development of the disease, disorder, and/or condition; (2) a genetic polymorphism associated with development of the disease, disorder, and/or condition; (3) increased and/or decreased expression and/or activity of a protein and/or nucleic acid associated with the disease, disorder, and/or condition; (4) habits and/or lifestyles associated with development of the disease, disorder, and/or condition; (5) a family history of the disease, disorder, and/or condition; and (6) exposure to and/or infection with a microbe associated with development of the disease, disorder, and/or condition. In some embodiments, an individual who is susceptible to a disease, disorder, and/or condition will develop the disease, disorder,

and/or condition. In some embodiments, an individual who is susceptible to a disease, disorder, and/or condition will not develop the disease, disorder, and/or condition.

[0001505] *Sustained release:* As used herein, the term "sustained release" refers to a pharmaceutical composition or compound release profile that conforms to a release rate over a specific period of time.

[0001506] *Synthetic:* The term "synthetic" means produced, prepared, and/or manufactured by the hand of man. Synthesis of polynucleotides or polypeptides or other molecules of the present invention may be chemical or enzymatic.

[0001507] *Targeted Cells:* As used herein, "targeted cells" refers to any one or more cells of interest. The cells may be found *in vitro*, *in vivo*, *in situ* or in the tissue or organ of an organism. The organism may be an animal, preferably a mammal, more preferably a human and most preferably a patient.

[0001508] *Therapeutic Agent:* The term "therapeutic agent" refers to any agent that, when administered to a subject, has a therapeutic, diagnostic, and/or prophylactic effect and/or elicits a desired biological and/or pharmacological effect.

[0001509] *Therapeutically effective amount:* As used herein, the term "therapeutically effective amount" means an amount of an agent to be delivered *(e.g.*, nucleic acid, drug, therapeutic agent, diagnostic agent, prophylactic agent, *etc.)* that is sufficient, when administered to a subject suffering from or susceptible to an infection, disease, disorder, and/or condition, to treat, improve symptoms of, diagnose, prevent, and/or delay the onset of the infection, disease, disorder, and/or condition.

[0001510] *Therapeutically effective outcome:* As used herein, the term "therapeutically effective outcome" means an outcome that is sufficient in a subject suffering from or susceptible to an infection, disease, disorder, and/or condition, to treat, improve symptoms of, diagnose, prevent, and/or delay the onset of the infection, disease, disorder, and/or condition.

[0001511] *Total daily dose:* As used herein, a "total daily dose" is an amount given or prescribed in 24 hr period. It may be administered as a single unit dose.

[0001512] *Totipotency:* As used herein, "totipotency" refers to a cell with a developmental potential to make all of the cells found in the adult body as well as the extra-embryonic tissues, including the placenta.

[0001513] *Transcription factor:* As used herein, the term "transcription factor" refers to a DNA-binding protein that regulates transcription of DNA into RNA, for example, by activation or repression of transcription. Some transcription factors effect regulation of transcription alone, while others act in concert with other proteins. Some transcription factor can both activate and

repress transcription under certain conditions. In general, transcription factors bind a specific target sequence or sequences highly similar to a specific consensus sequence in a regulatory region of a target gene. Transcription factors may regulate transcription of a target gene alone or in a complex with other molecules.

[0001514] *Transcription:* As used herein, the term "transcription" refers to methods to introduce exogenous nucleic acids into a cell. Methods of transfection include, but are not limited to, chemical methods, physical treatments and cationic lipids or mixtures.

[0001515] *Transdijferentiation:* As used herein, "transdifferentiation" refers to the capacity of differentiated cells of one type to lose identifying characteristics and to change their phenotype to that of other fully differentiated cells.

[0001516] *Treating:* As used herein, the term "treating" refers to partially or completely alleviating, ameliorating, improving, relieving, delaying onset of, inhibiting progression of, reducing severity of, and/or reducing incidence of one or more symptoms or features of a particular infection, disease, disorder, and/or condition. For example, "treating" cancer may refer to inhibiting survival, growth, and/or spread of a tumor. Treatment may be administered to a subject who does not exhibit signs of a disease, disorder, and/or condition and/or to a subject who exhibits only early signs of a disease, disorder, and/or condition for the purpose of decreasing the risk of developing pathology associated with the disease, disorder, and/or condition.

[0001517] *Unmodified:* As used herein, "unmodified" refers to any substance, compound or molecule prior to being changed in any way. Unmodified may, but does not always, refer to the wild type or native form of a biomolecule. Molecules may undergo a series of modifications whereby each modified molecule may serve as the "unmodified" starting molecule for a subsequent modification.

[0001518] *Unipotent:* As used herein, "unipotent" when referring to a cell means to give rise to a single cell lineage.

[0001519] *Vaccine:* As used herein, the phrase "vaccine" refers to a biological preparation that improves immunity to a particular disease.

[0001520] *Viralprotein:* As used herein, the pharse "viral protein" means any protein originating from a virus.

Equivalents and Scope

[0001521] Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments in accordance with the

invention described herein. The scope of the present invention is not intended to be limited to the above Description, but rather is as set forth in the appended claims.

[0001522] In the claims, articles such as "a," "an," and "the" may mean one or more than one unless indicated to the contrary or otherwise evident from the context. Claims or descriptions that include "or" between one or more members of a group are considered satisfied if one, more than one, or all of the group members are present in, employed in, or otherwise relevant to a given product or process unless indicated to the contrary or otherwise evident from the context. The invention includes embodiments in which exactly one member of the group is present in, employed in, or otherwise relevant to a given product or process. The invention includes embodiments in which more than one, or all of the group members are present in, employed in, or otherwise relevant to a given product or process.

[0001523] It is also noted that the term "comprising" is intended to be open and permits but does not require the inclusion of additional elements or steps. When the term "comprising" is used herein, the term "consisting of" is thus also encompassed and disclosed.

[0001524] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Methods and materials are described herein for use in the present disclosure; other, suitable methods and materials known in the art can also be used.

[0001525] Where ranges are given, endpoints are included. Furthermore, it is to be understood that unless otherwise indicated or otherwise evident from the context and understanding of one of ordinary skill in the art, values that are expressed as ranges can assume any specific value or subrange within the stated ranges in different embodiments of the invention, to the tenth of the unit of the lower limit of the range, unless the context clearly dictates otherwise.

[0001526] In addition, it is to be understood that any particular embodiment of the present invention that falls within the prior art may be explicitly excluded from any one or more of the claims. Since such embodiments are deemed to be known to one of ordinary skill in the art, they may be excluded even if the exclusion is not set forth explicitly herein. Any particular embodiment of the compositions of the invention *(e.g.,* any nucleic acid or protein encoded thereby; any method of production; any method of use; *etc.)* can be excluded from any one or more claims, for any reason, whether or not related to the existence of prior art.

[0001527] All cited sources, for example, references, publications, databases, database entries, and art cited herein, are incorporated into this application by reference, even if not expressly stated in the citation. In case of conflicting statements of a cited source and the instant application, the statement in the instant application shall control.

[0001528] Section and table headings are not intended to be limiting.

EXAMPLES

[0001529] Methods of cyclization and/or concatemerization are described in Example 13 of International Publication No. WO2015034928, the contents of which is herein incorporated by reference in its entirety.

[0001530] Methods of chimeric synthesis of RNA are described in Example 14 of US Provisional Application No. 62/025,985, filed July 17, 2014, entitled Terminal Modifications of Polynucleotides, the contents of which are herein incorporated by reference in its entirety.

Example 1. Manufacture of Polynucleotides

[0001531] According to the present invention, the manufacture of polynucleotides and or parts or regions thereof may be accomplished utilizing the methods taught in International Publication No. WO2014152027, filed March 14, 2014 (Attorney Docket number M500), the contents of which is incorporated herein by reference in its entirety.

[0001532] Purification methods may include those taught in International Publication No. WO2014152030, filed March 14, 2014 (Attorney Docket number M501); International Publication No. WO201415203 1, filed March 14, 2014 (Attorney Docket number M502); each of which is incorporated herein by reference in its entirety.

[0001533] Detection and characterization methods of the polynucleotides may be performed as taught in International Publication No. WO2014144039, filed March 14, 2014 (Attorney Docket number M505), each of which is incorporated herein by reference in its entirety.

[0001534] Characterization of the polynucleotides of the invention may be accomplished using a procedure selected from the group consisting of polynucleotide mapping, reverse transcriptase sequencing, charge distribution analysis, and detection of RNA impurities, wherein characterizing comprises determining the RNA transcript sequence, determining the purity of the RNA transcript, or determining the charge heterogeneity of the RNA transcript. Such methods are taught in, for example, International Publication No. WO201414471 1, filed March 14, 2014 (Attorney Docket number M506) and International Publication No. WO2014144767, filed March 14, 2014 (Attorney Docket number M507) the contents of each of which is incorporated herein by reference in its entirety.

Example 2. Chimeric polynucleotide synthesis; triphosphate route

Introduction

[0001535] According to the present invention, two regions or parts of a chimeric polynucleotide may be joined or ligated using triphosphate chemistry.

[0001536] According to this method, a first region or part of 100 nucleotides or less is chemically synthesized with a 5' monophosphate and terminal 3'desOH or blocked OH. If the region is longer than 80 nucleotides, it may be synthesized as two strands for ligation.

[0001537] If the first region or part is synthesized as a non-positionally modified region or part using in vitro transcription (IVT), conversion the 5'monophosphate with subsequent capping of the 3' terminus may follow.

[0001538] Monophosphate protecting groups may be selected from any of those known in the art.

[0001539] The second region or part of the chimeric polynucleotide may be synthesized using either chemical synthesis or IVT methods. IVT methods may include an RNA polymerase that can utilize a primer with a modified cap. Alternatively, a cap of up to 130 nucleotides may be chemically synthesized and coupled to the IVT region or part.

[0001540] It is noted that for ligation methods, ligation with DNA T4 ligase, followed by treatment with DNAse should readily avoid concatenation.

[0001541] The entire chimeric polynucleotide need not be manufactured with a phosphate-sugar backbone. If one of the regions or parts encodes a polypeptide, then it is preferable that such region or part comprise a phosphate-sugar backbone.

[0001542] Ligation is then performed using any known click chemistry, orthoclick chemistry, solulink, or other bioconjugate chemistries known to those in the art.

Synthetic route

[0001543] The chimeric polynucleotide is made using a series of starting segments. Such segments include:

[0001544] (a) Capped and protected 5' segment comprising a normal 3'OH (SEG. 1)

[0001545] (b) 5' triphosphate segment which may include the coding region of a polypeptide and comprising a normal 3'OH (SEG. 2)

[0001546] (c) 5' monophosphate segment for the 3' end of the chimeric polynucleotide (e.g., the tail) comprising cordycepin or no 3'OH (SEG. 3)

[0001547] After synthesis (chemical or IVT), segment 3 (SEG. 3) is treated with cordycepin and then with pyrophosphatase to create the 5'monophosphate.

[0001548] Segment 2 (SEG. 2) is then ligated to SEG. 3 using RNA ligase. The ligated polynucleotide is then purified and treated with pyrophosphatase to cleave the diphosphate. The treated SEG.2-SEG. 3 construct is then purified and SEG. 1 is ligated to the 5' terminus. A further purification step of the chimeric polynucleotide may be performed.

[0001549] Where the chimeric polynucleotide encodes a polypeptide, the ligated or joined segments may be represented as: 5'UTR (SEG. 1), open reading frame or ORF (SEG. 2) and 3'UTR+PolyA (SEG. 3).

[0001550] The yields of each step may be as much as 90-95%.

Example 3; PCR for cDNA Production

[0001551] PCR procedures for the preparation of cDNA are performed using 2x KAPA HIFI™ HotStart ReadyMix by Kapa Biosystems (Woburn, MA). This system includes 2x KAPA ReadyMixl2.5 µï; Forward Primer (10 uM) 0.75 µï; Reverse Primer (10 uM) 0.75 µï; Template cDNA -100 ng; and dH_2O diluted to 25.0 µï. The reaction conditions are at 95° C for 5 min. and 25 cycles of 98° C for 20 sec, then 58° C for 15 sec, then 72° C for 45 sec, then 72° C for 5 min. then 4° C to termination.

[0001552] The reverse primer of the instant invention incorporates a poly-T^o for a poly-A^o in the mRNA. Other reverse primers with longer or shorter poly(T) tracts can be used to adjust the length of the poly(A) tail in the polynucleotide mRNA.

[0001553] The reaction is cleaned up using Invitrogen's PURELINK™ PCR Micro Kit (Carlsbad, CA) per manufacturer's instructions (up to 5 µg). Larger reactions will require a cleanup using a product with a larger capacity. Following the cleanup, the cDNA is quantified using the NANODROP™ and analyzed by agarose gel electrophoresis to confirm the cDNA is the expected size. The cDNA is then submitted for sequencing analysis before proceeding to the *in vitro* transcription reaction.

Example 4. *In vitro* Transcription (TVT)

[0001554] The *in vitro* transcription reaction generates polynucletodies containing uniformly modified polynucleotides. Such uniformly modified polynucleotides may comprise a region or part of the polynucleotides of the invention. The input nucleotide triphosphate (NTP) mix is made in-house using natural and un-natural NTPs.

[0001555] A typical *in vitro* transcription reaction includes the following:

1 Template cDNA 1.0 µg

2 10x transcription buffer (400 mM Tris-HCl pH 8.0, 190 mM $MgCl_2$, 50 mM DTT, 10 mM Spermidine) 2.0 µï

3 CustomNTPs (25mM each) 7.2 µï

4 RNase Inhibitor 20 U

5 T7 RNA polymerase 3000 U

6 dH_20 Up to 20.0 µï. and

7 Incubation at 37° C for 3 hr-5 hrs.

[0001556] The crude IVT mix may be stored at 4° C overnight for cleanup the next day. 1 U of RNase- free DNase is then used to digest the original template. After 15 minutes of incubation at 37° C, the mRNA is purified using Ambion's MEGACLEAR™ Kit (Austin, TX) following the manufacturer's instructions. This kit can purify up to 500 µg of RNA. Following the cleanup, the RNA is quantified using the NanoDrop and analyzed by agarose gel electrophoresis to confirm the RNA is the proper size and that no degradation of the RNA has occurred.

Example 5. Enzymatic Capping

[0001557] Capping of a polynucleotide is performed as follows where the mixture includes: IVT RNA 60 µg-180 µg and dH_20 up to 72 µï. The mixture is incubated at 65° C for 5 minutes to denature RNA, and then is transferred immediately to ice.

[0001558] The protocol then involves the mixing of lOx Capping Buffer (0.5 M Tris-HCl (pH 8.0), 60 mM KC1, 12.5 mM $MgCl_2$) (10.0 µï); 20 mM GTP (5.0 µï); 20 mM S-Adenosyl Methionine (2.5 µï); RNase Inhibitor (100 U); 2'-0-Methyltransferase (400U); Vaccinia capping enzyme (Guanylyl transferase) (40 U); dH_20 (Up to 28 µï); and incubation at 37° C for 30 minutes for 60 µg RNA or up to 2 hours for 180 µg of RNA.

[0001559] The polynucleotide is then purified using Ambion's MEGACLEAR™ Kit (Austin, TX) following the manufacturer's instructions. Following the cleanup, the RNA is quantified using the NANODROP™ (ThermoFisher, Waltham, MA) and analyzed by agarose gel electrophoresis to confirm the RNA is the proper size and that no degradation of the RNA has occurred. The RNA product may also be sequenced by running a reverse-transcription-PCR to generate the cDNA for sequencing.

Example 6. PolyA Tailing Reaction

[0001560] Without a poly-T in the cDNA, a poly-A tailing reaction must be performed before cleaning the final product. This is done by mixing Capped IVT RNA (100 µï); RNase Inhibitor (20 U); lOx Tailing Buffer (0.5 M Tris-HCl (pH 8.0), 2.5 M NaCl, 100 mM $MgCl_2$)(12.0 µï); 20 mM ATP (6.0 µï); Poly-A Polymerase (20 U); dH_20 up to 123.5 µï and incubation at 37° C for 30 min. If the poly-A tail is already in the transcript, then the tailing reaction may be skipped and proceed directly to cleanup with Ambion's MEGACLEAR™ kit (Austin, TX) (up to 500 µg). Poly-A Polymerase is preferably a recombinant enzyme expressed in yeast.

[0001561] It should be understood that the processivity or integrity of the polyA tailing reaction may not always result in an exact size polyA tail. Hence polyA tails of approximately between 40-200 nucleotides, e.g, about 40, 50, 60, 70, 80, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 150-165, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164 or 165 are within the scope of the invention.

Example 7. Natural 5′ Caps and 5′ Cap Analogues

[0001562] 5'-capping of polynucleotides may be completed concomitantly during the *in vitro*-transcription reaction using the following chemical RNA cap analogs to generate the 5'-guanosine cap structure according to manufacturer protocols: 3'-0-Me-m7G(5')ppp(5') G [the ARCA cap];G(5')ppp(5')A; G(5')ppp(5')G; m7G(5')ppp(5')A; m7G(5')ppp(5')G (New England BioLabs, Ipswich, MA). 5'-capping of modified RNA may be completed post-transcriptionally using a Vaccinia Virus Capping Enzyme to generate the "Cap 0" structure: m7G(5')ppp(5')G (New England BioLabs, Ipswich, MA). Cap 1 structure may be generated using both Vaccinia Virus Capping Enzyme and a 2'-0 methyl-transferase to generate: m7G(5')ppp(5')G-2'-0-methyl. Cap 2 structure may be generated from the Cap 1 structure followed by the 2'-0-methylation of the 5'-antepenultimate nucleotide using a 2'-0 methyl-transferase. Cap 3 structure may be generated from the Cap 2 structure followed by the 2'-0-methylation of the 5'-preantepenultimate nucleotide using a 2'-0 methyl-transferase. Enzymes are preferably derived from a recombinant source.

[0001563] When transfected into mammalian cells, the modified mRNAs have a stability of between 12-18 hours or more than 18 hours, e.g., 24, 36, 48, 60, 72 or greater than 72 hours.

Example 8. Capping Assays

A. Protein Expression Assay

[0001564] Polynucleotides encoding a polypeptide, containing any of the caps taught herein can be transfected into cells at equal concentrations. 6, 12, 24 and 36 hours post-transfection the amount of protein secreted into the culture medium can be assayed by ELISA. Synthetic polynucleotides that secrete higher levels of protein into the medium would correspond to a synthetic polynucleotide with a higher translationally-competent Cap structure.

B. Purity Analysis Synthesis

[0001565] Polynucleotides encoding a polypeptide, containing any of the caps taught herein can be compared for purity using denaturing Agarose-Urea gel electrophoresis or HPLC analysis. Polynucleotides with a single, consolidated band by electrophoresis correspond to the higher purity product compared to polynucleotides with multiple bands or streaking bands. Synthetic polynucleotides with a single HPLC peak would also correspond to a higher purity product. The capping reaction with a higher efficiency would provide a more pure polynucleotide population.

C. Cytokine Analysis

[0001566] Polynucleotides encoding a polypeptide, containing any of the caps taught herein can be transfected into cells at multiple concentrations. 6, 12, 24 and 36 hours post-transfection the amount of pro-inflammatory cytokines such as TNF-alpha and IFN-beta secreted into the culture

medium can be assayed by ELISA. Polynucleotides resulting in the secretion of higher levels of pro-inflammatory cytokines into the medium would correspond to polynucleotides containing an immune-activating cap structure.

D. Capping Reaction Efficiency

[0001567] Polynucleotides encoding a polypeptide, containing any of the caps taught herein can be analyzed for capping reaction efficiency by LC-MS after nuclease treatment. Nuclease treatment of capped polynucleotides would yield a mixture of free nucleotides and the capped 5'-5-triphosphate cap structure detectable by LC-MS. The amount of capped product on the LC-MS spectra can be expressed as a percent of total polynucleotide from the reaction and would correspond to capping reaction efficiency. The cap structure with higher capping reaction efficiency would have a higher amount of capped product by LC-MS.

Example 9. Agarose Gel Electrophoresis of Modified RNA or RT PCR Products

[0001568] Individual polynucleotides (200-400 ng in a 20 µï volume) or reverse transcribed PCR products (200-400 ng) are loaded into a well on a non-denaturing 1.2% Agarose E-Gel (Invitrogen, Carlsbad, CA) and run for 12-15 minutes according to the manufacturer protocol.

Example 10. Nanodrop Modified RNA Quantification and UV Spectral Data

[0001569] Modified polynucleotides in TE buffer (1 µï) are used for Nanodrop UV absorbance readings to quantitate the yield of each polynucleotide from an chemical synthesis or *in vitro* transcription reaction.

Example 11. Formulation of Modified mRNA Using Lipidoids

[0001570] Polynucleotides are formulated for *in vitro* experiments by mixing the polynucleotides with the lipidoid at a set ratio prior to addition to cells. *In vivo* formulation may require the addition of extra ingredients to facilitate circulation throughout the body. To test the ability of these lipidoids to form particles suitable for *in vivo* work, a standard formulation process used for siRNA-lipidoid formulations may used as a starting point. After formation of the particle, polynucleotide is added and allowed to integrate with the complex. The encapsulation efficiency is determined using a standard dye exclusion assays.

Example 12. Method of Screening for Protein Expression

A. Electrospray Ionization

[0001571] A biological sample which may contain proteins encoded by a polynucleotide administered to the subject is prepared and analyzed according to the manufacturer protocol for electrospray ionization (ESI) using 1, 2, 3 or 4 mass analyzers. A biologic sample may also be analyzed using a tandem ESI mass spectrometry system.

Patterns of protein fragments, or whole proteins, are compared to known controls for a given protein and identity is determined by comparison.

B. Matrix-Assisted Laser Desorption/Ionization

[0001572] A biological sample which may contain proteins encoded by one or more polynucleotides administered to the subject is prepared and analyzed according to the manufacturer protocol for matrix-assisted laser desorption/ionization (MALDI).

[0001573] Patterns of protein fragments, or whole proteins, are compared to known controls for a given protein and identity is determined by comparison.

C. Liquid Chromatography-Mass spectrometry-Mass spectrometry

[0001574] A biological sample, which may contain proteins encoded by one or more polynucleotides, may be treated with a trypsin enzyme to digest the proteins contained within. The resulting peptides are analyzed by liquid chromatography-mass spectrometry-mass spectrometry (LC/MS/MS). The peptides are fragmented in the mass spectrometer to yield diagnostic patterns that can be matched to protein sequence databases via computer algorithms. The digested sample may be diluted to achieve 1 ng or less starting material for a given protein. Biological samples containing a simple buffer background (e.g. water or volatile salts) are amenable to direct in-solution digest; more complex backgrounds (e.g. detergent, non-volatile salts, glycerol) require an additional clean-up step to facilitate the sample analysis.

[0001575] Patterns of protein fragments, or whole proteins, are compared to known controls for a given protein and identity is determined by comparison.

Example 13. Effect of 5'UTR in modified nucleic acids

[0001576] BJ Fibroblast cells were seeded at a density of 20,000 cells per well in 100 ul cell culture medium (EMEM (Eagle's Minimum Essential Medium) +10% FBS (fetal bovine serum)). G-CSF mRNA having a synthetic 5'UTR as described in Table 6 (polyA tail of approximately 140 nucleotides not shown in sequence; 5'cap, Capl; fully modified with 5-methylcytosine and 1-methylpseudouridine) was transfected in triplicate at a concentration of 75 ng per well in 96 well plates. 24 hours, 48 hours and 72 hours after transfection, the supernatant was collected and expression of G-CSF was measured by ELISA, and the results are shown in Table 6. Moderate increases in protein expression were seen upon deletion of the KOZAK sequence, and deletion of up to 21 nucleotides from the 3' end of the 5'UTR were well tolerated (5'UTRs of 41, 38 and 26 nt in length, respectively.) Inclusion of Gtx sequences resulted in moderate increases in protein expression with more significant increases observed for Gtx sequences in combination with deletion of KOZAK sequences. By contrast, A→T substitution (point mutation(s)) at the 3' end of the 5'UTR resulted in decreased expression, correlating with

the degree of substitution. As such, sequence modification of the 5'UTR allows one to increase ("dial up") or decrease ("dial down") expression.

Table 6. G-CSF Expression

Construct	5'UTR SEQ ID NO	mRNA SEQ ID NO	G-CSF Expression (ng/ml)		
			24 hours	48 hours	72 hours
GCSF	42	43	194.1	337.2	176.2
GCSF-GTX	44	45	260.4	588.5	325.3
GCSF-GTX_9del5'	46	47	289.3	737.2	393.8
GCSF no Kozak	48	49	288.5	551.0	243.3
GCSF 3t5'	50	51	150.3	243.3	117.3
GCSF 9t5'	52	53	114.5	179.2	61.1
GCSF 9del5'	54	55	295.1	489.3	226.7
GCSF 21del5'	56	57	279.5	534.9	239.7
GCSF 6t5'	58	59	0.7	0.9	0.5

Example 14. Effect of 5'UTR in modified nucleic acids on Expression

A. G-CSF Expression

[0001577] Human hepatocytes, Mouse myoblasts and BJ Fibroblasts were seeded at a density of 40,000, 35,000 and 20,000 per well, respectively, in 100 ul cell culture medium (CP Culture Medium Cat#Z99029 replaced with HI Medium Cat#Z99009 plus a Torpedo Antibiotic Mix Cat# Z990000, DMEM+10%FBS or EMEM+ 10%FBS respectively). G-CSF mRNA having a synthetic 5'UTR as described in Table 7 (polyA tail of approximately 140 nucleotides not shown in sequence; 5'cap, Capl; fully modified with 5-methylcytosine and l-methylpseudouridine) was tested at a concentration of 75 ng per well in 96 well plates. 24 hours after transfection, the expression of G-CSF was measured by ELISA, and the results are shown in Table 7. Notably, deletion of a significant portion of the 3' end of the 5' UTR, resulting in a 5' UTR of only 13 nucleotides in length, had no effect on protein expression (no reduction.)

Table 7. G-CSF expression

Construct	5'UTR Sequence or SEQ ID NO	mRNA SEQ ID NO	Protein Expression (ng/mL)		
			Human hepatocytes	Mouse myoblasts	BJ fibroblast
GCSF control T80	42	43	440.0	358.0	271.0
GCSF 13nt5'	60	61	1116.4	528.2	318.4
GCSF GGG5'	GGG	62	99.1	51.8	58.9

Table 8 sets forth data from a second experiment showing no reduction of G-CSF expression (mRNA fully modified with 5-methylcytosine and l-methylpseudouridine) using a short, 13 nucleotide, 5' UTR.

Table 8. G-CSF expression

Construct	Expression (ng/ml)
EPO T80	494.0
EPO 13nt5’	1116.4
5’GGG	99.1

B. EPO Expression

[0001578] Human hepatocytes and BJ Fibroblasts were seeded at a density of 40,000 and 20,000 per well, respectively, in 100 ul cell culture medium (CP Culture Medium Cat#Z99029 replaced with HI Medium Cat#Z99009 plus a Torpedo Antibiotic Mix Cat#Z99000 or EMEM+10%FBS, respectively). EPO mRNA having a synthetic 5'UTR as described in Table 9 (polyA tail of approximately 140 nucleotides not shown in sequence for EPO GGG5' and EPO 13nt5' and 80 nucleotides not shown in sequence for EPO T80; 5'cap, Capl; fully modified with 5-methylcytosine and 1-methylpseudouridine) was tested at a concentration of 75 ng per well in 96 well plates. 24 hours after transfection, the expression of EPO was measured by ELISA, and the results are shown in Table 9. As was the case for GCSF expression, the 13 nt 5'UTR is sufficient to support significant protein expression.

Table 9. EPO expression

Construct	5’UTR Sequence or SEQ ID NO	mRNA SEQ ID NO	Protein Expression (ng/mL)	
			Human hepatocytes	BJ fibroblast
EPO T80	42	63	1486.2	376.1
EPO GGG5’	GGG	64	236.7	121.6
EPO 13nt5’	65	66	1519.5	520.0

Table 10 sets forth data from a second experiment showing no reduction of EPO expression (mRNA fully modified with 5-methylcytosine and 1-methylpseudouridine) using a short, 13 nucleotide, 5' UTR.

Table 10. EPO expression

Construct	Expression (ng/ml)
EPO T80	1486.2
EPO 13nt5’	1519.5
5’GGG	236.7

Example 15. Effect of a TEE in an Untranslated Region in Modified Nucleic Acids

[0001579] BJ Fibroblast cells and human haptocytes were seeded at a density of 20,000 or 40,000 per well, respectively, in 100 ul cell culture medium (EM+10%FBS and CP Culture Medium Cat#Z99029 replaced with HI Medium Cat#Z99009 plus a Torpedo Antibiotic Mix Cat#Z99000, respectively). G-CSF mRNA having a TEE sequence as described in Tables 11-12 (polyA tail of approximately 140 nucleotides not shown in sequence; 5'cap, Capl; fully modified with 5-methylcytosine and 1-methylpseudouridine) was transfected in triplicate at a concentration of 150 ng per well in 96 well plates. A control of G-CSF mRNA without a TEE sequence was also used (mRNA sequence shown in SEQ ID NO: 43; polyA tail of approximately 140 nucleotides not shown in sequence; 5'cap, Capl; fully modified with 5-methylcytosine and 1-methylpseudouridine). 24 hours, 48 hours and 72 hours after transfection, the supernatant was collected and expression of G-CSF was measured by ELISA, and the results are shown in Tables 11 and 12. The various TEEs did not produce significant increases in protein expression in this test system.

Table 11. G-CSF Expression in Human Hepatocytes

Construct	TEE SEQ ID NO	mRNA SEQ ID NO	G-CSF Expression (ng/ml)		
			24 hours	48 hours	72 hours
GCSF	N/A	43	70.7	46.7	15.3
TEE1	67	68	55.5	59.2	27.1
TEE2	69	70	45.0	26.3	4.4
TEE3	71	72	56.2	36.2	6.8
TEE4	73	74	69.2	52.4	13.8
TEE5	75	76	64.9	49.8	15.9
TEE6	77	78	32.5	37.1	14.8
TEE7	79	80	67.5	44.7	9.9
TEE8	81	82	88.9	66.1	21.0
TEE9	83	84	37.7	14.4	1.9
TEE10	85	86	101.0	67.3	15.3
TEE11	87	88	79.8	35.2	7.6
TEE12	89	90	59.0	25.8	5.0

Table 12. G-CSF Expression in BJ Fibroblasts

Construct	TEE SEQ ID NO	mRNA SEQ ID NO	G-CSF Expression (ng/ml)		
			24 hours	48 hours	72 hours
GCSF	N/A	43	94.9	139.6	85.3
TEE1	67	68	97.1	171.6	106.4
TEE2	69	70	64.8	46.2	8.0
TEE3	71	72	87.7	80.7	17.0
TEE4	73	74	107.2	131.8	36.9
TEE5	75	76	105.1	142.4	47.6
TEE6	77	78	39.8	77.0	41.0
TEE7	79	80	84.0	76.5	13.7
TEE8	81	82	117.5	164.8	62.2
TEE9	83	84	50.2	52.4	8.9
TEE 10	85	86	104.3	92.2	12.2
TEE 11	87	88	110.0	68.9	9.9
TEE 12	89	90	54.2	26.9	5.6

Example 16. Effect of a TEE in an Untranslated Region in an Uncapped Modified Nucleic Acid

[0001580] BJ Fibroblast cells were seeded at a density of 20,000 per well in 100 ul cell culture medium (EMEM+10% FBS). G-CSF mRNA having a TEE sequence as described in Table 13 (polyA tail of approximately 140 nucleotides not shown in sequence; 5'cap, Capl; fully modified with 5-methylcytosine and 1-methylpseudouridine) was transfected in triplicate at a concentration of 75 ng per well in 96 well plates. A control of G-CSF mRNA without a TEE sequence was also used (mRNA sequence shown in SEQ ID NO: 68; polyA tail of approximately 140 nucleotides not shown in sequence; 5'cap, fully modified with 5-methylcytosine and 1-methylpseudouridine). 24 hours after transfection, the supernatant was collected and expression of G-CSF was measured by ELISA, and the results are shown in Table 13. The various TEEs did not produce significant increases in protein expression in this test system.

Table 13. G-CSF Expression in Human Hepatocytes

Construct	TEE SEQ ID NO	mRNA SEQ ID NO	G-CSF Expression (ng/ml)
			24 hours
GCSF UC	N/A	43	0.96
TEE1	67	68	1.55
TEE2	69	70	0.35
TEE3	71	72	0.69
TEE4	73	74	0.44
TEE5	75	76	1.20
TEE6	77	78	1.38
TEE7	79	80	1.88
TEE8	81	82	1.65
TEE9	83	84	0.17
TEE10	85	86	0.66
TEE11	87	88	0.82
TEE12	89	90	1.16

Example 17. Effect of the Length of the 3'UTR in Modified Nucleic Acids on Expression

[0001581] Human hepatocytes and BJ Fibroblasts were seeded at a density of 40,000 and 20,000 cells per well, respectively, in 100 ul cell culture medium (CP Culture Medium Cat#Z99029 replaced with HI Medium Cat#Z99009 plus a Torpedo Antibiotic Mix Cat#Z990000 or EMEM+10%FBS, respectively). Luciferase mRNA having a 3'UTR or lack thereof as described in Table 14 (polyA tail of approximately 80 nucleotides not shown in sequence; 5'cap, Capl; fully modified with 5-methylcytosine and 1-methylpseudouridine) was tested at a concentration of 75 ng per well in 96 well plates. 24 hours after transfection, the expression of Luciferase was

measured by Luminescence (Luciferase Assay), and the results are shown in Table 14. The data show that there is no reduction of protein function with shortening or absence of the 3' UTR.

Table 14. Luciferase expression

Construct	3'UTR SEQ ID NO	mRNA SEQ ID NO	Protein Expression (RLU)	
			Human hepatocytes	BJ fibroblast
Normal	91	92	115953.7	133006.7
Short	93	94	146586	148484.3
No	N/A	95	109658.3	114675.3

Example 18. Effect of 3'UTR in modified nucleic acids on Protein Expression

A. G-CSF

[0001582] Human hepatocytes, HeLa cells and BJ Fibroblasts were seeded at a density of 40,000, 17,000 or 20,000 cells per well, respectively, in 100 ul cell culture medium (CP Culture Medium Cat #Z99029 replaced with HI Medium Cat # Z99009 plus a Torpedo Antibiotic Mix Cat # Z990000, DMEM+10%FBS and EMEM+10% FBS respectively). G-CSF mRNA having a 3'UTR or lack thereof as described in Tables 15-17 (polyA tail of approximately 80 nucleotides not shown in sequence; 5'cap, Capl; fully modified with 5-methylcytosine and 1-methylpseudouridine) was tested at a concentration of 75 ng per well in 96 well plates. 24 hours, 48 hours and 72 hours after transfection, the expression of G-CSF was measured by ELISA, and the results are shown in Tables 15-17. The data show that there is no reduction of protein expression with shortening or absence of the 3' UTR.

Table 15. G-CSF expression in HeLa Cells

Construct	3'UTR SEQ ID NO	mRNA SEQ ID NO	Protein Expression (ng/mL)		
			24 hours	48 hours	72 hours
Full length	96	43	640.8	606.1	277.6
Short	97	98	1195.6	1054.7	437.1
No	N/A	99	1287.7	852.0	291.0

Table 16. G-CSF expression in BJ Fibroblast Cells

Construct	3'UTR SEQ ID NO	mRNA SEQ ID NO	Protein Expression (ng/mL)		
			24 hours	48 hours	72 hours
Full length	96	43	83.1	52.2	14.3
Short	97	98	136.3	80.5	17.4
No	N/A	99	158.4	70.9	12.4

Table 17. G-CSF expression in Human Hepatocytes

Construct	3'UTR SEQ ID	mRNA SEQ ID NO	Protein Expression (ng/mL)		
			24 hours	48 hours	72 hours

	NO				
Full length	**96**	**43**	**78.4**	**77.0**	**33.6**
Short	**97**	**988**	**135.3**	**129. 1**	**40.9**
No	**N/A**	**99**	**191 .8**	**117.4**	**20.7**

B. EPO

[0001583] Human hepatocytes and BJ Fibroblasts were seeded at a density of 40,000 or 20,000 cells per well, respectively, in 100 ul cell culture medium (CP Culture Medium Cat #Z99029 replaced with HI Medium Cat # Z99009 plus a Torpedo Antibiotic Mix cat # Z990000 and EME+10%FBS, respectively). EPO mRNA having a 3'UTR or lack thereof as described in Table 18 (polyA tail of approximately 80 nucleotides not shown in sequence; 5'cap, Capl; fully modified with 5-methylcytosine and 1-methylpseudouridine) was tested at a concentration of 75 ng per well in 96 well plates. 24 hours after transfection, the expression of EPO was measured by ELISA, and the results are shown in Table 18. The data show that there is no reduction of protein expression with shortening or absence of the 3' UTR.

Table 18. EPO expression

Construct	3'UTR SEQ ID NO	mRNA SEQ ID NO	Protein Expression (ng/mL)	
			BJ Fibroblasts	Human Hepatocytes
Epo Hs3'UTR	100	63	280.3	423.8
EPO Short Hs 3'UTR	101	102	254.0	524.2
EPO No Hs 3'UTR	N/A	103	283.5	595.3

C. mCherry

[0001584] HeLa cells were seeded at a density of 17,000 per well in 100 ul cell culture medium (DMEM+10%FBS). mCherry mRNA having a 3'UTR or lack thereof as described in Table 19 (polyA tail of approximately 80 nucleotides not shown in sequence; 5'cap, Capl; fully modified with 5-methylcytosine and 1-methylpseudouridine) was tested at a concentration of 75 ng per well in 96 well plates. 24 hours after transfection, the expression of mCherry was measured by fluorescence reading on the plate reader (excitation at 585, emission 620, gain 100), and the results are shown in Table 19. The data show that there is no reduction of protein expression with shortening or absence of the 3' UTR.

Table 19. mCherry expression

Construct	3'UTR SEQ ID NO	mRNA SEQ ID NO	Percentage of Control
MC Hs 3'UTR	104	105	100
MC Short 3'UTR	106	107	92.4
MC no 3'UTR	N/A	108	157.8

D. GFP

[0001585] HeLa cells were seeded at a density of 17,000 per well in 100 ul cell culture medium (DMEM+10%FBS). GFP mRNA having a 3'UTR or lack thereof as described in Table 20 (polyA tail of approximately 80 nucleotides not shown in sequence; 5'cap, Capl; fully modified with 5-methylcytosine and 1-methylpseudouridine) was tested at a concentration of 75 ng per well in 96 well plates. 24 hours after transfection, the expression of GFP was measured by fluorescence reading on the plate reader (excitation at 485, emission 515, gain 100), and the results are shown in Table 20. The data show that there is no reduction of protein expression with shortening or absence of the 3' UTR.

Table 20. GFP expression

Construct	3'UTR SEQ ID NO	mRNA SEQ ID NO	Percentage of Control
GFP Hs 3'UTR	109	110	100
GFP Short 3'UTR	111	112	101.2
GFP no 3'UTR	N/A	113	91.1

Example 19. Effect of 3'UTR in modified nucleic acids on Protein Expression *In Vivo*

[0001586] Mice (n=4) (8 week old female Balb/c) were administered intravenously 2 ug in 100 ul GCSF mRNA as described in Table 21 (polyA tail of approximately 80 nucleotides not shown in sequence; 5'cap, Capl; fully modified with 5-methylcytosine and 1-methylpseudouridine) formulated in lipoplex or a control of vehicle only.

[0001587] 6 hours after administration blood was collected from the mice and G-CSF protein expression was determined by ELISA. The results are shown in Table 21. The data show that there is no reduction of protein function *in vivo* with shortening or absence of the 3' UTR.

Table 21. G-CSF expression

Construct	Expression (pg/ml)
3' UTR control	248.9
Short 3' UTR	472.5
No 3' UTR	197.8
Vehicle	12.2

The data set forth in Examples 18-21 demonstrate that the 3' UTR is not essential for mRNA drug design (as shown for multiple ORFs and cell types and *in vivo.* This further provides for an engineering opportunity to include other regulatory sequences, *e.g.*, miR binding sites, within UTR oligo sequences to more finely tune protein expression.

Example 20. Effect of miR recognition sequences in the 3'UTR in modified nucleic acids on Protein Expression

A. In Vitro

[0001588] Human hepatocytes and HeLa cells were seeded at a density of 40,000 per well in cell culture medium (CP Culture Medium Cat #Z99029 replaced with HI Medium Cat # Z99009 plus a Torpedo Antibiotic Mix Cat #Z990000). Luciferase mRNA with or without a miR-122 site as described in Tables 22-23 (polyA tail of approximately 140 nucleotides not shown in sequence; 5'cap, Capl; fully modified with 5-methylcytosine and 1-methylpseudouridine) was tested at a concentration of 75 ng per well in 96 well plates. A control of untreated and vehicle only was evaluated in HeLa cells. 24 hours after transfection, the expression of Luciferase was measured using Luciferase cell assay, and the results are shown in Tables 22-23. The data demonstrate that Luciferase activity can be suppressed by engineering miR-122 sites into the 3'UTR of an mRNA (87% reduction in Hs Hepatocytes) but that neither miR-122 seed nor seedless site sequences were sufficient to suppress protein activity.

Table 22. Luciferase expression in HeLa Cells

Construct	miR-122 Site Sequence	3'UTR SEQ ID NO	mRNA SEQ ID NO	Expression Relative Light Units (RLU)
Luc-miR-122	miR-122 site	114	115	59304.5
Luc-miR-122seed-mmM3	miR-122 site seed sequence	116	117	49761.5
Luc-miR-122seedless	miR-122 site without the seed sequence	118	119	62466
Luc-mM3	No miR-122 site	120	121	72994.5
L2000	N/A	N/A	N/A	108
Untreated	N/A	N/A	N/A	51

[0001589] Similar experiments were conducted using mRNA constructs (polyA tail of approximately 140 nucleotides not shown in sequence; 5'cap, Capl; fully modified with 5-methylcytosine and 1-methylpseudouridine) encoding GCSF or Factor IX in Hs Hepatocytes, with quantification of protein expression at 24, 48 and 72 hours. Use of a single miR regulatory (binding) site, when engineered into the 3' UTR, was sufficient to down regulate expression. Down regulation was seen for the miR binding site but not for seed or seedless sequences. GCSF @ 24 hr - decreased 71%; 48hr - decreased 97%; 72 hr - decreased 95%. Factor IX @ 24hr - decreased 71%, 48hr - decreased 96%, 72hr - decreased 100% (relative expression.) By contrast, a single miR site had only minimal impact in similar experiments conducted with Heb3B cells (which express very low or negligible levels of Heb3B as compared to hepatocytes.)

Table 23. Luciferase expression in Human Hepatocyte Cells

Construct	miR-122 Site	3'UTR SEQ	mRNA SEQ	Expression

	Sequence	ID NO	ID NO	Relative Light Units (RLU)
miR-122	miR-122 site	114	115	1355.2
miR-122 Seed	miR-122 site seed sequence	116	117	16452.3
miR-122 Seedless	miR-122 site without the seed sequence	118	119	22333.1
miR-less	No miR-122 site	120	121	10534

[0001590] A reduction of expression of about 87% was seen in the constructs containing a miR-122 sequence in the 3'UTR as compared to the control sequence whereas an increase in expression was seen in the miR-122 seed and miR-122 seedless constructs.

[0001591] In a repeat experiment, miR-mediated suppression was observed across all chemistries tested. In particular, mRNA fully modified with 5-methylcytosine and pseudouridine showed 69% down regulation; mRNA fully modified with 5-methylcytosine and 1-methylpseudouridine showed 80% down regulation; mRNA fully modified with 1-methylpseudouridine showed 86.7% down regulation and human hepatocytes treated with unmodified mRNA showed 63% down regulation.

B. In vivo

[0001592] Mice (n=6) (8 week old female Balb/c) were administered 100 ul intravenously of 0.05 mg/kg Luciferase mRNA as described in Table 24 (polyA tail of approximately 140 nucleotides not shown in sequence; 5'cap, Capl; fully modified with 5-methylcytosine and 1-methylpseudouridine) formulated in a lipid nanoparticle comprising the lipid DLin-KC2-DMA described in Tables 24 and 25 or a control of vehicle only.

Table 24. Lipid Nanoparticle Formulation

Ionizable Amino Lipid (Lipid/Mol%)	Non-Cationic Lipid (Lipid/Mol%)	Cholesterol (Mol%)	PEG Lipid (Lipid/Mol %)
Dlin-KC2-DMA 50	10	Cholesterol 38.5	PEG-DOMG 1.5

[0001593] 2 hours after administration the mice were anesthetized, injected with the luciferase substrate D-luciferin and bioluminescence imaging (BLI) from living animals was evaluated in an IVIS imager 20 minutes later. 6 hours after administration the liver and spleen were collected from the mice and Luciferase expression was determined by bioluminescence imaging (BLI) in an IVIS imager. The results in Flux (p/s) are shown in Table 25. The constructs with a miR-122 in the 3'UTR had a 92.9% knockdown compared to the constructs without a mir-122 sequence in liver and a 7.9% knockdown in the spleen.

Table 25. Luciferase expression in Liver and Spleen

Construct	miR-122 Site Sequence	3'UTR SEQ ID NO	mRNA SEQ ID NO	Expression (p/s)	
				Liver	Spleen
miR-122	miR-122 site	114	115	2.00E+05	2.77E+04
miR-122 Seed	miR-122 site seed seqeunce	116	117	2.49E+06	3.21E+04
miR-122 Seedless	miR-122 site without the seed sequence	118	119	3.03E+06	3.60E+04
miR-less	No miR-122 site	120	121	2.83E+06	3.01E+04
Vehicle	N/A	N/A	N/A	0	0

Example 21. Effect of mir-142 3p recognition sequences in the 3'UTR in modified nucleic acids on Expression

A. *Human EPO*

[0001594] RAW 264.7 cells were seeded at a density of 40,000 per well in lOOul cell culture medium DMEM (Dulbecco's Modified Eagle Medium). Human EPO mRNA with or without a miR-142 3p site as described in Table 26 (polyA tail of approximately 140 nucleotides not shown in sequence; 5'cap, Capl; fully modified with 5-methylcytosine and 1-methylpseudouridine) was tested at a concentration of 75 ng per well in 96 well plates. 24 hours after transfection, the expression of human EPO was measured by ELISA, and the results are shown in Table 30.

Table 26. Human EPO expression

Construct	miR-122 Site Sequence	3'UTR SEQ ID NO	mRNA SEQ ID NO	Expression (ng/ml)
miR-142 3p	miR-142 3p site	122	123	127.8
miR-142 3p seedless	miR-122 site without the seed sequence	124	125	446
miR-less	No miR-122 site	100	63	127.8

B. *Mouse EPO*

[0001595] RAW 264.7 cells were seeded at a density of 40,000 per well in 100 ul cell culture medium DMEM. Mouse EPO mRNA with or without a miR-142 3p site as described (polyA tail of approximately 140 nucleotides not shown in sequence; 5'cap, Capl; fully modified with 5-methylcytosine and 1-methylpseudouridine) was tested at a concentration of 75 ng per well in 96 well plates. 24 hours after transfection, the expression of mouse EPO was measured by ELISA, and the results are shown in Table 27. The data show 70% and 56% decreased in human and mouse EPO protein production, respectively, for mRNAs having miR-142 3P sites engineered into the 3' UTR.

Table 27. Mouse EPO expression

Construct	miR-122 Site Sequence	3'UTR SEQ ID NO	mRNA SEQ ID NO	Expression (ng/ml)
miR-142 3p	miR-142 3p site	126	127	1898
miR-142 3p seedless	miR-122 site without the seed sequence	128	129	5903
miR-less	No miR-122 site	130	131	4367

Example 22. Suppression of EPO Expression by engineering mir-142 3p sites in the 3'UTR

A. *Human EPO*

[0001596] Mice (n=4) (8 week old female Balb/c) were administered 100 ul intravenously 2 ug human EPO mRNA as described in Table 28 (polyA tail of approximately 140 nucleotides not shown in sequence; 5'cap, Capl; fully modified with 5-methylcytosine and 1-methylpseudouridine) formulated in lipoplex (2ug).

[0001597] 6 hours after administration the mice were anesthetized and serum was collected, the expression of human EPO was measured by EPO ELISA, and the results are shown in Table 28. The data show greater than 95%, *i.e.*, 95.8%, suppression for human EPO protein expression when mRNA having miR-142 3p sites engineered into the 3'UTR is administered *in vivo.*

Table 28. Human EPO expression

Construct	miR-122 Site Sequence	3'UTR SEQ ID NO	mRNA SEQ ID NO	Expression (pg/ml)
miR-142 3p	miR-142 3p site	122	123	156.5
miR-less	No miR-122 site sequence	100	63	3719.7

B. *Mouse EPO*

[0001598] Mice (n=4) (8 week old female Balb/c) were administered lOOul intravenously 2 ug mouse EPO mRNA as described in Table 29 (polyA tail of approximately 140 nucleotides not shown in sequence; 5'cap, Capl; fully modified with 5-methylcytosine and 1-methylpseudouridine) formulated in lipoplex. A control of vehicle only was also evaluated.

[0001599] 6 hours after administration serum was collected, the expression of mouse EPO was measured using EPO ELISA, and the results are shown in Table 29. The data show greater than 90%, *i.e.*, 91.7%, suppression for mouse EPO protein expression when mRNA having miR-142 3p sites engineered into the 3'UTR is administered *in vivo.*

Table 29. EPO expression

Construct	miR-122 Site Sequence	3'UTR SEQ ID NO	mRNA SEQ ID NO	Expression (pg/ml)
miR-142 3p	miR-142 3p site	126	127	1205.4
miR-less	**No** miR-122 site sequence	130	13 1	14501 .9

Vehicle	N/A	N/A	N/A	19.8

Example 23. Effect of miR site position in the 3'UTR of modified nucleic acids on Expression

A. *G-CSF*

[0001600] Human hepatocytes were seeded at a density of 40,000 per well in 100 ul cell culture medium (CP Culture Medium Cat #Z99029 replaced with HI Medium Cat # Z99009 plus a Torpedo Antibiotic Mix Cat # Z99000). G-CSF mRNA with or without a miR-122 sequence as described in Table 30 (polyA tail of approximately 140 nucleotides not shown in sequence; 5'cap, Capl; fully modified with 5-methylcytosine and 1-methylpseudouridine) was tested at a concentration of 75 ng per well in 96 well plates. 24 hours after transfection, the expression of G-CSF was measured by GCSF ELISA, and the results are shown in Table 30. miR sites were engineered into various positions within the 3' UTR, *i.e.*, near the 5' end, in the center, and at the 3' end of the 3' UTR. Varying the position of the miR site within the 3' UTR had only modest effect, with positioning at the 5' end of the 3' UTR showing the greatest suppression (70%) and positioning at the center or 3' end having more modest suppression (81% and 82% suppression, respectively.)

Table 30. G-CSF expression

Construct	miR-122 site Sequence	3'UTR SEQ ID NO	mRNA SEQ ID NO	Expression (ng/ml)
5' miR-122	miR-122 site at 5' end or 3' UTR	132	133	84.8
Center miR-122	miR-122 site at center or 3' UTR	134	135	87.9
3' miR-122	miR-122 site at 3' end of 3' UTR	136	137	157.8
miR-less	No miR-122 sequence	96	43	481.2

B. *Factor IX*

[0001601] Human hepatocytes were seeded at a density of 40,000 per well in 100 ul cell culture medium (CP Culture Medium Cat #Z99029 replaced with HI Medium Cat # Z99009 plus a Torpedo Antibiotic Mix Cat # Z99000). Factor IX mRNA with 1 miR-122 sequence, with 4 miR-122 sequences or without a miR-122 sequence as described in Table 31 (polyA tail of approximately 140 nucleotides not shown in sequence; 5'cap, Capl; fully modified with 5-methylcytosine and 1-methylpseudouridine) was tested at a concentration of 75 ng per well in 96 well plates. 24 hours after transfection, the expression of Factor IX was measured by Factor IX ELISA, and the results are shown in Table 31. Concaternation of miR sites within the 3' UTR resulted in modest increase in suppression (87% suppression.)

Table 31. Factor IX expression

Construct	miR-122 Sequence	3'UTR SEQ ID NO	mRNA SEQ ID NO	Expression (ng/ml)
miR-122	1x miR-122 sequence	138	139	16.5
miR-122 4x	4x miR-122 sequence	140	141	9.4
miR-less	No miR-122 sequence	142	143	74.7

Example 24. Effect of 3'UTR in modified nucleic acids on Protein Expression - The "Hetero-miR" Approach

A. *In vitro* testing

[0001602] It was postulated that engineering multiple independent miR sites into the 3' UTR might allow for down-regulation of protein expression in different cell types or organs, depending in the miR expression profile in said cells or organs. To test the hypothesis, human hepatocytes, HeLa cells and RAW264.7were seeded at a density of 40,000, 17,000 or 40,000 per well, respectively, in 100 ul cell culture medium (either DMEM or CP Culture Medium Cat #Z99029 replaced with HI Medium Cat # Z99009 plus a Torpedo Antibiotic Mix Cat # Z990000). Luciferase mRNAs were constructed having having a miR-122 site, a miR-142 3p site, or a combination of said sites engineered into the 3'UTR sequence as described in Tables 32-34 (polyA tail of approximately 140 nucleotides not shown in sequence; 5'cap, Capl; fully modified with 5-methylcytosine and 1-methylpseudouridine). Each mRNA was tested at a concentration of 75 ng per well in 96 well plates. The mRNAs were tested on cell types chosen based on their known miR expression profiles - human hepatocytes = miR-142 3p(-)/miR122(+); HeLa cells = miR-142 3p(-)/miR122(-) and RAW264.7 cells = miR-142 3p(+)/miR122(-). 24 hours after transfection, the expression of Luciferase was measured by Luciferase Cell Assay (Promega) and the results are shown in Tables 32-34.

Table 32. Luciferase expression in HeLa Cells

Construct	miR Sites	3'UTR SEQ ID NO	mRNA SEQ ID NO	Expression Relative Light Units (RLU)
miR-142 5Hs3UTR	miR-142 3p (SEQ ID **NO:** 144)	146	147	11232
miR-142 SL Hs3UTR	miR-142 3p (SEQ ID **NO:** 144)	148	149	14973.3
miR-142/122 Hs3UTR	miR-142 3p (SEQ ID **NO:** 144) miR-122 (SEQ ID **NO:** 145)	150	151	7205.7
miR-122mM3UTR	miR-122 (SEQ ID **NO:** 145)	152	153	12287
miR-less Hs 3UTR	**N/A**	154	155	8316

miR-less Mm-3UTR	**N/A**	156	157	10993.7

Table 33. Luciferase expression in RAW264.7 Cells

Construct	miR Sequences	3'UTR SEQ ID NO	mRNA SEQ ID NO	Expression Relative Light Units (RLU)
miR-142 5Hs3UTR	miR-142 3p (SEQ ID **NO:** 144)	146	147	435.7
miR-142 SL Hs3UTR	miR-142 3p (SEQ ID **NO:** 144)	148	149	1903
miR-142/122 Hs3UTR	miR-142 3p (SEQ ID **NO:** 144) miR-122 (SEQ ID **NO:** 145)	150	151	467.7
miR-122mM3UTR	miR-122 (SEQ ID **NO:** 145)	152	153	2037.7
miR-less Hs 3UTR	**N/A**	154	155	1121.3
miR-less Mm-3UTR	**N/A**	156	157	2220.3

Table 34. Luciferase expression in Human Hepatocyte Cells

Construct	miR Sequences	3'UTR SEQ ID NO	mRNA SEQ ID NO	Expression Relative Light Units (RLU)
miR-142 5Hs3UTR	miR-142 3p (SEQ ID **NO:** 144)	146	147	39123
miR-142 SL Hs3UTR	miR-142 3p (SEQ ID **NO:** 144)	148	149	44325.7
miR-142/122 Hs3UTR	miR-142 3p (SEQ ID **NO:** 144) miR-122 (SEQ ID **NO:** 145)	150	151	3472.7
miR-122mM3UTR	miR-122 (SEQ ID **NO:** 145)	152	153	3552.3
miR-less Hs 3UTR	**N/A**	154	155	36824
miR-less Mm-3UTR	**N/A**	156	157	39910

[0001603] Hs = human and Mm = mouse. The data demonstrate that cell-type specific miRs targeted the corresponding miR site when engineered into 3' UTRs with miR-142 3p suppressing protein expression in RAW264.7 cells (miR-142 3p site alone (61% suppression) or in combination with miR-122 site (58% suppression)) and with miR-122 suppressing protein expression in human hepatocytes (miR-122 site alone (90% suppression) or in combination with miR-142 3p site (90% suppression).) As predicted, miR-122 sites failed to suppress protein in cells devoid of miR-122 expression and miR-142 3p sites failed to suppress protein in cells devoid of miR-142 3p expression. These data show that it is possible to suppress protein expression in multiple unrelated cell types using the "Hetero-miR" approach.

B. *LNP Formulation*

[0001604] Further *in* vitro testing was performed using the above-described mRNA constructs and cells, but with the mRNAs being formulated in LNPs for delivery purposes. Human hepatocytes, HeLa cells and RAW264.7were seeded at a density of 40,000, 17,000 or 40,000 per well, respectively, in 100 ul cell culture medium (either DMEM or CP Culture Medium Cat #Z99029 replaced with HI Medium Cat # Z99009 plus a Torpedo Antibiotic Mix Cat # Z990000). Luciferase mRNA having a 3'UTR with at least one miR sequence or without a miR sequence as described in Tables 37-39 (polyA tail of approximately 140 nucleotides not shown in sequence; 5'cap, Capl; fully modified with 5-methylcytosine and 1-methylpseudouridine) formulated in a lipid nanoparticle (LNP) as described in Tables 35-36 was tested at a concentration of 75 ng per well in 96 well plates. 24 hours after transfection, the expression of Luciferase was measured by Luciferase Cell Assay (Promega), and the results are shown in Tables 37-39. As was the case in the previous Example, miR sites facilitated suppression of protein when mRNAs containing same (in 3' UTR sequences) were expressed in appropriate cell types, *i.e.,* cells expressing the corresponding miR.

Table 35. LNP Formulation

Ionizable Amino Lipid (Lipid/Mol%)	Non-Cationic Lipid (Lipid/Mol%)	Cholesterol (Mol%)	PEG Lipid (Lipid/Mol %)
DLin-KC2-DMA 50%	DSPC 10%	Cholesterol 38.5%	PEG-DOMG 1.5%

Table 36. LNP formulations characterization

Particle Size	Zeta Potential	Encapsulation Efficiency
76.1 nm	0.2 mV	98%

Table 37. Luciferase expression in HeLa Cells

Construct	miR Sequences	Expression Relative Light Units (RLU)
miR-122	miR-122 (SEQ ID NO: 145)	5577
miR-142 3p	miR-142 3p (SEQ ID NO: 144)	8385
miR-122/142 3p	miR-142 3p (SEQ ID NO: 144) miR-122 (SEQ ID NO: 145)	6579
miR-less	N/A	7241

Table 38. Luciferase expression in RAW264.7 Cells

Construct	miR Sequences	Expression Relative Light Units (RLU)

miR-122	miR-122 (SEQ ID NO: 145)	5883
miR-142 3p	miR-142 3p (SEQ ID NO: 144)	627
miR-122/142 3p	miR-142 3p (SEQ ID NO: 144) miR-122 (SEQ ID NO: 145)	909
miR-less	N/A	6292

Table 39. Luciferase expression in Human Hepatocyte Cells

Construct	miR Sequences	Expression Relative Light Units (RLU)
miR-122	miR-122 (SEQ ID NO: 145)	6406
miR-142 3p	miR-142 3p (SEQ ID NO: 144)	39003
miR-122/142 3p	miR-142 3p (SEQ ID NO: 144) miR-122 (SEQ ID NO: 145)	8515
miR-less	N/A	35217

[0001605] In a similar experiment testing mRNAs fully modified with 1-methylpsuedouridine, luciferase activity was again suppressed in diverse cell types in a cell type-specific manner, depending on endogenous miR expression. In particular, luciferase mRNA having a miR-142-3p binding site engineered into the 3'UTR was expressed in healthy hepatocytes but suppressed in antigen-presenting cells (APCs), i.e., Kupffer cells (80% suppression) and in liver cancer cells, i.e., RAW264.7 cells (89% suppression). Luciferase mRNA having a miR-122 binding site engineered into the 3'UTR was expressed in Kupffer cells and RAW264.7 cells, but suppressed in primary human hepatocytes (84% suppression). Luciferase mRNA having both a miR-142-3p and a miR-122 binding site engineered into the 3'UTR was suppressed in all three cell types, primary human hepatocytes (95% suppression), Kupffer cells (85% suppression) and RAW264.7 cells (87% suppression). Percent suppression was compared to miR-less mRNA control. All constructs expressed at about control levels in HeLa cells, as would be expected based on the miR expression profiled of these cells.

C. *In Vivo* Expression

[0001606] The "Hetero-miR" approach was next tested *in vivo.* Mice (n=6) (8 week old female Balb/c) were administered 100 ul, intravenously, 0.05 mg/kg Luciferase mRNA comprising at least one miR sequence as described above (polyA tail of approximately 140 nucleotides not shown in sequence; 5'cap, Capl; fully modified with 5-methylcytosine and 1-methylpseudouridine) formulated in a lipid nanoparticle (LNP) as described above.

[0001607] 6 hours after administration the mice were anesthetized, injected with the luciferase substrate D-luciferin and the liver and spleen were removed 20 minutes laters. Bioluminescence imaging (BLI) was evaluated in an IVIS imager and the results are shown in Table 40.

Table 40. Luciferase expression

Construct	miR Sequences	Liver	Spleen
		Flux (p/s)	Flux (p/s)
miR-122	miR-122 (SEQ ID NO: 145)	2.94E+06	4.46E+05
miR-142 3p	miR-142 3p (SEQ ID NO: 144)	2.86E+07	1.86E+05
miR-122/142 3p	miR-142 3p (SEQ ID NO: 144) miR-122 (SEQ ID NO: 145)	2.53E+06	8.02E+04
miR-less	N/A	2.33E+07	5.27E+05

[0001608] The data demonstrate that engineering appropriate miR sites into the 3' UTR of mRNAs allows for suppression in tissues where corresponding miRs are expressed. The data demonstrate simultaneous suppression of luciferase from chemically-modified mRNAs in multiple organs (Liver - 98.3% knockdown for the miR-122/142 3p construct and 90.5% knockdown for miR-122 construct; Spleen - 86.8 %knockdown for the miR-122/142 3p construct and 83.4% knockdown for miR-142 construct). In particular, the data demonstrate that a human alpha-globulin 3' UTR (Hs 3' UTR) with both miR-122 and miR-142-3p sites inhibits Luc (G2) activity in both liver and spleen simultaneously. Thus, multiple miR sites can be used to control off target effects simultaneously in multiple organs.

[0001609] In a similar experiment, the expression of EPO was suppressed 95.8% for the mir-122 construct and 98.5% for the miR-122/142 3p construct as compared to the miR-less construct expression. These data show that multiple miR sites can be used to control off-target effects for different constructs.

Example 25. Regulation of *In Vivo* Protein Expression using Muscle Specific miRs Female Balb/c Mice (n=3) were administered intramuscluarly 0.125 mg/kg Luciferase mRNA in 50 ul comprising at least one miR sequence as described in Tables 41-43(polyA tail of approximately 140 nucleotides not shown in sequence; 5'cap, Capl; fully modified with 5-methylcytosine and 1-methylpseudouridine) formulated in saline. A control of vehicle only was also evaluated.

[0001610] 24 hours after administration the mice were anesthetized, injected with the luciferase substrate D-luciferin and bioluminescence imaging (BLI) from living animals was evaluated in an IVIS imager 20 minutes later. The results are shown in Tables 41-43.

Table 41. miR-133

Construct	miR-133 Sequence	3'UTR SEQ ID NO	mRNA SEQ ID NO	Flux (P/s)
miR-133	miR-133	160	161	1.80E+06

	SEQ ID NO: 158			
miR-133 SL	miR-133 without the seed SEQ ID NO: 159	162	163	7.13E+06
Luc	none	--	--	5.76E+06

Table 42. miR-206

Construct	miR-133 Sequence	3'UTR SEQ ID NO	mRNA SEQ ID NO	Flux (p/s)
miR-206	miR-206 SEQ ID NO: 164	166	167	4.23E+05
miR-206 SL	miR-206 without the seed SEQ ID NO: 165	168	169	3.07E+06
Luc	none	--	--	5.76E+06

Table 43. miR-1

Construct	miR-133 Sequence	3'UTR SEQ ID NO	mRNA SEQ ID NO	Flux (p/s)
miR-1	miR-1 SEQ ID NO: 170	171	172	5.53E+05
miR-1 SL	miR-1 without the seed SEQ ID NO: 173	174	175	3.80E+06
Luc	none			5.76E+06

[0001611] The data demonstrate that protein expression upon IM injection can be suppressed in muscle by muscle-specific miRs *(e.g.,* miR-133, miR-206 and miR-1) when corresponding miR sites are engineered into mRNAs within the 3' UTR. Suppression was greater than 65%, and even greater than 90%. miR-133 suppressed protein expression by 68.7%, miR-206 by 92.7% and miR-1 by 90.4%.

Example 26. Effect of a miR recognition sequence in the Poly A tail of modified nucleic acids

[0001612] HeLa cells and RAW264.7were seeded at a density of 17,000 or 40,000 per well, respectively, in 100 ul cell culture medium (DMEM). Luciferase mRNA having at least one miR-142-3p sequence or without a miR-142-3p sequence as described in Table 44 (polyA tail of approximately 140 nucleotides not shown in sequence; 5'cap, Capl; fully modified with 5-methylcytosine and 1-methylpseudouridine) was tested at a concentration of 75 ng per well in 96 well plates. 24 hours after transfection, the expression of Luciferase was measured by Luciferase Cell Assay, and the results are shown in Table 44.

Table 44. Luciferase expression

Construct	miR-142-3p Sequences	3'UTR SEQ ID NO	mRNA SEQ ID NO	HeLa Relative Light Units	RAW264.7 Relative Light Units

				(RLU)	(RLU)
5’	miR-142-3p sequence at the beginning of the polyA tailing region	176	177	157112	45970.7
5’SL	miR-142-3p without the seed sequence at the beginning of the polyA tailing region	178	179	141960.7	200451
Center	miR-142-3p sequence in the center of the polyA tailing region	180	181	175247.7	186278
Center SL	miR-142-3p without the seed sequence in the center of the polyA tailing region	182	183	151144.7	214181.7
3’	miR-142-3p sequence at the end of the polyA tailing region	184	185	255973	196199.7
3’SL	miR-142-3p without the seed sequence at the end of the polyA tailing region	186	187	130239.3	160653
3p	miR in 3’UTR	--	--	176857	65124
Luc	N/A	--	--	194006.7	247120.3

Example 27. Regulation of *In Vitro* Protein Expression using Endothelial Specific miRs

A. Luciferase

[0001613] HeLa, MSI and Huvec cells were seeded at a density of 17,000, 30,000 or 30,000 per well, respectively, in 100 ul cell culture medium (DMEM and Endothelial basal medium with supplements (Lonza Cat #4176)). MSI = pancreatic islet endothelial cell line; HUVEC = human umbilical vein endothelial cell line; miR-126 = endothelial-specific miR. Luciferase mRNA having a 3'UTR with engineered to include a miR-126 regulatory sequence (or seedless subsequence) as described in Tables 45-47 (polyA tail of approximately 140 nucleotides not shown in sequence; 5'cap, Capl; fully modified with 5-methylcytosine and 1-methylpseudouridine) was tested at a concentration of 75 ng per well in 96 well plates. 24 hours after transfection, the expression of Luciferase was measured by Luciferase Cell Assay, and the results are shown in Table 45.

Table 45. Expression in HeLa cells

Construct	Expression Relative Light Units		
	HeLa	MS1	HUVEC
miR-126	55053.5	8629.1	44087.5
miR-126 SL	41834.1	29202.5	162802.9
Luc	51275.5	41154.6	31790.5

[0001614] The data show that engineering miR-126 regulatory sites into mRNA (within the 3' UTR) provide the ability to regulate protein expression in cells expressing the corresponding

miR *(i.e.,* endothelial cells.) At least 80% down regulation was observed (80% for MSI cells, 86% for HUVEC cells.)

B. EPO and GCSF

[0001615] HeLa, MSI and Huvec cells were seeded at a density of 17,000 or 30,000 per well in 100 ul cell culture medium (DMEM and Endothelial basal medium with supplements (Lonza Cat# 4176)). EPO or GCSF mRNA having a 3'UTR engineered to contain a miR-126 regulatory sequence (or seedless subsequence) as described above (polyA tail of approximately 140 nucleotides not shown in sequence; 5'cap, Capl; fully modified with 5-methylcytosine and 1-methylpseudouridine) was tested at a concentration of 75 ng per well in 96 well plates. 24 hours after transfection, the expression of EPO was measured by EPO ELISA, and the results are shown in Tables 46-47.

Table 46. EPO

Construct	Expression ng/ml		
	HeLa	MS1	HUVEC
miR-126	901.8	132.6	486.3
miR-126 SL	686.8	457.2	794.3
Luc	686.3	489.0	902.7

Table 47. GCSF Expression

Construct	Expression ng/ml		
	HeLa	MS1	HUVEC
miR-126	132.6	486.3	486.3
miR-126 SL	457.2	794.3	794.3
Luc	489.0	902.7	902.7

[0001616] These data show that engineering miR-126 regualtory sites into EPO or GCSF mRNAs (within the 3' UTR) provide the ability to regulate protein expression in cells expressing the corresponding miR *(i.e.,* endothelial cells.) Greater than 75% down regulation was observed for EPO (78% for MSI cells, 77% for HUVEC cells.) Greater than 45% down regulation was observed for GCSF (73% for MSI cells, 46% for HUVEC cells.)

[0001617] Collectively these data evidence miR-126 as an endothelium-specific miRNA and show the utility of *in vitro* systems to test effectiveness in regulation of target protein expression.

Example 28. Effect of Chemical Modification on miR-regulated Protein Expression *EPO Expression*

[0001618] HeLa cells were seeded at a density of 17,000 per well in 100 ul cell culture medium (DMEM). Human hepatocyte cells were seeded at a density of 40,000 per well in 100 ul cell culture medium (CP Culture Medium Cat# Z99029 replaced with HI Medium Cat# Z99009 plus a Torpedo Antibiotic Mix Cat# Z990000) . EPO mRNA having a 3'UTR containing a miR-122

regulatory sequence (or subsequence lacking the seed sequence) as described in Table 48 (polyA tail of approximately 140 nucleotides not shown in sequence; 5'cap, Capl) was tested at a concentration of 75 ng per well in 96 well plates. Various chemical modifications were tested in the engineered mRNAs, as described in the Table (no modifications; fully modified with 5-methylcytosine (m^5C) and pseudouridine (Ψ); fully modified with 5-methylcytosine (m^5C) and 1-methylpseudouridine ($m^1\Psi$); and fully modified with 1-methylpseudouridine ($m^1\Psi$).) 24 hours after transfection, the expression of EPO was measured by ELISA, and the results are shown in Table 48.

Table 48. Expression in HeLa cells

Construct	Description	mRNA SEQ ID NO	Chemical Modifications	Expression ng/ml	
				HeLa	Hs Hepatocytes
miR-122	EPO with miR-122 (SEQ ID NO: 145)	188	No modifications	9.5	0.1
			Fully Modified with m^5C and Ψ	146.2	4.3
			Fully Modified with m^5C and ($m^1\Psi$)	1094.7	58.1
			Fully Modified with ($m^1\Psi$)	2543.6	29.9
miR-122 SL	EPO with miR-122 without the seed	190	No modifications	18.6	0.7
			Fully Modified with m^5C and Ψ	145.8	13.3
			Fully Modified with m^5C and ($m^1\Psi$)	1204	149.3
			Fully Modified with ($m^1\Psi$)	2362.02	341.0
EPO no miR	No miR-122 sequence	189	No modifications	18.9	1.7
			Fully Modified with m^5C and Ψ	111.8	12.8
			Fully Modified with m^5C and ($m^1\Psi$)	1182	255.3

[0001619] The constructs comprising the miR-122 regulatory sequence exhibited decreased EPO expression in the human hepatocytes (which express miR-122) but not in HeLa cells (which lack miR-122.) Down-regulation was observed for unmodified mRNA and for all chemical modifications tested. In particular, unmodified mRNA showed 94% down-regulation; mRNA fully modified with m^5C and Ψ showed 68% down-regulation; mRNA fully modified with m^5C and $m^1\Psi$ showed 77% down regulation; and mRNA fully modified with $m^1\Psi$ showed 95% down-regulation (when expressed in Hs Hepatocytes).

Luciferase Expression

[0001620] HeLa cells were seeded at a density of 17,000 per well in 100 ul cell culture medium (DMEM). Human hepatocyte cells were seeded at a density of 40,000 per well in 100 ul cell

culture medium (CP Culture Medium Cat #Z99029 replaced with HI Medium Cat# Z99009 plus a Torpedo Antibiotic Mix Cat# Z990000). Luciferase mRNA having a 3'UTR engineered to include a miR-122 regulatory sequence (or seedless miR-122 sequence) as described in Table 49 (polyA tail of approximately 140 nucleotides not shown in sequence; 5'cap, Capl) was tested at a concentration of 75 ng per well in 96 well plates. Various chemical modifications were tested in the engineered mRNAs, as described in the Table (no modifications; fully modified with 5-methylcytosine (m^5C) and pseudouridine (Ψ); fully modified with 5-methylcytosine (m^5C) and 1-methylpseudouridine ($m^1\Psi$); and fully modified with 1-methylpseudouridine ($m^1\Psi$).) 24 hours after transfection, the expression of Luciferase was measured by Luciferase Cell Assay, and the results are shown in Table 49.

Table 49. Luciferase Expression

Construct	Description	Chemical Modifications	Expression - Relative Light Units	
			HeLa	Hs Hepatocytes
miR-122	miR-122 (SEQ ID NO: 145)	No modifications	7017	62.5
		Fully Modified with m^5C and Ψ	151493	3534
		Fully Modified with m^5C and $m^1\Psi$	240353	90722.6
		Fully Modified with $m^1\Psi$	1340605	63445.3
miR-122 SL	miR-122 without the seed	No modifications	6143	77
		Fully Modified with m^5C and Ψ	123800	20805
		Fully Modified with m^5C and $m^1\Psi$	2722493	404142
		Fully Modified with $m^1\Psi$	1524516	1278594.3
miR-less	No miR-122 sequence	No modifications	3315.8	34.5
		Fully Modified with m^5C and Ψ	126355	39533.3
		Fully Modified with m^5C and $m^1\Psi$	175430	6579686
		Fully Modified with $m^1\Psi$	1102603	1290583

[0001621] The constructs comprising the miR-122 regulatory sequence exhibited decreased Luciferase expression in the human hepatocytes (which express miR-122) but not in HeLa cells (which lack miR-122.) Down-regulation was observed for all chemical modifications tested. In particular, mRNA fully modified with m^5C and Ψ showed 91% down-regulation; mRNA fully modified with m^5C and $m^1\Psi$ showed 86% down regulation; and mRNA fully modified with $m^1\Psi$ showed 95% down-regulation (when expressed in Hs Hepatocytes). Hs Hepatocytes exhibited cell death when treated with unmodified mRNA in this particular experiment. Collectively,

these data show that miR-122 knocks down miR-122 site-containing target expression for a variety of chemically-modified mRNAs tested.

Example 29. *In Vivo* EPO Study for Minimal Viable UTR

[0001622] Balb/C Mice (n=4) were administered intravenously 0.05 mg/kg of EPO mRNA as described in Table 51 (polyA tail of approximately 140 nucleotides not shown in sequence; 5'cap, Capl; fully modified with 1-methylpseudouridine) formulated in lipid nanoparticles comprising the lipid DLin-KC2-DMA (Table 50). A control of EPO mRNA as described above (polyA tail of approximately 140 nucleotides not shown in sequence; 5'cap, Capl; fully modified with 5-methylcytosine and 1-methylpseudouridine (5mC/lmpU), formulated in lipid nanoparticles comprising the lipid DLin-KC2-DMA. An exemplary formulation is described in Table 50.

Table 50. LNP Formulation

Ionizable Amino Lipid (Lipid/Mol%)	Non-Cationic Lipid (Lipid/Mol%)	Cholesterol (Mol%)	PEG Lipid (Lipid/Mol %)
DLin-KC2-DMA 50	DSPC 10	Cholesterol 38.5	PEG-DOMG 1.5

[0001623] 6 hours and 24 hours after administration serum was collected and protein expression was evaluated using ELISA and the results are shown in Table 51.

Table 51. Protein Production

Construct	mRNA SEQ ID NO	Protein Production (ng/ml)	
		6 hours	24 hours
5mC/1mpU control	191	38635	15114.5
1mpU control	192	99623.5	43048.5
EPO MVU	193	163249	47180.25
EPO 13 nt5'	194	128804	63577.67
EPO Sh 3'	195	69789	27195.25

[0001624] These data show that mRNA constructs having a 5' UTR of only 13 nucleotides and a 3' UTR of only 31 nucleotides are sufficient to drive significant protein expression.

OTHER EMBODIMENTS

[0001625] It is to be understood that the words which have been used are words of description rather than limitation, and that changes may be made within the purview of the appended claims without departing from the true scope and spirit of the invention in its broader aspects.

[0001626] While the present invention has been described at some length and with some particularity with respect to the several described embodiments, it is not intended that it should be limited to any such particulars or embodiments or any particular embodiment, but it is to be construed with references to the appended claims so as to provide the broadest possible interpretation of such claims in view of the prior art and, therefore, to effectively encompass the intended scope of the invention.

[0001627] All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, section headings, the materials, methods, and examples are illustrative only and not intended to be limiting.

Claims

1. A polynucleotide comprising

(a) a first region of linked nucleosides encoding a polypeptide of interest;

(b) a first terminal region located 5' relative to said first region comprising a 5' untranslated region;

(c) a second terminal region located 3' relative to said first region; and

(d) a tailing region,

wherein the 5' untranslated region is a synthetic 5' untranslated region having a length of 3 - 13 nucleotides.

2. The polynucleotide of claim 1, wherein the length of the synthetic 5' untranslated region is 10-12 nucleotides in length.

3. The polynucleotide of claim 1, wherein the second terminal region comprising a 3' untranslated region.

4. The polynucleotide of claim 3, wherein the 3' untranslated region has a length of 20-50 nucleotides in length.

5. The polynucleotide of claim 4, wherein the length of the 3' untranslated region is 30 nucleotides.

6. The polynucleotide of claim 1 where the tailing region comprises a polyA tail of approximately 80 nucleotides in length.

7. The polynucleotide of claim 6 where the tailing region comprises at least one miR sequence.

8. The polynucleotide of claim 7 where the at least one miR sequence is located at a position selected from the group consisting of the beginning of the polyA tail, the middle of the polyA tail and the end of the polyA tail.

9. The polynucleotide of claim 1 where the second terminal region comprises at least one miR sequence.

10. The polynucleotide of claim 9 wherein the second terminal region comprises a 3' untranslated region and said 3' untranslated region comprises the at least one miR sequence.

11. The polynucleotide of claim 10, wherein the at least one miR sequence is selected from the group consisting of miR-142-3p, miR-122, miR-133, miR-1, miR-206, miR-126,

miR-132, miR-125, miR-124, miR-21, miR-484, miR-17, miR-34a and fragments thereof.

12. The polynucleotide of claim 10, wherein the at least one miR sequence is specific for a tissue selected from the group consisting of muscle, endothelium, lung, ovarian, colorectal, prostate, liver and spleen.

13. The polynucleotide of claim 12, wherein the tissue is muscle and the at least one miR sequence is selected from the group consisting of miR-133, miR-1 and miR-206.

14. The polynucleotide of claim 13, wherein the at least one miR sequence is miR-206.

15. The polynucleotide of claim 12, wherein the tissue is endothelium and the at least one miR sequence is miR- 126.

16. The polynucleotide of claim 12, wherein the tissue is lung and the at least one miR sequence is miR-21.

17. The polynucleotide of claim 12, wherien the tissue is ovarian and the at least one miR sequence is miR-484.

18. The polynucleotide of claim 12, wherein the tissue is colorectal and the at least one miR sequence is miR-17.

19. The polynucleotide of claim 12, wherein the tissue is prostate and the at least one miR sequence is miR-34a.

20. The polynucleotide of claim 10, wherien the at least one miR sequence is specific for the central nervous system.

21. The polynucleotide of claim 10, wherein the at least one miR sequence is selected from the group consisting of miR-132, miR-125 and miR-124.

22. The polynucleotide of claim 1 wherein the 5' untranslated region comprises at least one miR sequence.

23. The polynucleotide of claim 22, wherein the at least one miR sequence is miR-lOa.

23. The polynucleotide of claim 1 further comprising at least one chemical modification.

24. A method of producing a polypeptide of interest in a cell or tissue comprising contacting said cell or tissue with the polynucleotide of any of claims 1-23.

25. The method of claim 24 wherein the contacting is a route of administration selected from the group consisting of intramuscular, intravenous, intradermal, and subcutaneous.

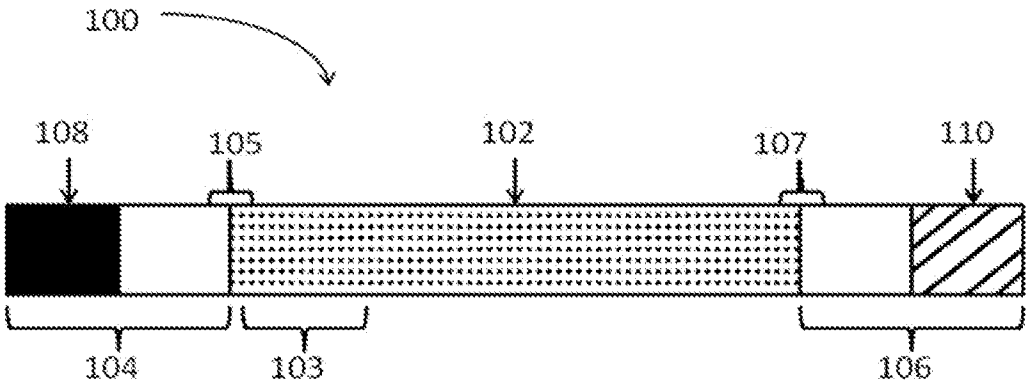
26. A method of reducing the expression a polypeptide of interest in a cell or tissue comprising contacting said cell or tissue with the polynucleotide of any of claims 13-23

27. The method of claim 26, wherein the contacting is a route of administration selected from the group consisting of intramuscular, intravenous, intradermal, and subcutaneous.

28. A pharmaceutical composition comprising the polynucleotide of any of claims 1-23 and a pharmaceutically acceptable excipient.

FIGURE 1

A.


100
108
105
102
107
110
104
103
106

B.

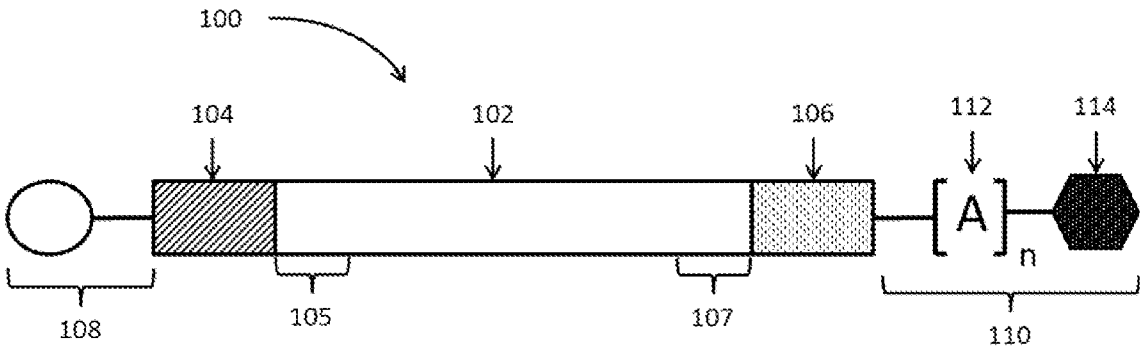

100
104
102
106
112
114
A
n
108
105
107
110

FIGURE 2

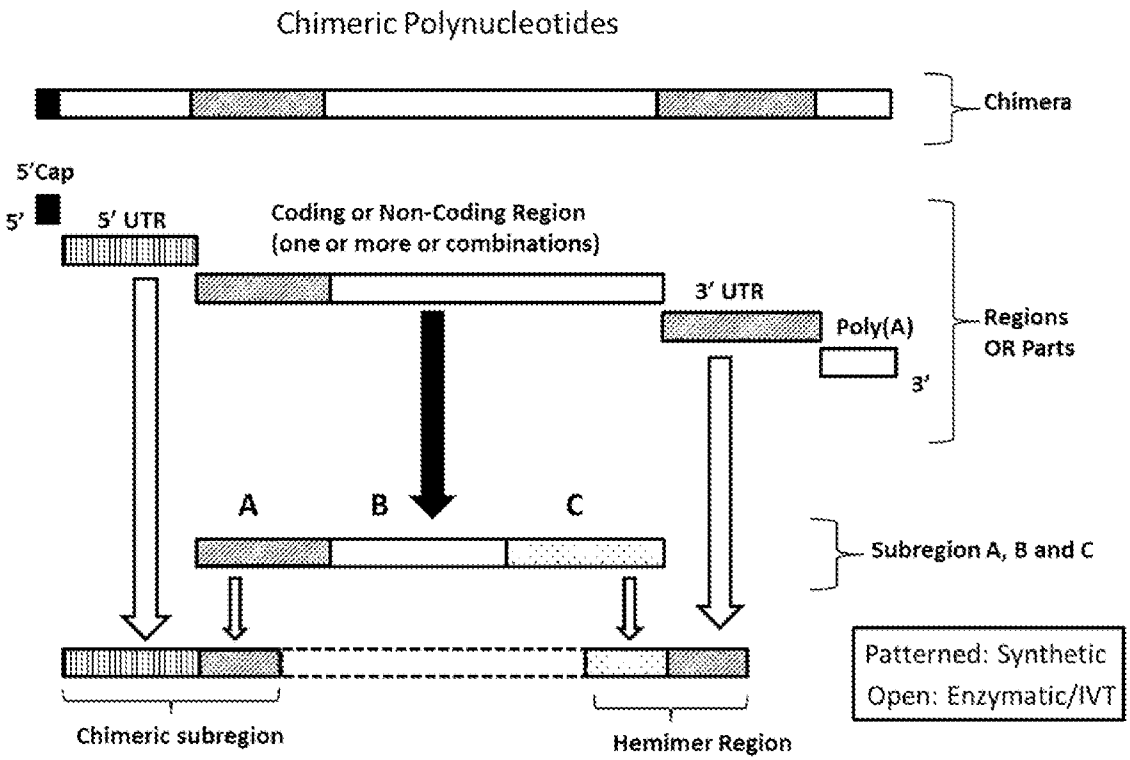

Chimeric Polynucleotides
Chimera
5'Cap
5'
5' UTR
Coding or Non-Coding Region
(one or more or combinations)
3' UTR
Poly(A)
3'
Regions
OR Parts
A
B
C
Subregion A, B and C
Patterned: Synthetic
Open: Enzymatic/IVT
Chimeric subregion
Hemimer Region

FIGURE 3

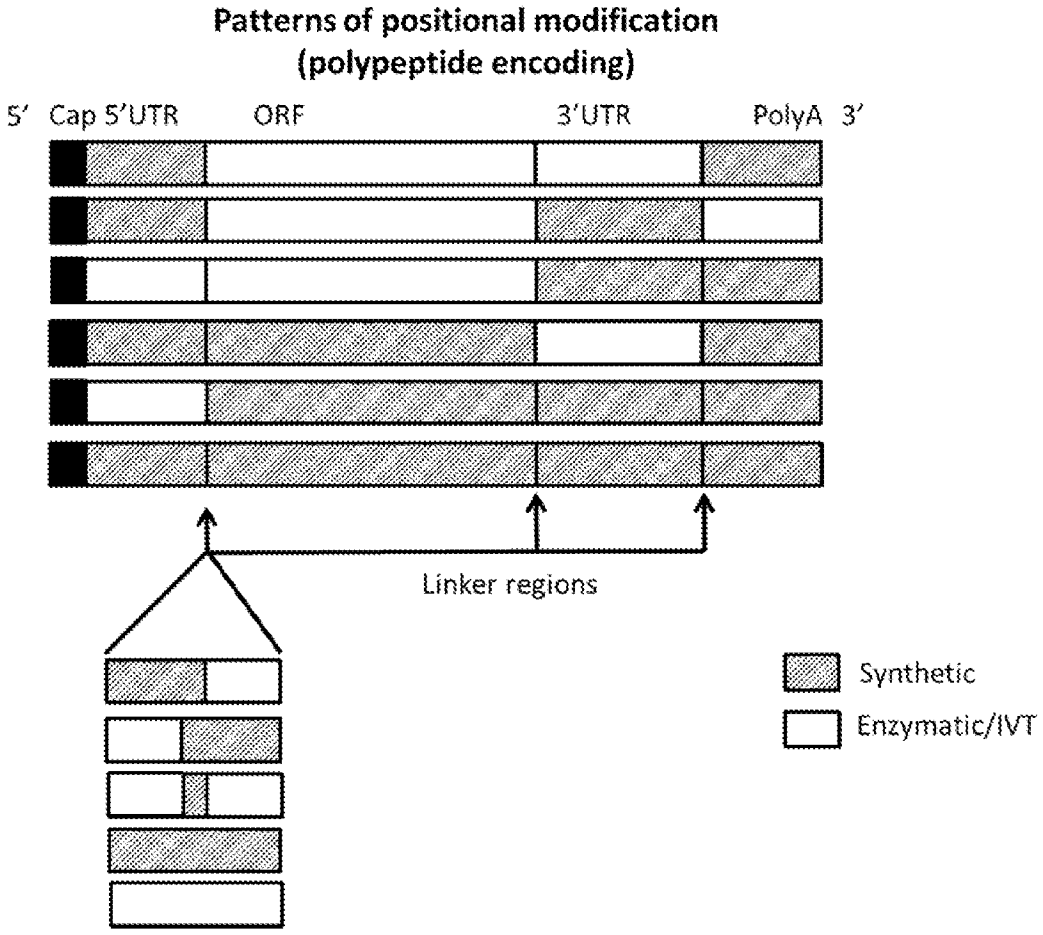

Patterns of positional modification
(polypeptide encoding)
5′ Cap 5′UTR
ORF
3′UTR
PolyA 3′
Linker regions
Synthetic
Enzymatic/IVT

FIGURE 4

Patterns of positional modification

A (L)	B (L)	C (L)

5′[NNNNN$_n$]$_x$ L1 [NNNNNNNNNNNN$_o$]$_y$ L2 [XXXXXXXXXXXX$_p$]$_z$ L3 3′

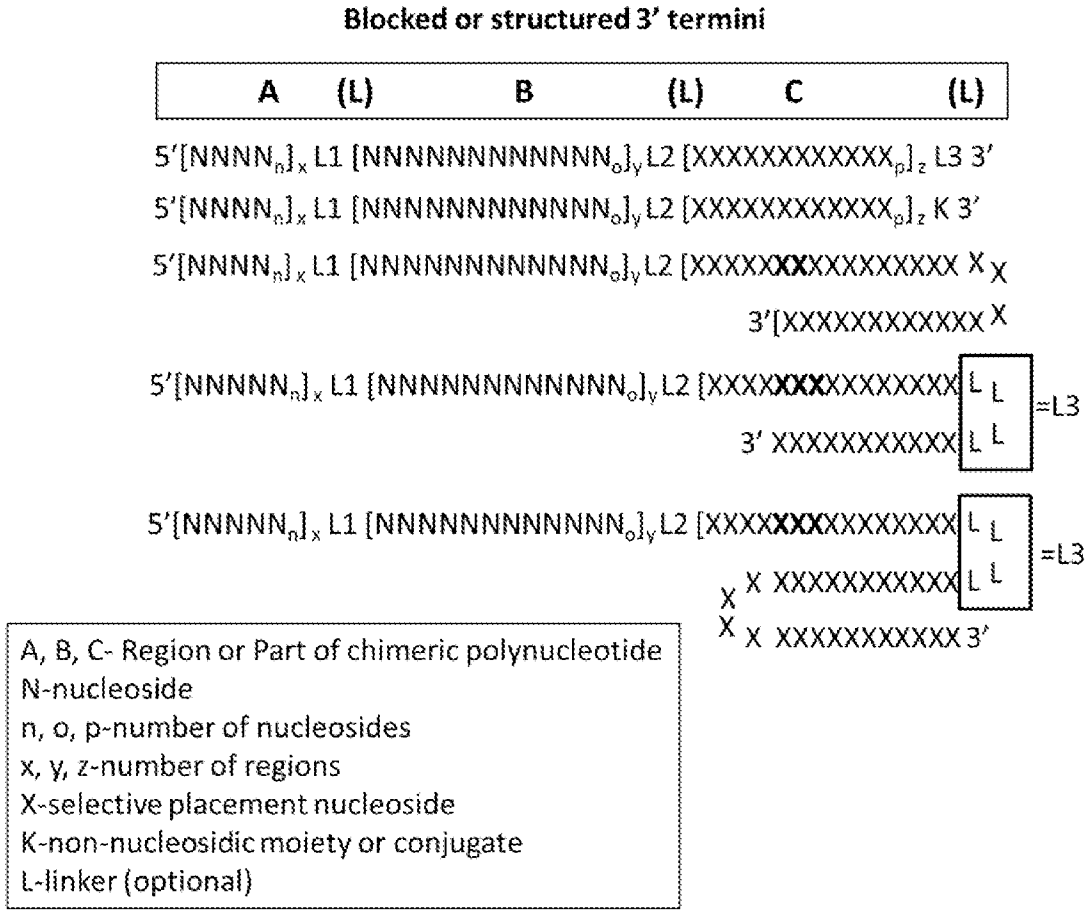
5′[NNNNN$_n$]$_x$ L1 [XXXXXXXXXXXXXXX$_o$]$_y$ L2 [XXXXXXXXXXXX$_p$]$_z$ L3 3′

5′[XXXXXX$_n$]$_x$ L1 [XXXXXXXXXXXXXXX$_o$]$_y$ L2 [XXXXXXXXXXXX$_p$]$_z$ L3 3′

5′[NNNNN$_n$]$_x$ L1 [NNNNNNNNNNNN$_o$]$_y$ L2 [XXXXXXXXXXXX$_p$]$_z$ L3 3′

A, B, C- Region or Part of chimeric polynucleotide
N-nucleoside
n, o, p-number of nucleosides
x, y, z-number of regions
X-selective placement nucleoside
L-linker (optional)

FIGURE 5


Blocked or structured 3' termini
A (L) B (L) C (L)
5'[NNNN$_n$]$_x$ L1 [NNNNNNNNNNNN$_o$]$_y$ L2 [XXXXXXXXXXX$_p$]$_z$ L3 3'
5'[NNNN$_n$]$_x$ L1 [NNNNNNNNNNNN$_o$]$_y$ L2 [XXXXXXXXXXX$_p$]$_z$ K 3'
5'[NNNN$_n$]$_x$ L1 [NNNNNNNNNNNN$_o$]$_y$ L2 [XXXXXXXXXXXXXXX X X
3'[XXXXXXXXXXX X
5'[NNNNN$_n$]$_x$ L1 [NNNNNNNNNNNN$_o$]$_y$ L2 [XXXXXXXXXXXXXXX L L
3' XXXXXXXXXXX L L
=L3
5'[NNNNN$_n$]$_x$ L1 [NNNNNNNNNNNN$_o$]$_y$ L2 [XXXXXXXXXXXXXXX L L
X X XXXXXXXXXXX L L
X X XXXXXXXXXXX 3'
=L3
A, B, C- Region or Part of chimeric polynucleotide
N-nucleoside
n, o, p-number of nucleosides
x, y, z-number of regions
X-selective placement nucleoside
K-non-nucleosidic moiety or conjugate
L-linker (optional)

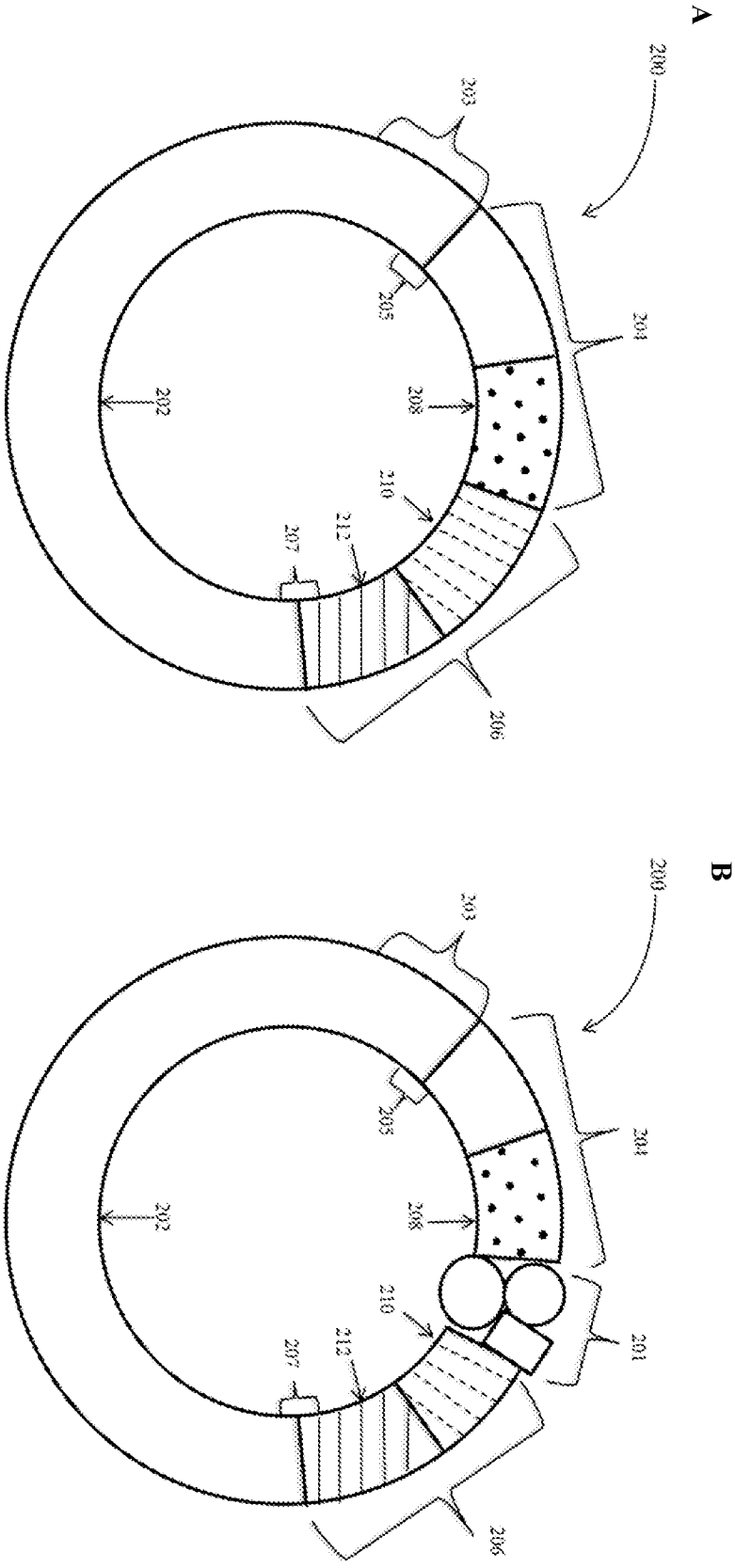

A
200
203
205
204
202
208
210
212
207
206
B
200
203
205
204
202
208
210
212
207
201
206

FIGURE 6

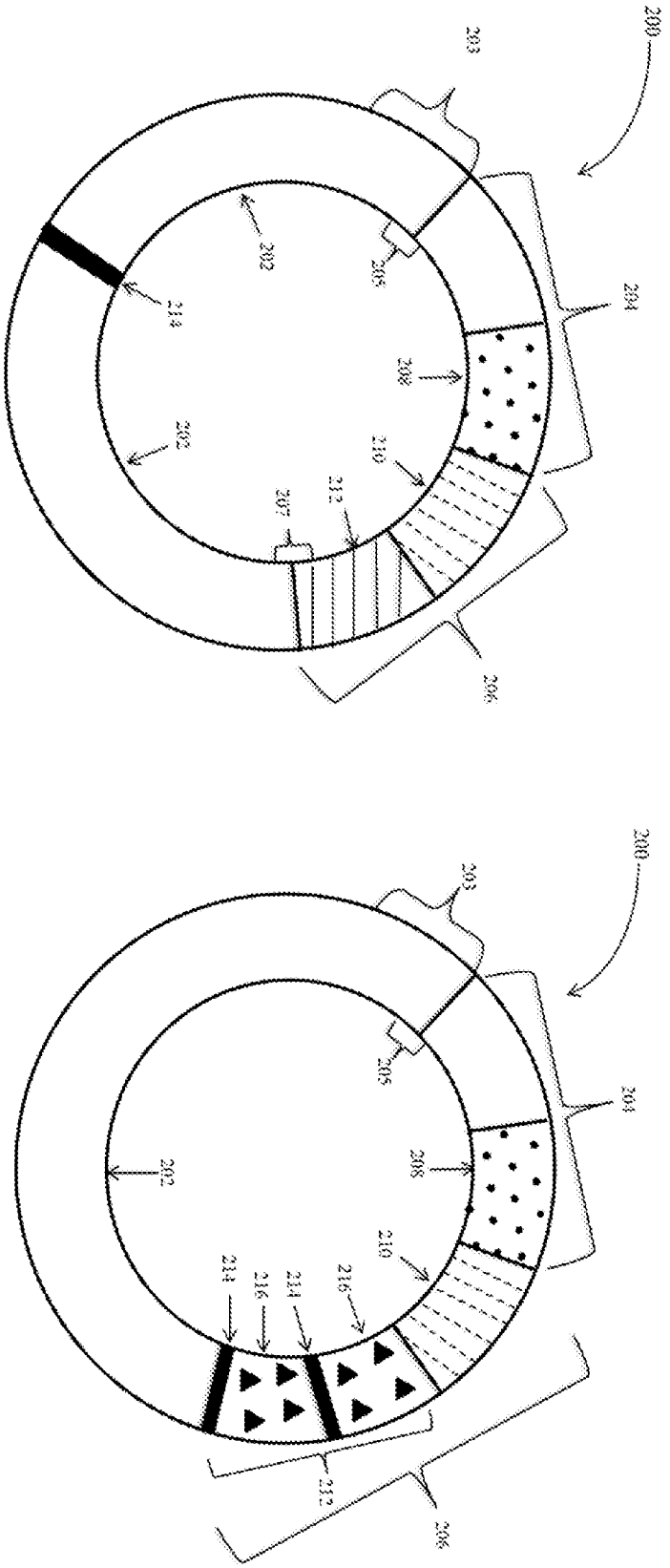
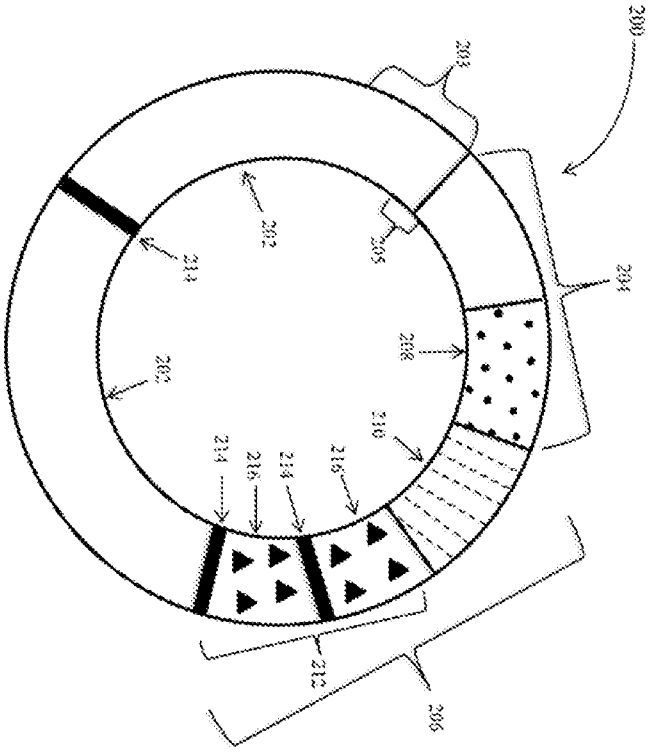


FIGURE 6, cont.



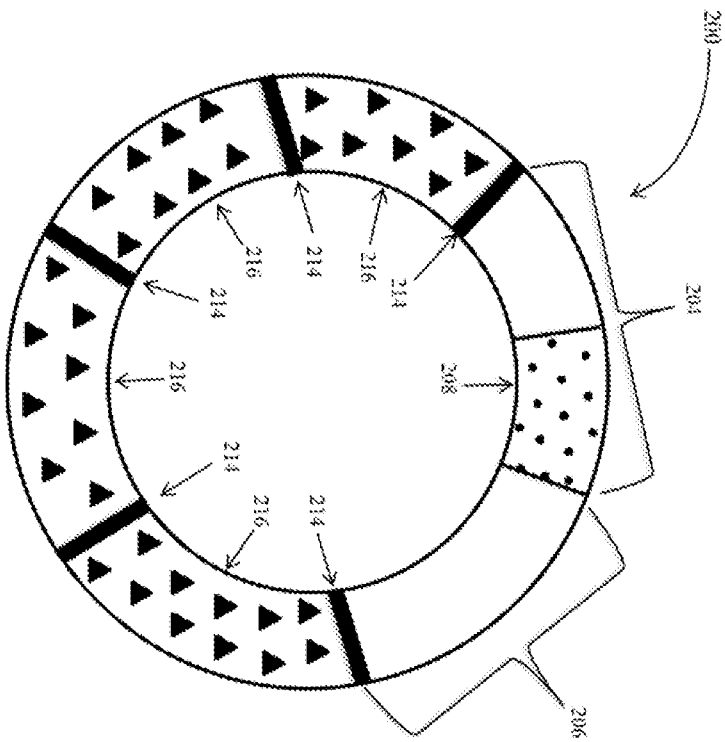


FIGURE 6, cont.

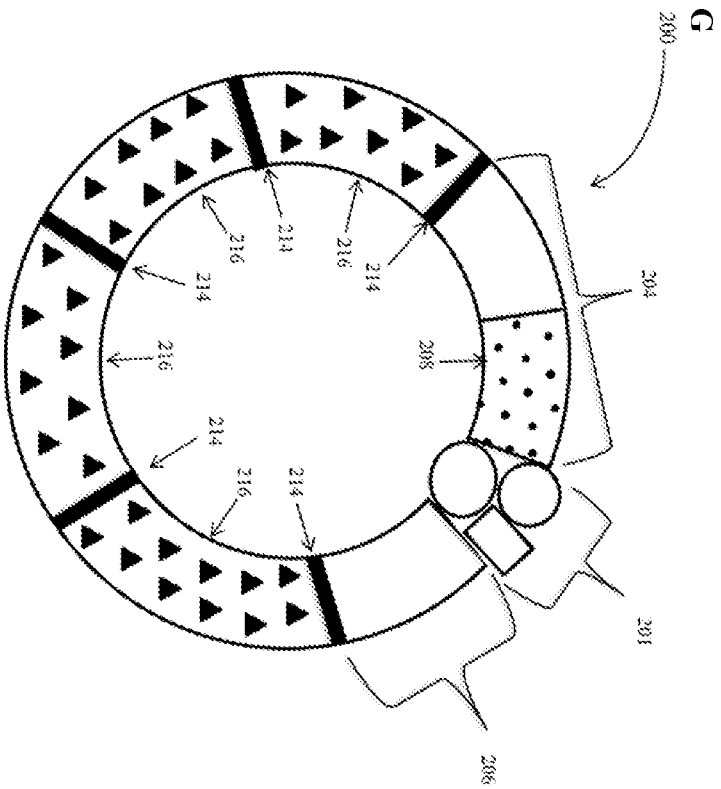


FIGURE 6, cont.