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ABSTRACT

Described herein are compositions comprising shortened fibers of poly-N-acetylglucosamine and/or a derivative thereof (“sNAG nanofibers”) and anti-bacterial applications of such compositions. The sNAG nanofibers may be formulated into compositions for the prevention and/or treatment of bacterial infections and diseases associated with such infections. Regimens employing such compositions are also described.

ANTI-BACTERIAL APPLICATIONS OF POLY-N-ACETYLGLUCOSAMINE NANOFIBERS

[0001] The present application is a divisional application of Australian Patent Application No. 2013202332, itself a divisional application of Australian Patent Application No. 2011239466 and claims priority to United States provisional application no. 61/324,657, the contents of all of which are incorporated herein by reference.

1. FIELD

[0002] Described herein are compositions comprising shortened fibers of poly-N-acetylglucosamine and/or a derivative thereof (“sNAG nanofibers”) and anti-bacterial applications of such compositions. The sNAG nanofibers may be formulated into compositions for the prevention and/or treatment of bacterial infections and diseases associated with such infections. Regimens employing such compositions are also described.

2. BACKGROUND

[0003] Currently, antibiotics are a standard therapy for bacterial infections. However, some individuals have an allergic reaction to certain antibiotics, others suffer from side effects associated with antibiotics, and the continued use of antibiotics often leads to a reduction in their efficacy. In addition, antibiotic therapy often leads to the emergence of antibiotic-resistant strains of bacteria. Accordingly, there is a continuing need for new anti-bacterial agents that are effective in fighting infection without generating resistance or reducing the efficacy overtime. There is a need for non-antibiotic anti-bacterial agents that can be used in clinical settings, *e.g.*, in the treatment of infectious diseases of the skin, digestive and respiratory tract, and in wound treatment.

[0004] Wound infection is one type of bacterial infection. Wound infection is a major complication, especially in patients with chronic disease such as diabetes or during immunosuppression. Such patients have disruptions in appropriate inflammatory responses, including the migration and recruitment of neutrophils and macrophages, which predisposes them to increased infection (Singer, A.J. and R.A. Clark, 1999, *N Engl J Med* 341(10): 738-46). In addition, bacterial infection can lead to impairment of wound healing and sepsis. Given the ineffectiveness of many current antibiotic treatments and the increased prevalence of antibiotic resistant bacteria such as MRSA (Methycillin-resistant *S. aureus*), new clinical treatments are in high demand.

3. SUMMARY

[0004a] In one aspect, the present invention particularly relates to a method for treating a disease or a condition associated with a bacterial imbalance in a subject in need thereof, comprising topically administering a composition comprising shortened fibers of poly- β -1 \rightarrow 4-N-acetylglucosamine (“sNAG nanofibers”) to a subject, wherein the sNAG nanofibers are less than 10 μm in length, wherein the sNAG nanofibers comprise 70% or more than 70% of N-acetylglucosamine monosaccharides, and wherein the sNAG nanofibers do not have an effect, or substantially have no effect, on bacterial growth or survival of *Staphylococcus aureus* bacterial cultures *in vitro*.

[0004b] In another aspect the present invention particularly relates to a method for treating a disease or a condition associated with a bacterial imbalance in a subject in need thereof, comprising topically administering a composition comprising shortened fibers of poly- β -1 \rightarrow 4-N-acetylglucosamine (“sNAG nanofibers”) to a subject, wherein the sNAG nanofibers are from about 1 μm to less than 10 μm in length, wherein the sNAG nanofibers comprise 70% or more than 70% of the N-acetylglucosamine monosaccharides, and wherein the sNAG nanofibers do not have an effect, or substantially have no effect, on bacterial growth or survival of *Staphylococcus aureus* bacterial cultures *in vitro*.

[0004c] In a still further aspect the present invention particularly relates to use of a composition comprising shortened fibers of poly- β -1 \rightarrow 4-N-acetylglucosamine (“sNAG nanofibers”) in the manufacture of a medicament for treating a disease or a condition associated with a bacterial imbalance in a subject in need thereof, said treating comprising topically administering the medicament to the subject, wherein the sNAG nanofibers are less than 10 μm in length, wherein the sNAG nanofibers comprise 70% or more than 70% of N-acetylglucosamine monosaccharides, and wherein the sNAG nanofibers do not have an effect, or substantially have no effect, on bacterial growth or survival of *Staphylococcus aureus* bacterial cultures *in vitro*.

[0004d] In a still further aspect, the present invention particularly relates to use of a composition comprising shortened fibers of poly- β -1 \rightarrow 4-N-acetylglucosamine (“sNAG

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nanofibers") in the manufacture of a medicament for treating a disease or a condition associated with a bacterial imbalance in a subject in need thereof, said treating comprising topically administering the medicament to the subject, wherein the sNAG nanofibers are from about 1 μm to less than 10 μm in length, wherein the sNAG nanofibers comprise 70% or more than 70% of the N-acetylglucosamine monosaccharides, and wherein the sNAG nanofibers do not have an effect, or substantially have no effect, on bacterial growth or survival of *Staphylococcus aureus* bacterial cultures *in vitro*.

[0004e] These and other aspects are described in further detail below. Aspects not the subject of the present invention are described herein for completeness and may form the subject of the parent applications AU2013202332, AU2011239466 or one or more divisional applications.

[0005] In one aspect, described herein are methods for treating and/or preventing a bacterial infection(s) and/or diseases associated with or caused by a bacterial infection in a subject.

[0006] In certain embodiments, described herein are methods for treating a bacterial infection in a subject comprising topically administering a composition comprising sNAG nanofibers to a subject. In some embodiments, the subject is diagnosed with the bacterial infection or displaying one or more symptoms of the bacterial infection. The methods of diagnosis of bacterial infection and symptoms of bacterial infection are those known in the art or described herein. The bacterial infection may be a skin infection, a gastrointestinal infection, a respiratory infection, a urinary tract infection, a reproductive tract infection, or infection of any other organ or tissue in the body of the subject as described herein. In one embodiment, the infection is a nosocomial infection, an MRSA infection, a *Pseudomonas* infection, or a *C. difficile* infection.

[0007] In certain embodiments, described herein are methods for treating and/or preventing a disease associated with a bacterial infection or a bacterial imbalance in a subject comprising topically administering a composition comprising sNAG nanofibers to the subject. In one such embodiment, the method involves treating and/or preventing a disease associated with a bacterial infection. In another embodiment, the method involves treating and/or preventing a disease

associated with a bacterial imbalance, for example, an imbalance in bacterial microbiota as described herein. In certain embodiments, the methods involve treating an existing bacterial infection. In some of these embodiments, the subject to be treated is diagnosed with a disease associated with a bacterial infection or displays one or more symptoms of such disease. In other embodiments, the subject to be treated is diagnosed with a disease associated with a bacterial imbalance or displays one or more symptoms of such imbalance. The disease may be a skin disease, a gastrointestinal disease, a respiratory disease, a urinary tract disease, a reproductive tract disease, or disease of any other organ or tissue in the body of the subject as described herein. In some embodiments, the disease is a skin disease or a gastrointestinal disease. In one embodiment, the disease is associated with a nosocomial infection, an MRSA infection, a *Pseudomonas* infection, or a *C. difficile* infection.

[0008] In some embodiments, described herein are methods for preventing a bacterial infection and/or a disease associated with a bacterial infection comprising topically administering a composition comprising sNAG nanofibers to a subject. In some embodiments, a

administering a composition comprising sNAG nanofibers to a subject. In some embodiments, a composition comprising sNAG nanofibers is administered to a subject at high risk of a bacterial infection to prevent a disease associated with a bacterial infection. In specific embodiments, a composition comprising sNAG nanofibers is administered to a subject with a wound or a subject who has undergone a surgery. In one embodiment, the composition is administered to an immunocompromised subject. In some embodiments, a composition comprising sNAG nanofibers is administered to a wound, where the wound is at high risk of bacterial infection. In certain embodiments, the wound is an open wound. The open wound may be a gunshot wound, a puncture wound, a laceration wound, an abrasion, a cut, a penetration wound, a surgical wound, or any other wound. In certain embodiments, the wound may be a puncture wound, for example, a puncture wound that is caused by a hemodialysis procedure or a catheterization procedure. In such embodiments, the subject to be treated may have been diagnosed with a hemodialysis-related or catheterization-related infection. In one embodiment, the bacterial infection and/or the disease associated with a bacterial infection to be prevented by a sNAG composition is not in a wound (e.g., an open wound) or is not associated with a wound. In one such embodiment, the bacterial infection and/or the disease associated with a bacterial infection is not at the site of a wound (e.g., not at the site of an open wound).

[0009] In some embodiments, described herein are methods for treating a bacterially infected wound in a subject, comprising topically administering a composition comprising sNAG nanofibers to the wound site in a subject. In some embodiments, the subject to be treated is diagnosed with a bacterial infection or displays one or more symptoms of the bacterial infection. In certain embodiments, the wound is an open wound. The open wound may be a gunshot wound, a puncture wound, a laceration wound, an abrasion, a cut, a penetration wound, a surgical wound, or any other wound. In certain embodiments, the wound may be a puncture wound, for example, a puncture wound that is caused by a hemodialysis procedure or a catheterization procedure. In such embodiments, the subject to be treated may have been diagnosed with a hemodialysis-related or catheterization-related infection.

[0010] Bacterial infections to be treated or prevented using the methods described herein include infections with bacteria of one or more of the following genera: *Bordetella*, *Barrelia*, *Brucella*, *Campylobacter*, *Chlamydia* and *Chlamydophylia*, *Clostridium*, *Corynebacterium*, *Enterococcus*, *Escherichia*, *Francisella*, *Haemophilus*, *Helicobacter*, *Legionella*, *Leptospira*,

Listeria, Mycobacterium, Mycoplasma, Neisseria, Pseudomonas, Rickettsia, Salmonella, Shigella, Staphylococcus, Streptococcus, Treponema, Vibria, and Yersinia. In some embodiments, a sNAG composition may be used to treat and/or prevent a disease associated with an infection by bacteria from one or more of the listed genera of bacteria, or one or more symptoms thereof.

[0011] Bacterial infections to be treated or prevented using the methods described herein also include infections with bacteria of one or more of the following species: *Bacillus anthracis, Bordetella pertussis, Borrelia burgdorferi, Brucella abortus, Brucella canis, Brucella melitensis, Brucella suis, Campylobacter jejuni, Chlamydia pneumonia, Chlamydia trachomatis, Clamidophila psittaci, Clostridium botulinum, Clostridium difficile, Clostridium perfringens, Clostridium tetani, Corynebacterium diphtheriae, Enterococcus faecalis, Enterococcus faecium, Escherichia coli, Francisella tularensis, Haemophilus influenzae, Helicobacter pylori, Legionella pneumophila, Leptospira pneumophila, Leptospira interrogans, Listeria monocytogenes, Mycobacterium leprae, Mycobacterium tuberculosis, Mycoplasma pneumoniae, Neisseria gonorrhoeae, Neisseria meningitidis, Pseudomonas aeruginosa, Proteus mirabilis, Rickettsia rickettsii, Salmonella typhi, Salmonella typhimurium, Shigella sonnei, Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus saprophyticus, Streptococcus agalactiae, Streptococcus pneumonia, Streptococcus pyogenes, Treponema pallidum, Vibrio cholerae, and Yersinia pestis.* In some embodiments, a sNAG composition may be used to treat and/or prevent a disease associated with an infection by bacteria from one or more of the listed species of bacteria, or one or more symptoms thereof.

[0012] In certain embodiments, the bacterial infection to be treated or prevented using the methods described herein is an MRSA infection, a *Pseudomonas* infection, or a *C. difficile* infection. In some embodiments, a sNAG composition may be used to treat and/or prevent a disease associated MRSA infection, a *Pseudomonas* infection, or a *C. difficile* infection, or one or more symptoms thereof symptom thereof.

[0013] In certain embodiments, the bacterial infection to be treated or prevented using the methods described herein is caused by bacteria that are known to one of ordinary skill in the art to be resistant to a standard anti-bacterial therapy, for example, resistant to one or more antibiotics. In one embodiment, the bacterial infection to be treated or prevented using the methods described herein is MRSA, e.g., a nosocomial MRSA. In some embodiments, a sNAG

composition may be used to treat and/or prevent a disease associated with an infection by bacteria resistant to one or more antibiotics. In one embodiment, a sNAG composition may be used to treat and/or prevent a disease associated with MRSA, e.g., associated with a nosocomial MRSA.

[0014] The subject to be treated using the methods described herein may be a mammal, preferably a human. The subject can also be a livestock animal (e.g., a chicken, a cow, a pig, a goat) or a pet (e.g., a dog or a cat), or any other animal.

[0015] The sNAG nanofibers contemplated in the methods described herein may be of varying lengths, widths and molecular weights as described in Section 5.1, *infra*. In certain embodiments, the majority (and in certain embodiments, at least or more than 60%, 70%, 80%, 90%, 95% or 99%) of the sNAG nanofibers, or 100% of the sNAG nanofibers, are between about 1 to 15 μm in length. In some embodiments, the majority (and in certain embodiments, at least or more than 60%, 70%, 80%, 90%, 95% or 99%) of the sNAG nanofibers, or 100% of the sNAG nanofibers, are between about 2 to 10 μm , or 4 to 7 μm in length. The sNAG nanofibers of the described length can be obtained, for example, as described below in Section 5.2, *infra*.

[0016] In certain embodiments, the sNAG nanofibers were produced by irradiation, e.g., gamma irradiation, of poly-N-acetylglucosamine or a derivative thereof. In some embodiments, the sNAG nanofibers are produced by irradiation of the poly- β -1 \rightarrow 4-N-acetylglucosamine in the form of dried fibers (e.g., at 500-2,000 kgy), or irradiation of the poly- β -1 \rightarrow 4-N-acetylglucosamine in the form of wet fibers (e.g., at 100-500 kgy).

[0017] In certain embodiments, the sNAG nanofibers are derived from microalgae. In another embodiment, the sNAG nanofibers are not derived from crustaceans. In yet another embodiment, the sNAG nanofibers may be derived from microalgae, crustaceans (e.g., shrimp), fungus or any other source.

[0018] In one embodiment, the sNAG nanofibers comprise N-acetylglucosamine monosaccharides and/or glucosamine monosaccharides, wherein more than 60%, 70%, 80%, 90%, 95%, or 99% of the monosaccharides of the sNAG nanofibers are N-acetylglucosamine monosaccharides. In another embodiment, the sNAG nanofibers comprise N-acetylglucosamine monosaccharides and/or glucosamine monosaccharides, wherein more than 70% of the monosaccharides of the sNAG nanofibers are N-acetylglucosamine monosaccharides.

[0019] In certain embodiments, the sNAG nanofibers used in the methods described herein do not have an effect on bacterial growth or survival of *Staphylococcus aureus* bacterial cultures *in vitro*, or substantially have no effect on bacterial growth or survival of *Staphylococcus aureus* bacterial cultures *in vitro*. In some embodiments, the sNAG nanofibers reduce bacterial growth or survival of bacterial cultures *in vitro* by less than 1 log, 0.75 log, 0.5 log, 0.25 log, 0.2 log or 0.1 log, e.g., when *Staphylococcus aureus* bacterial cultures are treated/incubated with the sNAG nanofibers *in vitro*. The tests for the effect of sNAG nanofibers on bacterial growth or survival and the evaluation of the test results are described, for example, in Section 5.1, Example 2 (e.g., Section 6.2.2.5) and Figure 11E, *infra*.

[0020] In certain embodiments, the sNAG nanofibers used in the methods described herein are non-reactive in a biocompatibility test or tests. For example, the sNAG nanofibers used in the methods described herein may be non-reactive when tested in an elution test, an intramuscular implantation test, an intracutaneous test, or a systemic test. In some embodiments, the compositions described herein are non-reactive when tested in an elution test, an intramuscular implantation test, an intracutaneous test, or a systemic test. In other embodiments, the sNAG nanofibers used in the methods described herein have Grade 0 or Grade 1 when tested in an elution test, an intramuscular implantation test, an intracutaneous test, or a systemic test. In yet another embodiment, the sNAG nanofibers used in the methods described herein are at most mildly reactive when tested in an elution test, an intramuscular implantation test, an intracutaneous test, or a systemic test. In one embodiment, the sNAG nanofibers or compositions comprising such nanofibers are non-reactive as determined by an intramuscular implantation test. In certain embodiments, the compositions described herein do not cause an allergenic reaction or an irritation, e.g., at the site of application. In other embodiments, the compositions described herein cause at most a mild allergenic reaction or a mild irritation, e.g., at the site of application.

[0021] The contemplated modes of administration of the compositions described herein are topical, e.g., topical on the skin; topical at the site of a wound, a surgery, a bacterial infection, or a symptom of an infection (e.g., a swelling); and topical to a body surface such as the skin, mucous membranes (e.g., vagina, anus, throat, eyes, ears), or the surface of other tissues. In certain embodiments, the sNAG nanofibers or compositions comprising such nanofibers are formulated as a dressing, a bandage, a mat, a spray, a liquid, a suspension, a membrane, a

powder, an ointment, a cream, a paste, a suppository, or a gel. In some embodiments, the sNAG nanofibers or compositions comprising such nanofibers are formulated as a cream, a gel, an ointment, a membrane, a powder, a spray, or a suppository.

[0022] In another aspect, described herein are compositions for use in the methods described herein. In a specific embodiment, the compositions comprise sNAG nanofibers. In certain embodiments, the compositions described herein comprise sNAG nanofibers and one or more additional active ingredients useful in preventing and/or treating a bacterial infection, a disease associated with a bacterial infection, or a symptom thereof. In some embodiments, the additional active ingredient is an anti-bacterial agent. Such additional anti-bacterial agent may be an antibiotic. In another embodiment, such additional anti-bacterial agent is zinc. In yet another embodiment, the compositions described herein do not comprise an antibiotic. In yet other embodiments, the compositions described herein do not comprise any additional anti-bacterial agent. In one embodiment, the compositions described herein comprise the sNAG nanofibers as the only active ingredient and do not comprise any additional active ingredients.

[0023] In specific embodiments, a composition comprises the sNAG nanofibers and an antibiotic. Examples of antibiotics that can be used in the compositions of the invention include microlides (e.g., erythromycin, azithromycin), aminoglycosides (e.g., amikacin, gentamicin, neomycin, streptomycin), cephalosporins (e.g., cefadroxil, cefaclor, cefotaxime, cefepime), fluoroquinolones (e.g., ciprofloxacin, levofloxacin), penicillins (e.g., penicillin, ampicillin, amoxicillin), tetracyclines (e.g., tetracycline, doxycycline), and/or carbapenems (e.g., meropenem, imipenem). The sNAG nanofibers and agents described herein may be used in such compositions. In some embodiments, a composition comprises the sNAG nanofibers and an agent effective to treat or prevent or commonly used to treat or prevent an *S. aureus* infection, MRSA infection, a *Pseudomonas* infection, or a *C. difficile* infection (e.g., an antibiotic effective against or commonly used against such infections).

[0024] In other embodiments, the compositions described herein are administered in conjunction with one or more additional anti-bacterial agents or any other suitable therapy. In some embodiments, the additional anti-bacterial agent or therapy is an antibiotic (e.g., a standard antibiotic therapy for the bacterial infection or a disease associated with a bacterial infection to be treated, as known in the art or described herein). In some embodiments, the additional anti-bacterial agent is an agent effective to treat or prevent or commonly used to treat or prevent an *S.*

aureus infection, MRSA infection, a *Pseudomonas* infection, or a *C. difficile* infection (e.g., an antibiotic effective against or commonly used against such infections). In some embodiments, the additional therapy is administered before, simultaneously with or after administration of a sNAG nanofiber composition. In yet another embodiment, the compositions described herein are not administered in conjunction with any other therapy, e.g., not administered in conjunction with an antibiotic.

3.1 Terminology

[0025] As used herein, the terms “sNAG nanofiber,” “sNAG,” “Taliderm,” or “Talymed” (formerly known as “Taliderm”) are used interchangeably to refer to shortened fibers of poly-N-acetylglucosamine and/or derivatives thereof.

[0026] As used herein, the term “about” means a range around a given value wherein the resulting value is the same or substantially the same (e.g., within 10%, 5% or 1%) as the expressly recited value. In one embodiment, “about” means within 10% of a given value or range. In another embodiment, the term “about” means within 5% of a given value or range. In another embodiment, the term “about” means within 1% of a given value or range.

[0027] As used herein, the terms “disease,” “disorder” or “condition” are used interchangeably to refer to a medical condition in a subject. In a specific embodiment, the disease is the pathological state associated with or caused by a bacterial infection.

[0028] As used herein, the term “bacterial infection” means the invasion by, multiplication and/or presence of bacteria in a cell or a subject.

[0029] As used herein, the numeric term “log” refers to log₁₀.

[0030] As used herein, the terms “therapies” and “therapy” can refer to any protocol(s), method(s), compositions, formulations, and/or agent(s) that can be used in the prevention and/or treatment of a bacterial infection or a symptom or condition associated therewith. In certain embodiments, the term “therapy” refers to a sNAG nanofiber(s) or a pharmaceutical composition comprising a sNAG nanofiber(s). In other embodiments, the term “therapy” refers to a therapy other than a sNAG nanofiber(s) or a pharmaceutical composition comprising a sNAG nanofiber(s). In specific embodiments, an “additional therapy” and “additional therapies” refer to a therapy other than a sNAG nanofiber(s) or a pharmaceutical composition comprising a sNAG nanofiber(s). In a specific embodiment, the therapy includes use of a sNAG nanofiber(s) or pharmaceutical composition comprising a sNAG nanofiber(s) as an adjuvant therapy; for

example, using a sNAG nanofiber composition in conjunction with a drug therapy, such as an antibiotic, and/or other therapies useful in treatment and/or prevention of a bacterial infection or a symptom or condition associated therewith.

[0031] As used herein, the term "effective amount" in the context of administering a sNAG nanofiber composition to a subject refers to the amount of a sNAG nanofiber that results in a beneficial or therapeutic effect. In specific embodiments, an "effective amount" of a sNAG nanofiber refers to an amount which is sufficient to achieve at least one, two, three, four or more of the following effects: (i) the clearance of a bacterial infection; (ii) the eradication of one or more symptoms associated therewith; (iii) the reduction of time required to clear a bacterial infection; (iv) the reduction or amelioration of the severity of a bacterial infection and/or one or more symptoms associated therewith; (v) the reduction in the duration of a bacterial infection and/or one or more symptoms associated therewith; (vi) the prevention or delay of the generation of a resistant strain or strains of bacteria or reduction of a number of resistant strains of bacteria generated; (vii) the prevention in the recurrence of a bacterial infection and/or one or more symptoms associated therewith; (viii) the reduction or elimination in the bacterial cell population; (ix) the reduction in the severity and/or duration of a condition caused by or associated with a bacterial infection; (x) the reduction in hospitalization of a subject; (xi) the reduction in hospitalization length; (xii) the increase in the survival of a subject; (xiii) the enhancement or improvement of the therapeutic effect of another therapy; (xiv) a reduction in mortality; (xv) the reduction or elimination in the spread of the bacteria from one subject to another subject, or one organ or tissue to another organ or tissue; (xvi) the prevention of an increase in the number of bacteria; (xvii) the prevention of the development or onset of a bacterial infection or one or more symptoms associated therewith; (xviii) the reduction in the number of symptoms associated with a bacterial infection; (xix) the reduction in the duration and/or severity of a condition caused by or associated with a bacterial infection; (xx) the inhibition or reduction in production of a bacterial toxin or toxins associated with a bacterial infection; (xxi) the stabilization or reduction of inflammation associated with a bacterial infection; (xxii) the induction of the expression of one or more defensin proteins and/or defensin-like proteins; (xxiii) the induction of the expression of one or more Toll-like receptors; (xxiv) the induction of the expression of one or more proteins that are beneficial for clearance or reduction in a bacterial infection or one or more symptoms associated therewith; (xxvi) the reduction in

organ failure associated with a bacterial infection or a disease associated therewith; (xxvii) the prevention of the onset, development or recurrence of a condition caused by or associated with a bacterial infection; and/or (xxviii) improvement in quality of life as assessed by methods well known in the art, e.g., a questionnaire. In specific embodiments, an “effective amount” of a sNAG nanofiber refers to an amount of a sNAG nanofiber composition specified herein, e.g., in Section 5.6, *infra*.

- [0032] As used herein, the term “elderly human” refers to a human 65 years or older.
- [0033] As used herein, the term “human adult” refers to a human that is 18 years or older.
- [0034] As used herein, the term “human child” refers to a human that is 1 year to 18 years old.
- [0035] As used herein, the term “human infant” refers to a newborn to 1 year old year human.
- [0036] As used herein, the term “premature human infant” refers to a newborn to 1 year old year human who was born of less than 37 weeks gestational age (e.g., before 37 weeks, 36 weeks, 35 weeks, 34 weeks, 33 weeks, 32 weeks, 31 weeks, 30 weeks, 29 weeks, 28 weeks, or less than 28 weeks of pregnancy).
- [0037] As used herein, the term “human toddler” refers to a human that is 1 year to 3 years old.
- [0038] As used herein, the term “majority” refers to greater than 50%, including, e.g., 50.5%, 51%, 55%, etc.
- [0039] As used herein, the term “subject” and “patient” are used interchangeably to refer to an animal (e.g., cow, horse, sheep, pig, chicken, turkey, quail, cat, dog, mouse, rat, rabbit, guinea pig, etc.). In a specific embodiment, the subject is a mammal such as a non-primate or a primate, e.g., a human. In specific embodiments, the subject is a human. See Section 5.5, *infra*, for more information concerning patients treated in accordance with the methods provided herein.
- [0040] As used herein, the term “low expression,” in the context of expression of a gene (e.g., based on the level of protein or peptide produced by the gene) refers to an expression that is less than the “normal” expression of the gene. In a specific embodiment, “low expression” refers to expression of a gene that is less than 90%, less than 80%, less than 75%, less than 70%, less than 65%, less than 60%, less than 55%, less than 50%, less than 45%, less than 40%, less than 35%, less than 30%, less than 25%, or less than 20% of the “normal” expression of the

gene. In another specific embodiment, "low expression" refers to expression of a gene that is about 20-fold, about 15-fold, about 10-fold, about 5-fold, about 4-fold, about 3-fold, about 2-fold, or about 1.5 fold less than the "normal" expression of the gene.

4. BRIEF DESCRIPTION OF FIGURES

- [0041] **Figure 1.** Nanofibers stimulate Akt 1 activation, an upstream regulator of Ets1. (A) Western blot analysis of phospho-Akt in response to sNAG and sNAG stimulation of serum starved EC. (B) RT-PCR analysis of EC infected either with scrambled control ("SCR") or Akt1 shRNA lentiviruses and assessed for expression of Ets1 and S26 as a loading control. (C) Schematic of a signal transduction pathway transducing a signal from sNAG nanofibers to Akt1, Ets1 and Defensins.
- [0042] **Figure 2.** Delayed wound healing in Akt1 null animals is partially rescued by Taliderm treatment. (A) Representative images of wounded WT and AKT1 null mice with and without treatment of Taliderm. (B) H&E staining of representative mouse skin sections from day 3 wounds.
- [0043] **Figure 3.** sNAG nanofibers stimulate cytokine and defensin expression in primary endothelial cells. (A) Immunohistochemistry of EC treated with or without sNAG using an antibody directed against α -defensin. (B) ELISA showing that nanofiber treatment of EC results in the secretion of α -defensins 1-3 (serum starved, treated with 5 μ g/ml or 10 μ g/ml sNAG).
- [0044] **Figure 4.** sNAG nanofibers stimulate defensin expression in primary endothelial cells in an Akt1 dependent manner. (A) and (B) Quantitative RT-PCR analyses of serum starved EC ("ss") treated with or without sNAG ("snag"), with or without PD98059 (MAPK inhibitor, "PD"), Wortmannin (PI3K inhibitor, "wtm") or infected with a scrambled control ("SCR"), or Akt1 ("AKT1") shRNA lentiviruses and assessed for expression of the genes indicated.
- [0045] **Figure 5.** sNAG nanofibers stimulate β -defensin 3 expression in mouse keratinocytes. (A) Immunofluorescent staining with β -defensin 3 (visible as bright staining in the upper right hand panel; see, e.g., thick white arrows) and Involucrin antibodies of paraffin embedded mouse cutaneous wound sections from WT and Akt1 null animals on Day 3. (B) Quantification of β -defensin 3 immunofluorescent staining using NIHImageJ software (TX=Taliderm; Akt1=Akt1 null). (C) Immunofluorescent staining of WT and Akt1 null treated and untreated keratinocytes with β -Defensin 3 (visible as bright staining; see, e.g., thick white

arrows) and TOPRO-3 (nuclei staining; see, e.g., thin white arrows). Notice the increase in β -Defensin 3 staining in WT and Akt1 Taliderm treated wounds.

[0046] **Figure 6.** Akt1 dependent transcription factor binding sites. Schematic of Akt1 dependent transcription factor binding sites. Using Genomatix software, 500 bp upstream of the transcription start site was analyzed for conserved sites on the mRNA of DEF1, 4, and 5 (ETS-black ovals; FKHD-striped ovals; CREB-white ovals; NFkB-checkered ovals).

[0047] **Figure 7.** sNAG treatment results in expression and secretion of defensins *in vitro*. (A) RTPCR analysis of serum starved ("SS") primary endothelial cells treated with sNAG (50 μ g/ml) for the times indicated and assessed for expression of β -defensin 3 and α -defensin 1. (B) Immunofluorescent labeling of endothelial cells either serum starved (untreated) or treated with sNAG nanofibers (10 μ g/ml for 5hrs). Antibodies are directed against α -defensin 5 (FITC, upper left hand panel), β -defensin 3 (Texas Red, upper right hand panel). Nuclei are stained with TOPRO-3 (Blue, lower left hand panel). Lower right hand panel represents triple overlay. (C) Immunofluorescent labeling of keratinocytes (HaCat) that are either serum starved (untreated) or treated with sNAG nanofibers (10 μ g/ml for 5 hours). Antibodies are directed against α -defensin 5 (FITC, upper left hand panel), β -defensin 3 (Texas Red, upper right hand panel). Nuclei are stained with TOPRO-3 (Blue, lower left hand panel).

[0048] **Figure 8.** sNAG induced defensin expression is dependent on Akt1. (A) Quantitative RT-PCR analyses using primers directed against α -defensin 1 from total RNA isolated from serum starved endothelial cells treated with or without sNAG for 3 hours, with or without pretreatment with PD98059 ("PD") (50 μ M), wortmannin ("WTM") (100nm). Quantitation is relative to the S26 protein subunit. (B) Quantitation of β -defensin 3 expression from total RNA isolated from serum starved endothelial cells treated with or without sNAG for 3 hours, with or without PD98059 (50 μ M), wortmannin (100nm) and shown as relative to S26. (C) Western Blot analysis of phospho-Akt in serum starved endothelial cells (SS) stimulated with sNAG for the times indicated. Line indicates where lanes have been removed (D) Quantitative RT-PCR analyses of serum starved endothelial cells infected with a scrambled control (SCR) or Akt1 shRNA lentiviruses, treated with or without sNAG and assessed for α -defensin 4 expression. Quantitation is shown relative to S26. (E) Quantitation of β -defensin 3 expression from total RNA isolated from serum starved endothelial cells infected with a scrambled control (SCR) or Akt1 shRNA lentiviruses, treated with or without sNAG. Quantitation is shown

relative to S26. All experiments were done in at least triplicate and repeated at least three independent times and p values are shown.

[0049] **Figure 9.** sNAG induced defensin expression *in vivo* requires Akt1. (A) Paraffin embedded sections of cutaneous wounds harvested on day 3 post wounding from both WT (n=3) and Akt1 mice. Wounds were either untreated or treated with sNAG membrane. Immunofluorescence was performed using antibodies directed against β -defensin 3 (green, visible as bright staining in the upper right hand panel; see, e.g., white thick arrows), Involucrin (Red), and Topro (Blue, nuclei staining; see, e.g., white thin arrows). (B) Paraffin embedded section from WT treated with sNAG harvested on day 3. Immunofluorescence was performed using antibodies directed against β -defensin 3 (green, visible as bright staining; see, e.g., thick white arrows), Involucrin (Red), and Topro (Blue, nuclei staining; see, e.g., thin white arrows). This lower magnification (20x) is included to better illustrate the epidermal layers expressing β -defensin 3. Scale bars = 50 μ m. (C) Quantitation of β -defensin 3 expression from paraffin embedded sections was performed using NIH ImageJ software. Experiments were repeated three independent times and p values are shown.

[0050] **Figure 10.** sNAG treatment increases wound closure in wild type mice. H&E staining of wound tissue sections derived from C57Bl6 wild type animals either untreated or treated with sNAG membrane. The day post-wound is indicated to the left of each panel. The solid black line follows the keratinocyte cell layer indicating wound closure. Black arrows indicate the margin of the wound bed.

[0051] **Figure 11.** sNAG treatment reduces bacterial infection in an Akt1 dependent manner. (A) Tissue gram staining of *S. aureus* infected wounds from WT mice. WT mice were wounded using a 4 mm biopsy punch. Immediately after wounding mice were inoculated with 1 \times 10⁸ cfu/ml. 30 minutes post-infection, mice in the treated group were treated with Taliderm. Skin samples were taken 5 days post-treatment and sectioned for analysis. Tissue gram staining was performed. Dark purple staining indicates gram-positive bacteria and neutrophils that have engulfed bacteria. Sections under 20x and 40x magnification are shown. (B) Tissue gram staining of paraffin embedded *S. aureus* infected wounds from WT and Akt1 null mice (n=3). Infected wounds were either untreated or treated with sNAG membrane and wound beds were harvested on day 3 and day 5 for analysis. Dark purple staining indicates the presence of gram positive bacteria in the wound bed. Black arrows indicate examples of gram positive staining.

Note the accumulation of positive staining in untreated WT that is lacking in WT animals treated with sNAG. Scale bars = 50 μ m. (C) CFUs derived from day 5 post wounding were quantitated from *S. aureus* infected wounds using both treated and untreated WT (n= 3) and Akt1 mice (n=3). Wild type mice that were sNAG treated show a significant ($p<.01$) decrease in bacteria load in the wound beds as compared to Akt1 null animals. All experiments were repeated three independent times and the p values are shown. (D) CFU quantitated from infected wounds at day 3 post wounding in a similar fashion described in (C). sNAG treatment of infected wounds shows a significant decrease in CFU of both WT and Akt1 null animals on day 3, but the WT animals show an approximate 10 fold difference compared to a 2 fold difference in Akt1 animals. (E) Quantitation of CFUs in *S. aureus* cultures that were either untreated or treated with various amounts of sNAG nanofibers. Each experiment was performed three independent times and p values are shown. (F) Tissue gram staining of *S. aureus* infected wounds harvested on day 3 post wound from WT mice (n=3) that were treated with or without β - defensin 3 peptide (1.0 uM). Note the decrease in gram positive staining in infected wounds that were treated with β -defensin 3 peptide. (G) Quantitation of CFUs from *S. aureus* infected WT mice (n=3) treated with or without β -defensin 3 peptide. Infected wounds that were treated with peptide show a significant decrease ($p <.05$) in CFU. Scale bars = 50 μ m. Each experiment was performed three independent times and p values are shown.

[0052] **Figure 12.** Rapid induction of defensin expression by sNAG treatment of *S. aureus* infected wounds. (A) Paraffin embedded tissue sections from *S. aureus* infected wounds, harvested on day 3, were subjected to immunofluorescence using antibodies directed against β -defensin 3 (green, visible as bright staining in the upper right hand panel and in the lower panel in the middle; see, e.g., thick white arrows), Involucrin (red) to mark the keratinocyte layer, and Topro (blue, nuclei staining; see, e.g., thin white arrows) from both sNAG treated WT (n=3) and untreated WT mice (n=3). Non specific staining of keratin is indicated by the no primary control which was stained with secondary antibody only. Scale bar = 50 μ m. (B) Quantitation of β -defensin 3 expression from paraffin embedded sections using NIH ImageJ software. *S. aureus* infected wounds that were treated with sNAG show a significant increase ($p<.05$) in β -defensin 3 staining. Experiments were repeated three independent times and p values are shown.

[0053] **Figure 13.** Antibodies against β -defensin 3 impedes antibacterial effects of sNAG treatment. (A) Tissue gram staining of paraffin embedded *S. aureus* infected wounds treated

with sNAG from WT mice ($n=3$) that were harvested on Day 3. sNAG treated wounds were treated with either β -defensin 3 antibody or isotype control goat IgG antibody prior to sNAG treatment. Representative images show increased accumulation gram positive staining (black arrows) in the wound beds of mice treated with an antibody directed against β -defensin 3. Scale bar = 20 μ m. (B) Quantitation of CFUs from *S. aureus* infected WT mice treated either β -defensin 3 antibody ($n=3$) or control IgG antibody ($n=3$) prior to sNAG treatment. β -defensin 3 application significantly increased ($p < .05$) CFU.

[0054] **Figure 14.** sNAG treatment reduces bacterial infection by *Pseudomonas aeruginosa*. Mice were wounded using a 4 mm biopsy punch, inoculated with 1.5×10^9 cfu/ml *P. aeruginosa*, infected wounds were either untreated ($n=6$) or treated ($n=6$) with sNAG membrane ($n=6$) 30 min post-infection, wound beds were harvested on day 3 for analysis, cultured for 30 minutes, plated, and CFUs of the untreated and treated infected wounds were quantitated. sNAG treated mice show a significant ($p < .05$) decrease in bacteria load in the wound beds as compared to untreated animals.

[0055] **Figure 15.** Effect of irradiation on chemical and physical structure of pGlcNAc fibers. (A) Correlation between molecular weight of pGlcNAc and irradiation level/formulation for irradiation. (B) Infrared (IR) spectrum of non-irradiated pGlcNAc slurry (top line), pGlcNAc slurry irradiated at 100 kGy (bottom line), and pGlcNAc slurry irradiated at 200 kGy (middle line). (C) Scanning electron microscopic (SEM) analyses of pGlcNAc. (D) Scanning electron microscopic (SEM) analyses of sNAG.

[0056] **Figure 16.** pGlcNAc did not affect metabolic rate. For each time period (*i.e.*, at 24 and 48 hours), the identity for each of the four bars (from left to right) is as follows: serum starvation (SS), VEGF, and pGlcNAc (NAG) at 50 and 100 μ g/ml.

[0057] **Figure 17.** pGlcNAc protected human umbilical vein endothelial cell (EC) from cell death induced by serum deprivation. For each time period (*i.e.*, at 24, 48 and 72 hours), the identity for each of the five bars (from left to right) is as follows: serum starvation (SS), VEGF, and pGlcNAc (NAG) at 50, 100, and 250 μ g/ml.

[0058] **Figure 18.** sNAG induced marked increase in metabolic rate. Identity for each of the five bars (from left to right) is as follows: serum starvation (SS), VEGF, and sNAG at 50, 100 and 200 μ g/ml.

[0059] **Figure 19.** sNAG did not protect EC from cell death induced by serum deprivation. For each time period (i.e., at 24 and 48 hours), the identity for each of the five bars (from left to right) is as follows: serum starvation (SS), VEGF, and sNAG at 50, 100 and 200 µg/ml.

5. DETAILED DESCRIPTION

[0060] The inventors have discovered that sNAG nanofibers decrease bacterial infection of cutaneous wounds infected with *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Without being bound by any specific mechanism of action, data presented in Section 6.2 suggests that the antibacterial effect of sNAG is not due to a direct interaction of sNAG with the bacteria but is due to downstream affects such as, for example, the regulation of defensins by Akt1 activation. Specifically, data show that treatment of bacterial cultures with sNAG nanofibers *in vitro* does not affect bacterial count indicating that sNAG nanofibers do not directly inhibit bacterial growth. In a specific example described in Section 6.2.2.5 and illustrated in Figure 11E, sNAG nanofibers do not have a direct effect on growth or survival of *Staphylococcus aureus*. The test described in Section 6.2.2.5, *infra*, may be used to test the lack of a direct effect of sNAG nanofibers on bacterial growth or survival. In this example, *S. aureus* cultures in solution were treated with varying concentrations of sNAG nanofibers for three hours, cultures were then plated overnight at 37°C and bacterial CFU/ml determined. As shown in Figure 11E, no effect on bacterial growth or survival was observed.

[0061] The inventors of the present invention have found that sNAG nanofibers can stimulate expression of defensins, which may boost the innate anti-bacterial response. It is widely accepted that defensins are important players in innate immunity and function in anti-bacterial activities. As demonstrated in the examples presented in Sections 6.1 and 6.2, *infra*, the inventors of the present invention have found that sNAG nanofibers can increase the expression of both α- and β-type defensins in endothelial cells and β-type defensins in keratinocytes *in vitro* and in a wound healing model *in vivo*.

[0062] Further, as demonstrated in the examples presented in Sections 6.1 and 6.2, *infra*, but without being bound by any specific mechanism of action, Akt1 appears to be important for sNAG-dependent defensin expression *in vitro* and *in vivo*, in a wound healing model. Consistently, sNAG treatment decreased bacterial infection of cutaneous wounds infected with *Staphylococcus aureus* in wild type control animals but not in similarly treated Akt1 null animals.

[0063] The inventors of this invention have also found that a number of Toll-like receptors can be up-regulated by sNAG treatment of human endothelial cells. Toll-like receptors ("TLRs" or "TLR") are highly conserved receptors that recognize specific molecular patterns of bacterial components leading to activation of innate immunity. Recent work has linked human defensin expression to TLR activation. In particular, stimulation of TLRs can lead to increased defensin synthesis. Thus, without being bound by any mechanism of action, sNAG nanofibers may act as a stimulator of innate immunity and bacterial clearance via the activation of Akt1.

[0064] Accordingly, described herein is the use of sNAG nanofibers as a novel method for preventing and/or treating bacterial infections and diseases associated therewith. In certain embodiments, treatment of bacterial infections with sNAG nanofibers decreases the bacterial load in patients. In specific embodiments, the use of sNAG nanofibers enhances wound closure while simultaneously eradicating, decreasing or preventing bacterial infection of the wound.

5.1 sNAG Nanofibers

[0065] Described herein are sNAG nanofiber compositions. The sNAG nanofibers comprise fibers of poly-N-acetylglucosamine and/or a derivative(s) thereof, the majority of which are less than 30 microns in length and at least 1 micron in length as measured by any method known to one skilled in the art, for example, by scanning electron microscopy ("SEM"). Such sNAG nanofibers may be obtained, for example, as described herein.

[0066] In certain embodiments, the majority (and in certain embodiments, at least 60%, 70%, 80%, 90%, 95%, 98%, 99%, 99.5%, 99.8%, 99.9%, or 100%, or between 55% to 65%, 55% to 75%, 65% to 75%, 75% to 85%, 75% to 90%, 80% to 95%, 90% to 95%, or 95% to 99%) of the sNAG nanofibers are less than about 30, 25, 20, 15, 12, 10, 9, 8, 7, 6, 5, 4, or 3 microns in length, and at least 1 micron in length as measured by any method known to one skilled in the art, for example, by SEM. In specific embodiments, the majority (and in certain embodiments, at least 60%, 70%, 80%, 90%, 95%, 98%, 99%, 99.5%, 99.8%, 99.9%, or 100%, or between 55% to 65%, 55% to 75%, 65% to 75%, 75% to 85%, 75% to 90%, 80% to 95%, 90% to 95%, or 95% to 99%) of the sNAG nanofibers are less than about 15 microns or less than about 12 microns in length, and at least 1 micron in length as measured by any method known to one skilled in the art, for example, by SEM. In specific embodiments, all (100%) of the sNAG nanofibers are less than about 15 microns in length, and at least 1 micron in length as measured by any method known to one skilled in the art, for example, by SEM. In certain embodiments,

the majority (and in certain embodiments, at least 60%, 70%, 80%, 90%, 95%, 98%, 99%, 99.5%, 99.8%, 99.9%, or 100%, or between 55% to 65%, 55% to 75%, 65% to 75%, 75% to 85%, 75% to 90%, 80% to 95%, 90% to 95%, or 95% to 99%) of the sNAG nanofibers are equal to or less than 14, 13, 12, 11, 10, 9, 8 or 7 microns in length, and at least 1 micron in length as measured by any method known to one skilled in the art, for example, by SEM. In some embodiments, the majority (and in certain embodiments, at least 60%, 70%, 80%, 90%, 95%, 98%, 99%, 99.5%, 99.8%, 99.9%, or 100%, or between 55% to 65%, 55% to 75%, 65% to 75%, 75% to 85%, 75% to 90%, 80% to 95%, 90% to 95%, or 95% to 99%) of the sNAG nanofibers are between 1 to 15, 2 to 15, 2 to 14, 1 to 12, 2 to 12, 1 to 10, 2 to 10, 3 to 12, 3 to 10, 1 to 9, 2 to 9, 3 to 9, 1 to 8, 2 to 8, 3 to 8, 4 to 8, 1 to 7, 2 to 7, 3 to 7, 4 to 7, 1 to 6, 1 to 5, 1 to 4, or 1 to 3 microns in length as measured by any method known to one skilled in the art, for example, by SEM.

[0067] In a specific embodiment, the majority (and in certain embodiments, at least 60%, 70%, 80%, 90%, 95%, 98%, 99%, 99.5%, 99.8%, 99.9%, or 100%, or between 55% to 65%, 55% to 75%, 65% to 75%, 75% to 85%, 75% to 90%, 80% to 95%, 90% to 95%, or 95% to 99%) of the sNAG nanofibers are about 8, 7, 6, 5, 4, 3 or 2 microns in length as measured by any method known to one skilled in the art, for example, by SEM. In another specific embodiment, the majority (and in certain embodiments, at least 60%, 70%, 80%, 90%, 95%, 98%, 99%, 99.5%, 99.8%, 99.9%, or 100%, or between 55% to 65%, 55% to 75%, 65% to 75%, 75% to 85%, 75% to 90%, 80% to 95%, 90% to 95%, or 95% to 99%) of the sNAG nanofibers are between about 2 to about 10 microns, about 3 to about 8 microns, or about 4 to about 7 microns in length as measured by any method known to one skilled in the art, for example, by SEM. In another specific embodiment, all (100%) of the sNAG nanofibers are between about 2 to about 10 microns, about 3 to about 8 microns, or about 4 to about 7 microns in length as measured by any method known to one skilled in the art, for example, by SEM.

[0068] In certain embodiments, the sNAG nanofibers fibers are in a range between 0.005 to 5 microns in thickness and/or diameter as determined by electron microscopy. In specific embodiments, the sNAG nanofibers are about 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, 0.1, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45, 0.5, 0.55, 0.6, 0.65, 0.7, 0.75, 0.8, 0.85, 0.9, 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2, 2.2, 2.4, 2.6, 2.8, 3 or 4 microns in thickness and/or diameter on average, or any range in between (e.g., 0.02 to 2 microns, 0.02 to 1 microns, 0.02 to 0.75

microns, 0.02 to 0.5 microns, 0.02 to 0.5 microns, 0.05 to 1 microns, 0.05 to 0.75 microns, 0.05 to 0.5 microns, 0.1 to 1 microns, 0.1 to 0.75 microns, 0.1 to 0.5 microns, etc.). In specific embodiments, the majority (and in certain embodiments, at least 60%, 70%, 80%, 90%, 95%, 98%, 99%, 99.5%, 99.8%, 99.9%, or 100%, or between 55% to 65%, 55% to 75%, 65% to 75%, 75% to 85%, 75% to 90%, 80% to 95%, 90% to 95%, or 95% to 99%) of the sNAG nanofibers have a thickness or diameter of about 0.02 to 1 microns. In other specific embodiments, the majority (and in certain embodiments, at least 60%, 70%, 80%, 90%, 95%, 98%, 99%, 99.5%, 99.8%, 99.9%, or 100%, or between 55% to 65%, 55% to 75%, 65% to 75%, 75% to 85%, 75% to 90%, 80% to 95%, 90% to 95%, or 95% to 99%) of the sNAG nanofibers have a thickness or diameter of about 0.05 to 0.5 microns. In specific embodiments, all (100%) of the sNAG nanofibers have a thickness or diameter of about 0.02 to 1 microns or about 0.05 to 0.5 microns. In certain embodiments, the majority (and in certain embodiments, at least 60%, 70%, 80%, 90%, 95%, 98%, 99%, 99.5%, 99.8%, 99.9%, or 100%, or between 55% to 65%, 55% to 75%, 65% to 75%, 75% to 85%, 75% to 90%, 80% to 95%, 90% to 95%, or 95% to 99%) of the sNAG nanofibers have a thickness or diameter of about 0.02 to 2 microns, 0.02 to 1 microns, 0.02 to 0.75 microns, 0.02 to 0.5 microns, 0.02 to 0.5 microns, 0.05 to 1 microns, 0.05 to 0.75 microns, 0.05 to 0.5 microns, 0.1 to 1 microns, 0.1 to 0.75 microns, or 0.1 to 0.5 microns.

[0069] In certain embodiments, the majority (and in certain embodiments, at least 60%, 70%, 80%, 90%, 95%, 98%, 99%, 99.5%, 99.8%, 99.9%, or 100%, or between 55% to 65%, 55% to 75%, 65% to 75%, 75% to 85%, 75% to 90%, 80% to 95%, 90% to 95%, or 95% to 99%) of the sNAG nanofibers are between 1 and 15 microns in length and have a thickness or diameter of about 0.02 to 1 microns.

[0070] In certain embodiments, the molecular weight of the sNAG nanofibers is less than 100kDa, 90kDa, 80kDa, 75kDa, 70 kDa, 65 kDa, 60kDa, 55kDa, 50kDa, 45 kDA, 40kDa, 35kDa, 30kDa, or 25kDa. In certain embodiments, the majority (and in certain embodiments, at least 60%, 70%, 80%, 90%, 95%, 98%, 99%, 99.5%, 99.8%, 99.9%, or 100%, or between 55% to 65%, 55% to 75%, 65% to 75%, 75% to 85%, 75% to 90%, 80% to 95%, 90% to 95%, or 95% to 99%) of the sNAG nanofibers have a molecular weight of less than 100kDa, 90kDa, 80kDa, 75kDa, 70 kDa, 65 kDa, 60kDa, 55kDa, 50kDa, 45 kDA, 40kDa, 35kDa, 30kDa, or 25kDa. In other embodiments, the majority (and in certain embodiments, at least 60%, 70%, 80%, 90%, 95%, 98%, 99%, 99.5%, 99.8%, 99.9%, or 100%, or between 55% to 65%, 55% to 75%, 65% to

75%, 75% to 85%, 75% to 90%, 80% to 95%, 90% to 95%, or 95% to 99%) of the sNAG nanofibers have a molecular weight between about 5kDa to 100kDa, about 10kDa to 100kDa, about 20kDa to 100kDa, about 10kDa to 80kDa, about 20kDa to 80kDa, 20kDa to 75kDa, about 25kDa to about 75kDa, about 30kDa to about 80kDa, about 30kDa to about 75kDa, about 40kDa to about 80kDa, about 40kDa to about 75kDa, about 40kDa to about 70kDa, about 50kDa to about 70kDa, or about 55kDa to about 65kDa. In one embodiment, the majority (and in certain embodiments, at least 60%, 70%, 80%, 90%, 95%, 98%, 99%, 99.5%, 99.8%, 99.9%, or 100%, or between 55% to 65%, 55% to 75%, 65% to 75%, 75% to 85%, 75% to 90%, 80% to 95%, 90% to 95%, or 95% to 99%) of the sNAG nanofibers have a molecular weight of about 60kDa.

[0071] In certain embodiments, 1% to 5%, 5% to 10%, 5% to 15%, 20% to 30% or 25% to 30% of the sNAG nanofibers are deacetylated. In some embodiments, 1%, 5%, 10%, 15%, 20%, 25%, or 30% of the sNAG nanofibers are deacetylated. In other embodiments, less than 30%, 25%, 20%, 15%, 10%, 5%, 4%, 3%, 2% or 1% of the sNAG nanofibers are deacetylated. In some embodiments, equal to or more than 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 99%, or all (100%), of the sNAG nanofibers are deacetylated. In other embodiments, less than 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99%, or 100% of the sNAG nanofibers are deacetylated.

[0072] In certain embodiments, 70% to 80%, 75% to 80%, 75% to 85%, 85% to 95%, 90% to 95%, 90% to 99% or 95% to 100% of the sNAG nanofibers are acetylated. In some embodiments, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99% or 100% of the sNAG nanofibers are acetylated. In other embodiments, more than 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99%, 99.5% or 99.9% of the sNAG nanofibers are acetylated. In some embodiments, equal to or more than 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 99%, or all (100%), of the sNAG nanofibers are acetylated. In other embodiments, less than 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99%, or 100% of the sNAG nanofibers are acetylated.

[0073] In some embodiments, the sNAG nanofibers comprise at least one glucosamine monosaccharide, and may further comprise at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or 99% of the N-acetylglucosamine monosaccharides. In other embodiments, the sNAG nanofibers comprise at least one N-acetylglucosamine monosaccharide, and may

further comprise at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or 99% of glucosamine monosaccharides.

[0074] In one aspect, the sNAG nanofibers increase the metabolic rate of serum-starved human umbilical cord vein endothelial cells ("EC") in a MTT assay. A MTT assay is a laboratory test and a standard colorimetric assay (an assay which measures changes in color) for measuring cellular proliferation (cell growth). Briefly, yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) is reduced to purple formazan in the mitochondria of living cells. This reduction takes place only when mitochondrial reductase enzymes are active, and therefore conversion can be directly related to the number of viable (living) cells. The metabolic rate of cells may be determined by other techniques commonly known to the skilled artisan.

[0075] In another aspect, the sNAG nanofibers do not rescue apoptosis of serum-starved EC in a trypan blue exclusion test. A trypan blue exclusion test is a dye exclusion test used to determine the number of viable cells present in a cell suspension. It is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue, Eosin, or propidium, whereas dead cells do not. The viability of cells may be determined by other techniques commonly known to the skilled artisan.

[0076] In certain embodiments, compositions comprising the sNAG nanofibers are described, wherein the sNAG nanofibers increase the metabolic rate of serum-starved human umbilical cord vein endothelial cells in a MTT assay and/or do not rescue apoptosis of serum-starved human umbilical cord vein endothelial cells in a trypan blue exclusion test. In some embodiments, the sNAG nanofibers increase the metabolic rate of serum-starved human umbilical cord vein endothelial cells in a MTT assay and do not rescue apoptosis of serum-starved human umbilical cord vein endothelial cells in a trypan blue exclusion test.

[0077] In a specific embodiment, the sNAG nanofibers are biocompatible. Biocompatibility may be determined by a variety of techniques, including, but not limited to such procedures as the elution test, intramuscular implantation, or intracutaneous or systemic injection into animal subjects. Such tests are described in U.S. Patent No. 6,686,342 (see, e.g., Example 10), which is incorporated by reference herein in its entirety.

[0078] In certain embodiments, the sNAG nanofibers used in the methods described herein are non-reactive in a biocompatibility test or tests. For example, the sNAG nanofibers used in the methods described herein may be non-reactive when tested in an elution test, an intramuscular implantation test, an intracutaneous test, and/or a systemic test. In other embodiments, the sNAG nanofibers used in the methods described herein have Grade 0 or Grade 1 test score when tested in an elution test, an intramuscular implantation test, an intracutaneous test, or a systemic test. In yet another embodiment, the sNAG nanofibers used in the methods described herein are at most mildly reactive when tested in an elution test, an intramuscular implantation test, an intracutaneous test, and/or a systemic test. In certain embodiments, the compositions described herein do not cause an allergenic reaction or an irritation. In other embodiments, the compositions described herein cause at most a mild allergenic reaction or a mild irritation, e.g., at the site of application. The relevant tests and evaluation of test results are described in, e.g., U.S. Patent No. 6,686,342, which is incorporated herein by reference in its entirety, and in Section 6.8, *infra*.

[0079] In a specific embodiment, the sNAG nanofibers are non-reactive when tested in an intramuscular implantation test. In one aspect, an intramuscular implantation test is an intramuscular implantation test – ISO 4 week implantation, as described in Section 6.8.3, *infra*. In certain embodiments, the sNAG nanofibers display no biological reactivity as determined by an elution test (Elution Test Grade = 0). In some embodiments, the sNAG nanofibers have a test score equal to “0” and/or are at most a negligible irritant as determined by intracutaneous injection test. In some embodiments, the sNAG nanofibers elicit no intradermal reaction (i.e., Grade 1 reaction) in Kligman test and/or have a weak allergenic potential as determined by Kligman test.

[0080] In certain aspects, the sNAG nanofibers are immunoneutral (i.e., they do not elicit an immune response).

[0081] In some embodiments, the sNAG nanofibers are biodegradable. The sNAG nanofibers preferably degrade within about 1 day, 2 days, 3 days, 5 days, 7 days (1 week), 8 days, 10 days, 12 days, 14 days (2 weeks), 17 days, 21 days (3 weeks), 25 days, 28 days (4 weeks), 30 days, 1 month, 35 days, 40 days, 45 days, 50 days, 55 days, 60 days, 2 months, 65 days, 70 days, 75 days, 80 days, 85 days, 90 days, 3 months, 95 days, 100 days or 4 months after administration or implantation into a patient.

[0082] In certain embodiments, the sNAG nanofibers do not cause a detectable foreign body reaction. A foreign body reaction, which may occur during wound healing, includes accumulation of exudate at the site of injury, infiltration of inflammatory cells to debride the area, and the formation of granulation tissue. The persistent presence of a foreign body can inhibit full healing. Rather than the resorption and reconstruction that occurs in wound healing, the foreign body reaction is characterized by the formation of foreign body giant cells, encapsulation of the foreign object, and chronic inflammation. Encapsulation refers to the firm, generally avascular collagen shell deposited around a foreign body, effectively isolating it from the host tissues. In one embodiment, treatment of a site (e.g., a wound or a site of a bacterial infection in a wound) with the sNAG nanofibers does not elicit a detectable foreign body reaction in 1 day, 3 days, 5 days, 7 days, 10 days or 14 days after treatment. In one such embodiment, treatment of a site (e.g., a wound) with the sNAG nanofibers does not elicit a foreign body encapsulations in 1 day, 3 days, 5 days, 7 days, 10 days or 14 days after treatment.

[0083] In some embodiments, the sNAG nanofibers (i) comprise fibers, wherein majority of the fibers are between about 1 and 15 microns in length, and (ii) (a) increase the metabolic rate of serum-starved EC in a MTT assay and/or do not rescue apoptosis of serum-starved EC in a trypan blue exclusion test, and (b) are non-reactive when tested in an intramuscular implantation test. In certain embodiments, the sNAG nanofibers (i) comprise fibers, wherein majority of the fibers are between about 1 and 12 microns in length, and (ii) (a) increase the metabolic rate of serum-starved EC in a MTT assay and/or do not rescue apoptosis of serum-starved EC in a trypan blue exclusion test, and (b) are non-reactive when tested in an intramuscular implantation test. In certain embodiments, the sNAG nanofibers (i) comprise fibers, wherein majority of the fibers are between about 4 and 7 microns in length, and (ii) (a) increase the metabolic rate of serum-starved EC in a MTT assay and/or do not rescue apoptosis of serum-starved EC in a trypan blue exclusion test, and (b) are non-reactive when tested in an intramuscular implantation test.

[0084] In certain embodiments, the sNAG nanofibers do not have a direct effect on the growth or survival of bacteria, such as *S. aureus*, as determined by one skilled in the art. In other embodiments, sNAG nanofibers do not have a direct effect on the growth or survival of bacteria, such as *S. aureus*, as determined by the methods set forth in Section 6.2.2.5, *infra*. In some embodiments, the sNAG nanofibers do not have a direct effect *in vitro* on bacterial growth or

survival. In one embodiment, the sNAG nanofibers do not have a direct effect (e.g., *in vitro*) on growth or survival of gram-negative bacteria. In another embodiment, the sNAG nanofibers do not have a direct effect (e.g., *in vitro*) on growth or survival of gram-positive bacteria. In yet another embodiment, the sNAG nanofibers do not have a direct effect (e.g., *in vitro*) on growth or survival of either gram-positive or gram-negative bacteria. In some embodiments, the sNAG nanofiber or a sNAG nanofiber composition does not bind bacteria (e.g., gram-positive bacteria, gram-negative bacteria, or both types of bacteria). In some embodiments, incubation of a bacterial culture with the sNAG nanofibers (e.g., 50-500 µg of sNAG nanofibers) *in vitro* does not reduce bacterial load in 10 minutes, 30 minutes, 1 hour, 2 hours, 3 hours, 6 hours, 12 hours, 24 hours, 48 hours, 72 hours or 96 hours of incubation (wherein the bacterial culture may be gram-positive and/or a gram-negative). In some embodiments, incubation of a *S. aureus* culture with sNAG nanofibers (e.g., about 80µg-300µg, or about 100-200 µg of sNAG naofibers) *in vitro* does not reduce bacterial load in 2 hours, 3 hours, 6 hours or 24 hours of incubation. In yet other embodiments, the sNAG nanofibers reduce bacterial growth or survival *in vitro* by less than 1 log, 0.9 log, 0.8 log, 0.75 log, 0.7 log, 0.6 log, 0.5 log, 0.4 log, 0.3 log, 0.25 log, 0.2 log, 0.1 log, 0.05 log, or 0.025 log, for example, when *Staphylococcus aureus* bacterial cultures are treated/incubated with the sNAG nanofibers *in vitro*. In some embodiments, the sNAG nanofibers reduce bacterial growth or survival *in vitro* by less than 1×10^4 , 2×10^4 , 3×10^4 , 4×10^4 , 5×10^4 , 6×10^4 , 7×10^4 , 8×10^4 , 9×10^4 , or 10×10^4 cfu/ml, for example, when *Staphylococcus aureus* bacterial cultures are treated/incubated with the sNAG nanofibers *in vitro*. The tests of the effect of sNAG nanofibers on bacterial growth or survival and the evaluation of the test results are described, for example, in Example 2 (e.g., Section 6.2.2.5) and Figure 11E, *infra*.

[0085] In some embodiments, the sNAG nanofibers (i) comprise fibers, wherein majority of the fibers are between about 1 and 15 microns, 1 and 12 microns, or 4 and 7 microns in length, (ii) do not have an effect on bacterial growth or survival of *Staphylococcus aureus* bacterial cultures *in vitro*, and (iii) are non-reactive when tested in a biocompatibility test (e.g., an intramuscular implantation test).

[0086] In certain embodiments, the sNAG nanofibers induce a certain pattern of gene expression (RNA or protein expression as determined by, e.g., RT-PCR, microarray or ELISA) in a cell, tissue or organ treated with or exposed to a sNAG nanofiber composition. Specifically,

in some embodiments, the sNAG nanofibers or a composition comprising the sNAG nanofibers induce expression of one or more defensin proteins, one or more defensin-like proteins, and/or one or more Toll-like receptors. In yet other embodiments, the sNAG nanofibers or a composition comprising the sNAG nanofibers induce expression of one or more proteins that are known to have an anti-bacterial effect.

[0087] In certain embodiments, the sNAG nanofibers or a composition comprising the sNAG nanofibers induce expression of one or more α -defensins (e.g., DEFA1 (i.e., α -defensin 1), DEFA1B, DEFA3, DEFA4, DEFAS, DEFA6), one or more β -defensins (e.g., DEFB1 (i.e., β -defensin 1), DEFB2, DEFB4, DEFB103A, DEFB104A, DEFB105B, DEFB107B, DEFB108B, DEFB110, DEFB112, DEFB114, DEFB118, DEFB119, DEFB123, DEFB124, DEFB125, DEFB126, DEFB127, DEFB128, DEFB129, DEFB131, DEFB136), and/or one or more θ -defensins (e.g., DEFT1P). In some embodiments, the sNAG nanofibers or a composition comprising the sNAG nanofibers induce expression of one or more of DEFA1, DEFA3, DEFA4, DEFAS, DEFB1, DEFB3, DEFB103A, DEFB104A, DEFB108B, DEFB112, DEFB114, DEFB118, DEFB119, DEFB123, DEFB124, DEFB125, DEFB126, DEFB128, DEFB129 and DEFB131. In certain embodiments, the sNAG nanofibers or a composition comprising the sNAG nanofibers induce expression of one or more Toll receptors (e.g., TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10, TLR11, and/or TLR12). In other embodiments, the sNAG nanofibers or a composition comprising the sNAG nanofibers induce expression of one or more of IL-1, CEACAM3, SPAG11, SIGIRR (IL1-like receptor), IRAK1, IRAK2, IRAK4, TBK1, TRAF6 and IKK1. In some embodiments, the sNAG nanofibers or a composition comprising the sNAG nanofibers induce expression of one or more of IRAK2, SIGIRR, TLR1, TLR2, TLR4, TLR7, TLR8, TLR10 and TRAF6. In one embodiment, the sNAG nanofibers or a composition comprising the sNAG nanofibers induce expression of at least one of the above-listed gene products.

[0088] In some embodiments, the sNAG nanofibers or a composition comprising the sNAG nanofibers induce expression of one or more of the above-listed genes in the amount equal to or more than about 0.25 fold, 0.5 fold, 1 fold, 1.5 fold, 2 fold, 2.5 fold, 3 fold, 3.5 fold, 4 fold, 4.5 fold, 5 fold, 6 fold, 7 fold, 8 fold, 9 fold, 10 fold, 12 fold, 15 fold or 20 fold as compared to the level of expression of the one or more of the above-listed genes in a cell, tissue or organ of a subject before treatment with the sNAG nanofibers (e.g., a known average level of expression of

the one or more of the above-listed genes). In some embodiments, the sNAG nanofibers or a composition comprising the sNAG nanofibers induce expression of one or more of the above-listed genes in the amount equal to or more than about 10%, 25%, 50%, 75%, 100%, 125%, 150%, 175%, 200%, 225%, 250%, 275%, 300%, 350%, 400%, 450%, 500%, 550%, 600%, 650%, 700%, 750%, 800%, 900% or 1000% the level of expression of the one or more of the above-listed genes in a cell, tissue or organ of a subject before treatment with the sNAG nanofibers (e.g., a known average level of expression of the one or more of the above-listed genes).

[0089] In some embodiments, the sNAG nanofibers but not long poly-N-acetylglucosamine, chitin and/or chitosan induce expression of the one or more genes listed above, as determined by a method known to one skilled in the art, or described herein. In some of these embodiments, long poly-N-acetylglucosamine, chitin and/or chitosan do not induce expression of the one or more genes listed above or induce lower level (e.g., more than 1.25 fold, 1.5 fold, 2 fold, 2.5 fold, 3 fold, 3.5 fold, 4 fold, 4.5 fold, 5 fold, 6 fold, 7 fold, 8 fold, 9 fold, or 10 fold lower) of expression of the one or more genes listed above as compared to the level of expression of the one or more genes listed above induced by the sNAG nanofibers, as determined by a method known to one skilled in the art, or described herein.

[0090] In certain embodiments, the sNAG nanofibers or a composition comprising the sNAG nanofibers induce a gene expression profile that is consistent with, similar to, about the same as, or equivalent to one or more gene expression profiles demonstrated in Tables I, II, III, V, VIII and IX, Sections 6.2-6.5, *infra*. In some embodiments, the sNAG nanofibers or a composition comprising the sNAG nanofibers induce expression of one or more of the genes shown to be upregulated by sNAG treatment in Tables I, II, III, V, VIII and IX, Sections 6.2-6.5, *infra*. In some embodiments, the sNAG nanofibers or a composition comprising the sNAG nanofibers induce expression of the majority or all of the genes shown to be upregulated by sNAG treatment in Tables I, II, III, V, VIII and IX, Sections 6.2-6.5, *infra*. In some of these embodiments, gene expression levels are measured at 1 hour, 2 hours, 4 hours, 5 hours, 6 hours, 8 hours, 10 hours, 12 hours, 14 hours, 16 hours, 18 hours, 20 hours, 24 hours, 48 hours, 3 days or 5 days after treatment of a cell, tissue or organ with a sNAG nanofiber composition by a method known to one skilled in the art, or described herein.

[0091] In certain embodiments, the sNAG nanofibers or a composition comprising the sNAG nanofibers induce a gene expression profile that differs from the profile induced by long poly-N-acetylglucosamine polymers or fibers. In specific embodiments, a gene expression profile induced by the sNAG nanofibers is consistent with, similar to, about the same as, or equivalent to that shown in Tables I, II, III, V, VIII and IX, Sections 6.2-6.5, *infra*, whereas gene expression profile induced by long poly-N-acetylglucosamine polymers or fibers is consistent with, similar to, about the same with, or equivalent to that shown in Table VIII and/or IX, Section 6.5, *infra*. In other embodiments, the sNAG nanofibers or a composition comprising the sNAG nanofibers induce a gene expression profile that differs from the gene expression profile induced by chitin or chitosan.

[0092] In a specific embodiment, the sNAG nanofibers are obtained by irradiating poly-N-acetylglucosamine and/or a derivative thereof. See Section 5.1.1, *infra*, regarding poly-N-acetylglucosamine and derivatives thereof and Section 5.2, *infra*, regarding methods for producing the sNAG nanofibers using irradiation. Irradiation may be used to reduce the length of poly-N-acetylglucosamine fibers and/or poly-N-acetylglucosamine derivative fibers to form shortened poly- β -1 \rightarrow 4-N-acetylglucosamine fibers and/or shortened poly-N-acetylglucosamine derivative fibers, i.e. sNAG nanofibers. Specifically, irradiation may be used to reduce the length and molecular weight of poly-N-acetylglucosamine or a derivative thereof without disturbing its microstructure. The infrared spectrum (IR) of sNAG nanofibers is similar to, about the same as, or equivalent to that of the non-irradiated poly- β -1 \rightarrow 4-N-acetylglucosamine or a derivative thereof.

[0093] In one embodiment, the sNAG nanofibers are not derived from chitin or chitosan. Whereas in another embodiment, the compositions described herein may be derived from chitin or chitosan, or the sNAG nanofibers may be derived from chitin or chitosan.

5.1.1 Poly-N-Acetylglucosamine and Derivatives Thereof

[0094] U.S. Patent Nos. 5,622,834; 5,623,064; 5,624,679; 5,686,115; 5,858,350; 6,599,720; 6,686,342; 7,115,588 and U.S. Patent Pub. 2009/0117175 (each of which is incorporated herein by reference) describe the poly-N-acetylglucosamine and derivatives thereof, and methods of producing the same. In some embodiments, the poly-N-acetylglucosamine has a β -1 \rightarrow 4 configuration. In other embodiments, the poly-N-acetylglucosamine has a α -1 \rightarrow 4 configuration.

The poly-N-acetylglucosamine and derivatives thereof may be in the form of a polymer or in the form of a fiber.

[0095] Poly-N-acetylglucosamine can, for example, be produced by, and may be purified from, microalgae, preferably diatoms. The diatoms which may be used as starting sources for the production of the poly-N-acetylglucosamine include, but are not limited to members of the *Coscinodiscus* genus, the *Cyclotella* genus, and the *Thalassiosira* genus. Poly-N-acetylglucosamine may be obtained from diatom cultures via a number of different methods, including the mechanical force method and chemical/biological method known in the art (see, e.g., U.S. Patent Nos. 5,622,834; 5,623,064; 5,624,679; 5,686,115; 5,858,350; 6,599,720; 6,686,342; and 7,115,588, each of which is incorporated herein by reference in its entirety). In certain embodiments, the poly-N-acetylglucosamine is not derived from one or more of the following: a shell fish, a crustacean, an insect, a fungi or yeasts.

[0096] In one embodiment, poly- β -1 \rightarrow 4-N-acetylglucosamine is derived from a process comprising a) treating a microalgae comprising a cell body and a poly- β -1 \rightarrow 4-N-acetylglucosamine polymer fiber with a biological agent (such as hydrofluoric) capable of separating the N-acetylglucosamine polymer fiber from the cell body for a sufficient time so that the poly- β -1 \rightarrow 4-N-acetylglucosamine polymer fiber is released from the cell body; b) segregating the poly- β -1 \rightarrow 4-N-acetylglucosamine polymer fiber from the cell body; and c) removing contaminants from the segregated poly- β -1 \rightarrow 4-N-acetylglucosamine polymer fiber, so that the poly- β -1 \rightarrow 4-N-acetylglucosamine polymer is isolated and purified.

[0097] In other embodiments, the poly- β -1 \rightarrow 4-N-acetylglucosamine may be derived from one or more of the following: a shell fish, a crustacean, an insect, a fungi or yeasts. In certain embodiments, the compositions described herein do not comprise chitin or chitosan.

[0098] One or more of the monosaccharide units of the poly-N-acetylglucosamine may be deacetylated. In certain embodiments, 1% to 5%, 5% to 10%, 5% to 15%, 20% to 30% or 25% to 30% of the poly-N-acetylglucosamine is deacetylated. In some embodiments, 1%, 5%, 10%, 15%, 20%, 25%, or 30% of the poly-N-acetylglucosamine is deacetylated. In other embodiments, less than 30%, 25%, 20%, 15%, 10%, 5%, 4%, 3%, 2% or 1% of the poly-N-acetylglucosamine is deacetylated. In some embodiments, equal to or more than 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%

95% or 99%, or all (100%), of the poly-N-acetylglucosamine is deacetylated. In other embodiments, less than 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99%, or 100% of the poly-N-acetylglucosamine is deacetylated.

[0099] In certain embodiments, a poly-N-acetylglucosamine composition comprises 70% to 80%, 75% to 80%, 75% to 85%, 85% to 95%, 90% to 95%, 90% to 99% or 95% to 100% of acetylated glucosamine (i.e., N-acetylglucosamine) monosaccharides. In some embodiments, a poly-N-acetylglucosamine composition comprises 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99% or 100% of acetylated glucosamine (i.e., N-acetylglucosamine) monosaccharides. In other embodiments, a poly-N-acetylglucosamine composition comprises more than 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99%, 99.5% or 99.9% of the acetylated glucosamine. In some embodiments, a poly-N-acetylglucosamine composition comprises equal to or more than 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 99%, or all (100%), of the acetylated glucosamine. In other embodiments, a poly-N-acetylglucosamine composition comprises less than 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99%, or 100% of the acetylated glucosamine.

[0100] In some embodiments, a poly-N-acetylglucosamine composition comprises at least one glucosamine monosaccharide, and may further comprise at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or 99% of N-acetylglucosamine monosaccharides. In other embodiments, a poly-N-acetylglucosamine composition comprises at least one N-acetylglucosamine monosaccharide, and may further comprise at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or 99% of glucosamine monosaccharides.

[0101] Derivatives of poly-N-acetylglucosamine may also be used in a composition described herein. Derivatives of poly-N-acetylglucosamine and methods of making such derivatives are described in U.S. Patent No. 5,623,064 (see, e.g., Section 5.4), which is incorporated by reference herein in its entirety. Derivatives of poly-N-acetylglucosamine may include, but are not limited to, partially or completely deacetylated poly-N-acetylglucosamine, or its deacetylated derivatives. Further, poly-N-acetylglucosamine may be derivatized by being sulfated, phosphorylated and/or nitrated. Poly-N-acetylglucosamine derivatives include, e.g., sulfated poly-N-acetylglucosamine derivatives, phosphorylated poly-N-acetylglucosamine

derivatives, or nitrated poly-N-acetylglucosamine derivatives. Additionally, one or more of the monosaccharide units of the poly-N-acetylglucosamine may contain one or more sulfonyl groups one or more O-acyl groups. In addition, one or more of the monosaccharides of the deacetylated poly-N-acetylglucosamine may contain an N-acyl group. One or more of the monosaccharides of the poly-N-acetylglucosamine or of its deacetylated derivative, may contain an O-alkyl group. One or more of the monosaccharide units of the poly-N-acetylglucosamine may be an alkali derivative. One or more of the monosaccharide units of the deacetylated derivative of poly-N-acetylglucosamine may contain an N-alkyl group. One or more of the monosaccharide units of the deacetylated derivative of poly-N-acetylglucosamine may contain at least one deoxyhalogen derivative. One or more of the monosaccharide units of the deacetylated derivative of poly-N-acetylglucosamine may form a salt. One or more of the monosaccharide units of the deacetylated derivative of poly-N-acetylglucosamine may form a metal chelate. In a specific embodiment, the metal is zinc. One or more of the monosaccharide units of the deacetylated derivative of poly-N-acetylglucosamine may contain an N-alkylidene or an N-arylidene group. In one embodiment, the derivative is an acetate derivative. In another embodiment, the derivative is not an acetate derivative. In one embodiment the poly-N-acetylglucosamine or deacetylated poly-N-acetylglucosamine is derivatized with lactic acid. Wherein, in another embodiment, the derivative is not derivatized with lactic acid.

5.2 Methods of Making sNAG Nanofibers

[00102] The poly-N-acetylglucosamine polymers or fibers, and any derivatives of poly-N-acetylglucosamine polymers or fibers described above, can be irradiated as dry polymers or fibers or polymer or fiber membranes. Alternatively, poly-N-acetylglucosamine polymers or fibers, and any derivatives of poly-N-acetylglucosamine polymers or fibers described above, can be irradiated when wet. The methods of making sNAG nanofibers by irradiation and the sNAG nanofibers so produced have been described in U.S. Patent Pub. No. 2009/0117175, which is incorporated by reference herein in its entirety.

[00103] In certain embodiments, the poly-N-acetylglucosamine polymers or fibers are formulated into a suspension/slurry or wet cake for irradiation. Irradiation can be performed prior to, concurrently with or following the formulation of the polymers or fibers into its final formulation, such as a dressing. Generally, the polymer or fiber content of suspensions/slurries and wet cakes can vary, for example from about 0.5 mg to about 50 mg of polymer or fiber per 1

ml of distilled water are used for slurries and from about 50 mg to about 1000 mg of polymer or fiber per 1 ml of distilled water are used for wet cake formulations. The polymer or fiber may first be lyophilized, frozen in liquid nitrogen, and pulverized, to make it more susceptible to forming a suspension/slurry or wet cake. Also, the suspensions/slurries can be filtered to remove water such that a wet cake is formed. In certain aspects, the polymer or fiber is irradiated as a suspension comprising about 0.5 mg, 1 mg, 2 mg, 3 mg, 4 mg, 5 mg, 6 mg, 7 mg, 8 mg, 9 mg, 10 mg, 12 mg, 15 mg, 18 mg, 20 mg, 25 mg or 50 mg of polymer or fiber per ml of distilled water, or any range in between the foregoing embodiments (e.g., 1-10 mg/ml, 5-15 mg/ml, 2-8 mg/ml, 20-50 mg/ml, etc.). In other aspects, the polymer or fiber is irradiated as a wet cake, comprising about 50-1,000 mg polymer or fiber per 1 ml of distilled water. In specific embodiments, the wet cake comprises about 50, 100, 200, 300, 400, 500, 600, 700, 800, 900 or 1000 mg of polymer or fiber per 1 ml distilled water, or any range in between (e.g., 100-500 mg/ml, 300-600 mg/ml, 50-1000 mg/ml, etc.).

[00104] The irradiation is preferably in the form of gamma radiation, e-beam radiation, or x-rays. Two sources of irradiation are preferred: radioactive nuclides and electricity. In specific embodiment, the radioactive nuclides are cobalt-60 and cesium-137. Both of these nuclides emit gamma rays, which are photons containing no mass. The gamma rays have energies from 0.66 to 1.3 MeV. Using electricity, electrons are generated and accelerated to energies up to 10 MeV or higher. When irradiating polymers or fibers to reduce their size, a consideration to take into account is that the depth of penetration of materials with densities similar to water by 10 MeV electrons is limited to about 3.7 cm with one-sided exposure or about 8.6 cm with two-sided exposure. Depth of penetration decreases at lower electron energies. Electron energy can be converted to x-rays by placing a metal (usually tungsten or tantalum) target in the electron beam path. Conversion to x-rays is limited to electrons with energies up to 5 MeV. X-rays are photons with no mass and can penetrate polymers or fibers similar to gamma rays. There is only about 8% efficiency in the conversion of electron energy to x-ray energy. High powered electron beam machines are needed in x-ray production facilities to account for the low conversion efficiency.

[00105] In a specific embodiment, the irradiation is gamma irradiation.

[00106] The absorbed dose of radiation is the energy absorbed per unit weight of product, measured in gray (gy) or kilogray (kgy). For dried polymers or fibers, the preferred absorbed

dose is about 500-2,000 kgy of radiation, most preferably about 750-1,250 kgy or about 900-1,100 kgy of radiation. For wet polymers or fibers, the preferred absorbed dose is about 100-500 kgy of radiation, most preferably about 150-250 kgy or about 200-250 kgy of radiation.

[00107] The dose of radiation can be described in terms of its effect on the length of the polymers or fibers. In specific embodiments, the dose of radiation used preferably reduces the length of the polymer or fiber by anywhere from about 10% to 90% of the starting length of the polymer or fiber, respectively. In specific embodiments, the average length is reduced by about 10%, by about 20%, by about 30%, by about 40%, by about 50%, by about 60%, by about 70%, by about 80%, or by about 90%, or any range in between (e.g., 20-40%, 30-70%, and so on and so forth). Alternatively, the dose of radiation used preferably reduces the length of the polymer or fiber to anywhere from 1 to 100 microns. In specific embodiments, and depending on the starting fiber length, the average length of the polymer or fiber is reduced to less than about 15 microns, less than about 14 microns, less than about 13 microns, less than about 12 microns, less than about 11 microns, less than about 10 microns, less than about 8 microns, less than about 7 microns, less than about 5 microns, less than about 4 microns, less than about 3 microns, less than 2 microns, or less than 1 microns. In certain embodiments, the length of the majority (and in certain embodiments, at least 60%, 70%, 80%, 90%, 95%, 98%, 99%, 99.5%, 99.8%, 99.9%, or 100%, or between 55% to 65%, 55% to 75%, 65% to 75%, 75% to 85%, 75% to 90%, 80% to 95%, 90% to 95%, or 95% to 99%) of the polymers or fibers is reduced to no greater than about 20 microns, no greater than about 15 microns, no greater than about 12 microns, no greater than about 10 microns, no greater than about 8 microns, no greater than about 7 microns, or no greater than about 5 microns. In certain embodiments, irradiation of the polymers or fibers reduces the length of the majority (and in certain embodiments, at least 60%, 70%, 80%, 90%, 95%, 98%, 99%, 99.5%, 99.8%, 99.9%, or 100%, or between 55% to 65%, 55% to 75%, 65% to 75%, 75% to 85%, 75% to 90%, 80% to 95%, 90% to 95%, or 95% to 99%) of the fibers to anywhere between about 1 to 20 microns, between about 1 to 15 microns, between about 2 to 15 microns, between about 1 to 12 microns, between about 2 to 12 microns, between about 1 to 10 microns, between about 2 to 10 microns, between about 1 to 8 microns, between about 2 to 8 microns, between about 1 to 7 microns, between about 2 to 7 microns, between about 3 to 8 microns, between about 4 to 7 microns, between about 1 to 5 microns, between about 2 to 5 microns,

between about 3 to 5 microns, between about 4 to 10 microns, or any ranges between the foregoing lengths, which are also encompassed.

[00108] The dose of radiation can also be described in terms of its effect on the molecular weight of the polymer or fiber. In specific embodiments, the dose of radiation used preferably reduces the molecular weight of the polymer or fiber by anywhere from about 10% to 90% of the starting weight of the polymer or fiber. In specific embodiments, the average molecular weight is reduced by about 10%, by about 20%, by about 30%, by about 40%, by about 50%, by about 60%, by about 70%, by about 80%, or by about 90%, or any range in between (e.g., 20-40%, 30-70%, and so on and so forth). Alternatively, the dose of radiation used preferably reduces the molecular weight of the polymer or fiber to anywhere from 1,000 to 1,000,000 daltons. In specific embodiments, and depending on the starting molecular weight, the average molecular weight of the polymer or fiber is reduced to less than 1,000,000 daltons, less than 750,000 daltons, less than 500,000 daltons, less than 300,000 daltons, less than 200,000 daltons, less than 100,000 daltons, less than 90,000 daltons, less than 80,000 daltons, less than 70,000 daltons, less than 60,000 daltons, less than 50,000 daltons, less than 25,000 daltons, less than 10,000 daltons, or less than 5,000 daltons. In certain embodiments, the average molecular weight is reduced to no less than 500 daltons, no less than 1,000 daltons, no less than 2,000 daltons, no less 3,500 daltons, no less than 5,000 daltons, no less than 7,500 daltons, no less than 10,000 daltons, no less than 25,000 daltons, no less than 50,000 daltons, no less than 60,000 daltons or no less than 100,000 daltons. Any ranges between the foregoing average molecular weights are also encompassed; for example, in certain embodiments, irradiation of the polymer or fiber reduces the average molecular weight to anywhere between 10,000 to 100,000 daltons, between 1,000 and 25,000 daltons, between 50,000 and 500,000 daltons, between 25,000 and 100,000 daltons, between 30,000 and 90,000 daltons, between about 40,000 and 80,000 daltons, between about 25,000 and 75,000 daltons, between about 50,000 and 70,000 daltons, or between about 55,000 and 65,000 daltons and so on and so forth. In certain embodiments, irradiation of the polymers or fibers reduces the molecular weight of the majority and in certain embodiments, at least 60%, 70%, 80%, 90%, 95%, 98%, 99%, 99.5%, 99.8%, 99.9%, or 100%, or between 55% to 65%, 55% to 75%, 65% to 75%, 75% to 85%, 75% to 90%, 80% to 95%, 90% to 95%, or 95% to 99%) of the fibers to anywhere between about 20,000 and 100,000 daltons, about 25,000 and 75,000 daltons, about 30,000 and 90,000 daltons, about 40,000 and 80,000 daltons, about 50,000

and 70,000 daltons, or about 55,000 and 65,000 daltons. In certain embodiments, irradiation of the polymers or fibers reduces the molecular weight of the majority and in certain embodiments, at least 60%, 70%, 80%, 90%, 95%, 98%, 99%, 99.5%, 99.8%, 99.9%, or 100%, or between 55% to 65%, 55% to 75%, 65% to 75%, 75% to 85%, 75% to 90%, 80% to 95%, 90% to 95%, or 95% to 99% of the fibers to about 60,000 daltons.

[00109] Following irradiation, slurries can be filtered and dried, and wet cakes can be dried, to form compositions (e.g., dressings and other compositions described herein) that are useful in the practice of the invention.

5.3 Compositions Comprising sNAG Nanofibers

[00110] The sNAG nanofibers may be formulated in a variety of compositions for topical administration as described herein.

[00111] A composition comprising the sNAG nanofibers may be formulated as a cream, a membrane, a film, a liquid solution, a suspension, a powder, a paste, an ointment, a suppository, a gelatinous composition, an aerosol, a gel, or a spray. In one embodiment, a composition comprising the sNAG nanofibers is formulated as an ultra-thin membrane. In some embodiments, a composition comprising the sNAG nanofibers is formulated as a dressing, a mat, or a bandage. Solid formulations suitable for solution in, or suspension in, liquids prior to administration are also contemplated. It is also possible that such compositions are incorporated in or coated on implantable devices, such as orthopedic implants (for hip, knee, shoulder; pins, screws, etc.), cardiovascular implants (stents, catheters, etc.) and the like where the antibacterial activity would be of benefit.

[00112] A composition comprising the sNAG nanofibers may include one or more of pharmaceutically acceptable excipients. Suitable excipients may include water, saline, salt solution, dextrose, glycerol, ethanol and the like, or combinations thereof. Suitable excipients also include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, oil (including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like), talc, sodium chloride, dried skim milk, propylene, glycol and the like. In addition, a composition comprising the sNAG nanofibers may include one or more of wetting agents, emulsifying agents, pH buffering agents, and other agents. The sNAG nanofiber compositions may also be incorporated

in a physiologically acceptable carrier, for example in a physiologically acceptable carrier suitable for topical application. The term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin.

[00113] The final amount of the sNAG nanofibers in a composition may vary. For example, the amount of the sNAG nanofibers in a composition (e.g., prepared for administration to a patient) may be greater than or equal to about 50%, about 60%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 98%, or about 99% weight by volume. In one embodiment, the amount of the sNAG nanofibers in a composition is about 95%, about 98%, about 99, or about 100%. Also, the amount of the sNAG nanofibers in a composition (e.g., prepared for administration to a patient) may be about 50%-100%, about 60%-100%, about 70%-100%, about 75%-100%, about 80%-100%, about 90%-100%, about 95%-100%, about 70%-95%, about 75%-95%, about 80%-95%, about 90%-95%, about 70%-90%, about 75%-90%, or about 80%-90% weight/volume. A composition may comprise more than 30%, 40%, 50%, 60%, 70%, 75%, 80%, 90%, 95% or 99% solution of the sNAG nanofibers.

[00114] A sNAG nanofiber composition may be formulated into a wound dressing. In certain embodiments, a sNAG nanofiber composition is formulated as a wound dressing in the form of a barrier, a membrane, or a film. Alternatively, a sNAG nanofiber composition may be added to dressing backings, such as barriers, membranes, or films. A barrier, membrane, or film can be supplied in a variety of standard sizes, which can be further cut and sized to the area being treated. The backing can be a conventional dressing material, such as a bandage or gauze to which a polymer or fiber is added or coated on, prior to application to the patient. Alternatively, the sNAG nanofibers can be formulated as a barrier, membrane, or film made out of strings, microbeads, microspheres, or microfibrils, or the composition can be formulated as a barrier-forming mat. In certain embodiments, at least 75%, at least 85%, at least 90%, or at least 95% of a dressing is composed of the sNAG nanofibers. In certain aspects, a dressing does not contain a conventional dressing material such as a gauze or bandage. In such embodiments, the sNAG nanofiber itself is formulated as a wound dressing.

[00115] A composition comprising the sNAG nanofibers may further comprise any suitable natural or synthetic polymers or fibers. Examples of suitable polymers or fibers include cellulose polymers, xanthan, polyaramides, polyamides, polyimides, polyamide/imides, polyamidehydrazides, polyhydrazides, polyimidazoles, polybenzoxazoles, polyester/amide, polyester/imide, polycarbonate/amides, polycarbonate/imides, polysulfone/amides, polysulfone imides, and the like, copolymers and blends thereof. Other suitable classes of polymers or fibers include polyvinyledene fluorides and polyacrylonitriles. Examples of these polymers or fibers include those described in U.S. Patent Nos. RE 30,351; 4,705,540, 4,717,393; 4,717,394; 4,912,197; 4,838,900; 4,935,490; 4,851,505; 4,880,442; 4,863,496; 4,961,539; and European Patent Application 0 219 878, all of which are incorporated by reference. The polymers or fibers can include at least one of either of cellulose polymers, polyamides, polyaramides, polyamide/imides or polyimides. In certain embodiments, the polymers or fibers include polyaramides, polyester, urethan and polytetrafluoroethylene. In one embodiment, the compositions described herein comprise more than one type of polymer (e.g., the sNAG nanofiber and cellulose).

[00116] In certain aspects, the sNAG nanofiber is the only active ingredient in a composition.

[00117] In other embodiments, a composition comprises one or more additional active ingredients, e.g., to promote an anti-bacterial effect and/or healing (e.g., wound healing). In some embodiments, the additional active ingredient is one or more anti-bacterial agents (e.g., an antibiotic, a defensin peptide, a defensin-like peptide, or a Toll-receptor-like peptide), or a growth factor. In specific embodiments, the additional active ingredient is a growth factor such as one or more of PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC, PDGF-DD, FGF-1, FGF-2, FGF-5, FGF-7, FGF-10, EGF, TGF- α , (HB-EGF), amphiregulin, epiregulin, betacellulin, neuregulins, epigen, VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, placenta growth factor (PLGF), angiopoietin-1, angiopoietin-2, IGF-I, IGF-II, hepatocyte growth factor (HGF), and macrophage-stimulating protein (MSP). In other embodiments, the additional active ingredient is an agent that boost the immune system, a pain relief agent, or a fever relief agent.

[00118] In certain embodiments, the additional active ingredient is an antibiotic of one of the following classes of antibiotics: macrolides (e.g., erythromycin, azithromycin), aminoglycosides (e.g., amikacin, gentamicin, neomycin, streptomycin), cephalosporins (e.g., cefadroxil, cefaclor, cefotaxime, cefepime), fluoroquinolones (e.g., ciprofloxacin, levofloxacin), penicillins (e.g.,

penicillin, ampicillin, amoxicillin), tetracyclines (e.g., tetracycline, doxycycline), and carbapenems (e.g., meropenem, imipenem). In some specific embodiments, the additional active ingredient is one or more of vancomycin, sulfa drug (e.g., co-trimoxazole/trimethoprim-sulfamethoxazole), tetracycline (e.g., doxycycline, minocycline), clindamycin, oxazolidinones (e.g., linezolid), daptomycin, teicoplanin, quinupristin/dalfopristin (synercid), tigecycline, alicin, bacitracin, nitrofurantoin, hydrogen peroxide, novobiocin, netilmicin, methylglyoxal, bee defensin-1, tobramycin, chlorhexidine digluconate, chlorhexidine gluconate, levofloxacin, zinc, and silver. In some embodiments, a composition comprises the sNAG nanofibers and an additional active ingredient effective to treat or prevent or commonly used to treat or prevent an *S. aureus* infection, MRSA infection, a *Pseudomonas* infection, or a *C. difficile* infection (e.g., an antibiotic effective against or commonly used against such infections).

[00119] A sNAG nanofiber composition may contain collagen, although in certain aspects a sNAG nanofiber composition does not contain collagen.

[00120] In certain embodiments, a sNAG nanofiber composition does not comprise any additional therapy. In certain embodiments, a sNAG nanofiber composition does not comprise any additional anti-bacterial agent, a defensin peptide, a defensin-like peptide, a Toll-receptor-like peptide, or a growth factor. In some embodiments, a sNAG nanofiber composition does not comprise an antibiotic. In yet other embodiments, a sNAG nanofiber composition may comprise an additional therapy (e.g., an antibiotic). In one such embodiment, the additional therapy (e.g., an antibiotic) is not encapsulated, immobilized or formulated in the sNAG nanofibers.

[00121] In other aspects, a sNAG nanofiber composition does not comprise a significant amount of protein material. In specific embodiments, the protein content of a sNAG nanofiber composition is no greater than 0.1%, 0.5% or 1% by weight. In other embodiments, the protein content of the composition is undetectable by Coomassie staining.

[00122] In one embodiment, zinc is also included in a sNAG nanofiber composition. In addition to its antimicrobial properties, zinc also plays a role in wound healing (see Andrews *et al.*, 1999, *Adv Wound Care* 12:137-8). The zinc is preferably added in the form of a salt, such as zinc oxide, zinc sulphate, zinc acetate or zinc gluconate.

5.4 Anti-Bacterial Uses of sNAG Compositions

[00123] A wide variety of bacterial infections and diseases associated therewith may be treated and/or prevented by the administration of the sNAG nanofiber compositions described herein (see, e.g., Sections 5.4.1 and 5.4.2, *infra*). In one embodiment the compositions described herein are bacteriostatic. In another embodiment, the compositions described herein are bactericidal. In an embodiment, the compositions described herein may be used to treat and/or prevent infections by Gram-positive bacteria and/or any diseases associated therewith. In another embodiment, the compositions described herein may be used to treat and/or prevent infections by Gram-negative bacteria and/or any diseases associated therewith. In yet another embodiment, the compositions described herein may be used to treat and/or prevent infections by both Gram-negative bacteria and Gram-positive bacteria and/or any diseases associated therewith.

[00124] Bacterial infections that may be treated and/or prevented using compositions described herein include infections by bacteria of the *Aquaspirillum* family, *Azospirillum* family, *Azotobacteraceae* family, *Bacteroidaceae* family, *Bartonella* species, *Bdellovibrio* family, *Campylobacter* species, *Chlamydia* species (e.g., *Chlamydia pneumoniae*), clostridium, *Enterobacteriaceae* family (e.g., *Citrobacter* species, *Edwardsiella*, *Enterobacter aerogenes*, *Erwinia* species, *Escherichia coli*, *Hafnia* species, *Klebsiella* species, *Morganella* species, *Proteus vulgaris*, *Providencia*, *Salmonella* species, *Serratia marcescens*, and *Shigella flexneri*), *Gardinella* family, *Huemophilus influenzae*, *Halobacteriaceae* family, *Helicobacter* family, *Legionallaceae* family, *Listeria* species, *Methylococcaceae* family, mycobacteria (e.g., *Mycobacterium tuberculosis*), *Neisseriaceae* family, *Oceanospirillum* family, *Pasteurellaceae* family, *Pneumococcus* species, *Pseudomonas* species, *Rhizobiaceae* family, *Spirillum* family, *Spirosomaceae* family, *Staphylococcus* (e.g., methicillin resistant *Staphylococcus aureus* and *Staphylococcus pyrogenes*), *Streptococcus* (e.g., *Streptococcus enteritidis*, *Streptococcus fasciae*, and *Streptococcus pneumoniae*), *Vampirovibr* *Helicobacter* family, and/or *Vampirovibrio* family. In a specific embodiment, diseases caused by or associated with infections by such bacteria may also be prevented and/or treated using the compositions described herein.

[00125] Bacterial infections that may be treated and/or prevented using compositions described herein also include infections by bacteria of the following genuses: *Bordetella*, *Borrelia*, *Brucella*, *Campylobacter*, *Chlamydia* and *Clamidophylia*, *Clostridium*,

Corynebacterium, Enterococcus, Escherichia, Francisella, Haemophilus, Helicobacter, Legionella, Leptospira, Listeria, Mycobacterium, Mycoplasma, Neisseria, Pseudomonas, Rickettsia, Salmonella, Shigella, Staphylococcus, Streptococcus, Treponema, Vibria, and/or Yersinia. In a specific embodiment, diseases caused by or associated with infections by such bacteria may also be prevented and/or treated using the compositions described herein.

[00126] Bacterial infections that may be treated and/or prevented using compositions described herein include infections by bacteria of the following species: *Bacillus anthracis, Bordetella pertussis, Borrelia burgdorferi, Brucella abortus, Brucella canis, Brucella melitensis, Brucella suis, Campylobacter jejuni, Chlamydia pneumonia, Chlamydia trachomatis, Clamidophila psittaci, Clostridium botulinum, Clostridium difficile, Clostridium perfringens, Clostridium tetani, Corynebacterium diphtheriae, Enterococcus faecalis, Enterococcus faecium, Escherichia coli, Francisella tularensis, Haemophilus influenzae, Helicobacter pylori, Legionella pneumophila, Leptospira pneumophila, Leptospira interrogans, Listeria monocytogenes, Mycobacterium leprae, Mycobacterium tuberculosis, Mycoplasma pneumoniae, Neisseria gonorrhoeae, Neisseria meningitidis, Pseudomonas aeruginosa, Proteus mirabilis, Rickettsia rickettsii, Salmonella typhi, Salmonella typhimurium, Shigella sonnei, Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus saprophyticus, Streptococcus agalactiae, Streptococcus pneumonia, Streptococcus pyogenes, Treponema pallidum, Vibrio cholerae, and/or Yersinia pestis.* In a specific embodiment, diseases caused by or associated with infections by such bacteria may also be prevented and/or treated using the compositions described herein.

[00127] In some embodiments, the compositions described herein may be used to treat and/or prevent infections by aerobic bacteria and/or diseases associated therewith. In other embodiments, the compositions described herein may be used to treat and/or prevent infections by anaerobic bacteria and/or diseases associated therewith.

[00128] In certain embodiments, the compositions described herein are used to treat and/or prevent *Pseudomonas aeruginosa* infections. *Pseudomonas* is a gram-negative aerobic bacteria found in soil, water, other moist environments, plants and animals, clinical isolates of which produce the blue-green pigment pyocyanin and a characteristic sweet odor. *Pseudomonas aeruginosa* is known to cause urinary tract infections, pneumonia, respiratory system infections, dermatitis, soft tissue infections, bacteremia, bone and joint infections, gastrointestinal infections

and a variety of systemic infections. It is known to be an important cause of infections, particularly in patients with burns, patients with cystic fibrosis, patients who are immunosuppressed (e.g., AIDS and cancer patients), and in patients who have been hospitalized for longer than 1 week. It is a frequent cause of nosocomial infections such as but not limited to pneumonia, urinary tract infections and bacteremia. Any one or all of these infections may be prevented and/or treated by the compositions described herein.

[00129] In some embodiments, the compositions described herein are used to treat and/or prevent *Staph* infections, and particularly, *Staphylococcus aureus* infections. Use of a sNAG nanofiber composition in this embodiment and other embodiments described herein may preclude the generation of resistant organisms as well as allow for the antibiotic-independent clearance of a bacterial infection.

[00130] In certain embodiments, the compositions described herein may be used to combat bacteria that are resistant to one or more anti-bacterial agents. For example, the compositions described herein may be used to treat bacteria that are resistant to one or more antibiotics, for example resistant to conventional antibiotics such as MRSA (methicillin-resistant *Staphylococcus aureus*), VRSA (Vancomycin-resistant *S. aureus*), VRE (Vancomycin-resistant *Enterococcus*), Penicillin-resistant *Enterococcus*, PRSP (Penicillin-resistant *Streptococcus pneumoniae*), isoniazid/rifampin-resistant *Mycobacterium tuberculosis* and other antibiotic-resistant strains of bacteria (e.g., resistant strains of *E. coli*, *Salmonella*, *Campylobacter*, and *Streptococci*). In one embodiment, the compositions disclosed herein may be used to treat multiple drug resistant bacteria.

[00131] In some specific embodiments, the compositions described herein may be used to treat and/or prevent Methicillin-resistant *Staphylococcus aureus* ("MRSA"; it may also be called multidrug-resistant *Staphylococcus aureus* or oxacillin-resistant *Staphylococcus aureus* ("ORSA")). MRSA is any strain of *Staphylococcus aureus* that has developed resistance to beta-lactam antibiotics, which include but are not limited to the penicillins (penicillin, methicillin, dicloxacillin, nafcillin, oxacillin, etc.) and the cephalexins. Some of the known strains of MRSA include EMRSA15 and EMRSA16 (also known as MRSA252), which are resistant to erythromycin and ciprofloxacin; CC8 (also known as ST8:USA300); ST1:USA400; ST8:USA500; ST59:USA1000; ST93 strains; ST80 strains; and ST59 strains. MRSA is responsible for a number of infections in humans. MRSA is a serious health concern, causing

approximately 50% of health-care associated staph infections. In the U.S., more than 94,000 people develop serious MRSA infection and about 19,000 die from infection each year. Especially prevalent MRSA is in hospitals; where risk factors for MRSA infection include prior antibiotic exposure (e.g., quinolone antibiotics), admission to an intensive care unit, surgery and exposure to an MRSA-colonized patient. Patients with open wounds, immunocompromised patients (due to, e.g., HIV/AIDS, cancer, transplant procedure, severe asthma), young children (e.g., human infant and human toddler), and the elderly (e.g., elderly human) are at high risk of developing an MRSA infection. Higher risk rates for MRSA infection are also observed in injection drug users, persons with diabetes, patients with dermatologic conditions, patients with invasive devices (e.g., intravascular catheters), and health care workers and other people who spend time in confined spaces (prison inmates, soldiers, patients in long-term healthcare facilities, such as nursing homes). *S. aureus* most commonly colonizes the anterior nares (the nostrils), although the rest of the respiratory tract, opened wounds, intravenous catheters and urinary tract are also potential sites for infection. Most of community-associated MRSA infections are localized to the skin and soft tissue. The initial symptoms of MRSA include red bumps that resemble pimples, spider bites or boils that may be accompanied by fever and rashes; the bumps may later develop into pus-filled boils. Common manifestations of community-associated MRSA are skin infections such as necrotizing fasciitis or pyomyositis, necrotizing pneumonia, infective endocarditis, bone or joint infections. Some MRSA leads to sepsis and toxic shock syndrome, which may be due to toxins carried by such strains (e.g., PVL, PSM). MRSA may cause cellulitis. Any of the above-listed or known in the art strains of MRSA, patients diagnosed with MRSA, symptoms of MRSA, patient populations at risk of MRSA and/or diseases associated with MRSA may be treated with the compositions described herein. In some embodiments, the compositions described herein prevent onset or development of one or more of the symptoms of MRSA, or reduce duration and/or severity of one or more of these symptoms (e.g., symptoms described herein).

[00132] The compositions described herein may be used as bactericidal agents to kill or damage unwanted bacteria. For example, the compositions described herein may be used to treat established bacterial infections, prophylactically for the prevention of bacterial infections, or administered topically to areas of a subject that are susceptible to infection or to areas of the

body that are likely sites for bacterial growth (e.g., the gums, open wounds, bed sores, and vaginal or groin areas).

[00133] In certain embodiments, the compositions described herein reduce bacterial growth and/or bacterial survival by more than about 0.1 log, 0.2 log, 0.25 log, 0.3 log, 0.4 log, 0.5 log, 0.6 log, 0.7 log, 0.75 log, 0.8 log, 0.9 log, 1 log, 1.25 log, 1.5 log, 1.75 log, 2 log, 2.25 log, 2.5 log, 2.75 log, 3 log, 3.25 log, 3.5 log, 3.75 log, 4 log, 4.5 log, 5 log, 5.5 log, 6 log, 6.5 log, 7 log, 7.5 log, 8 log, 8.5 log, 9 log, 9.5 log, 10 log, 10.5 log, 11 log, 11.5 log, 12 log, 12.5 log, 13 log, 13.5 log, 14 log, 14.5 log, or 15 log of colony forming units (CFU)/mL. In certain embodiments, the compositions described herein reduce bacterial growth and/or bacterial survival by about 0.2 log to 15 log, 0.2 log to 10 log, 0.2 log to 5 log, 0.5 log to 15 log, 0.5 log to 10 log, 0.5 log to 5 log, 0.5 log to 3 log, 1 log to 15 log, 1 log to 12 log, 1 log to 10 log, 1 log to 7 log, 1 log to 5 log, 1 log to 3 log, 1.5 log to 5 log, 2 log to 15 log, 2 log to 10 log, 2 log to 5 log, 3 log to 15 log, 3 log to 10 log, 3 log to 5 log, 4 log to 10 log, 2 log to 8 log, 3 log to 8 log, 4 log to 8 log, 2 log to 7 log, 3 log to 7 log, 2 log to 6 log of colony forming units (CFU)/mL, and any value in between these values. In certain embodiments, the compositions described herein reduce bacterial growth and/or bacterial survival by equal to or more than 1×10^{10} , 0.5×10^{11} , 1×10^{11} , 1.5×10^{11} , 2×10^{11} , 2.5×10^{11} , 3×10^{11} , 4×10^{11} , 5×10^{11} , 7×10^{11} , 1×10^{12} , 1.5×10^{12} , 2×10^{12} , 3×10^{12} , 5×10^{12} , 7×10^{12} , 8×10^{12} , 1×10^{13} , 1.5×10^{13} , or 2×10^{13} (CFU)/mL, or any range of values in between these values. In some embodiments, such reduction in bacterial growth and/or survival is achieved in less than about 30 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, 15 hours, 18 hours, 20 hours, 22 hours, 24 hours, 36 hours, 48 hours, 60 hours, 72 hours, one day, two days, three days, four days, five days, seven days, ten days, one week, two weeks, three weeks, four weeks, 1 month or 2 months after treatment of a bacterial infection with a single application/dose or multiple application/doses of a sNAG nanofiber composition.

[00134] In one embodiment, the infection to be treated with a sNAG nanofiber composition is not a viral infection, a fungal infection, a parasite infection, or an yeast infection.

[00135] A variety of diseases or disease conditions associated with bacterial infections may be treated and/or prevented with the sNAG nanofiber compositions described herein (see, e.g., Section 5.4.2, *infra*). In one embodiment, methods for treating an existing bacterial infection or a disease associated with a bacterial infection are contemplated.

[00136] In some embodiments, the compositions described herein may be used to treat wounds (see Section 5.4.1, *infra*). In specific embodiments, the compositions described herein are used to treat bacterially infected wounds. In other embodiments, the compositions described herein are used to prevent bacterial infection of wounds. In some embodiments, the compositions described herein are used to treat bacteria known to be associated with wound infections, and specifically used to treat *Staphylococcus aureus*/MRSA, *Streptococcus pyogenes*, *Enterococci* and/or *Pseudomonas aeruginosa* infections, and diseases associated with such infections.

[00137] In other embodiments, the compositions described herein are not used to treat wounds. In one embodiment, the compositions described herein are not used to treat chronic wounds. In another embodiment, the compositions described herein are not used to treat burn wounds. In yet another embodiment, the compositions described herein are not used to treat surgical wounds. In one embodiment, the compositions described herein are not used to treat chronic wounds, burn wounds and surgical wounds. In another embodiment, the compositions described herein are not used to treat a wound and/or a burn. In yet another embodiment, the compositions described herein are not used to treat un-infected wounds.

[00138] In some embodiments, the compositions described herein are not used to treat a bacterially infected wound. In another embodiment, the compositions described herein are not used to treat a bacterial infection associated with or caused by a wound. In some embodiments, the compositions described herein are not used to treat bacteria known to be associated with wound infections such as *Staphylococcus aureus*/MRSA, *Streptococcus pyogenes*, *Enterococci* and/or *Pseudomonas aeruginosa*.

[00139] In some embodiment, the compositions described herein may be used to treat or prevent a variety of bacterial infections and diseases caused by or associated with bacterial infections, which are not associated with a wound (see Section 5.4.2, *infra*).

[00140] In certain embodiments, treatment of a disease associated with a bacterial infection comprises administration of one of the compositions described herein to a subject or a population of subjects to treat the disease or to obtain a beneficial or therapeutic effect. In specific embodiments, such treatment achieves one, two, three, four, five or more of the following effects in a subject or a population of subjects: (i) reduction or amelioration of the severity of a disease or a symptom associated therewith; (ii) reduction of the duration of a disease or a symptom

associated therewith; (iii) prevention of the progression of a disease or a symptom associated therewith; (iv) regression of a disease or a symptom associated therewith; (v) prevention of the development or onset of a symptom associated therewith; (vi) prevention of the recurrence of a symptom associated therewith; (vii) prevention or reduction of the spread of a disease from the subject or population of subjects to another subject or population of subjects; (viii) reduction in organ failure associated with a disease; (ix) reduction of the incidence of hospitalization; (x) reduction of the hospitalization length; (xi) an increase in the survival; (xii) elimination of a disease; (xiii) enhancement or improvement of the prophylactic or therapeutic effect(s) of another therapy; (xiv) improvement in quality of life as assessed by methods well known in the art, e.g., a questionnaire; (xv) reduction of the number of symptoms of a disease; and/or (xvi) reduction in mortality. In some embodiments, treatment comprises any therapy using compositions described herein.

[00141] In some embodiments treatment of a bacterial infection comprises administration of one of the compositions described herein to a subject or a population of subjects to treat the bacterial infection or a symptom of a bacterial infection. In specific embodiments, such treatment achieves one, two, three, four, five or more of the following effects in a subject or a population of subjects: (i) the clearance of a bacterial infection; (ii) the eradication of one or more symptoms associated with a bacterial infection; (iii) the reduction of time required to clear a bacterial infection; (iv) the reduction or amelioration of the severity of a bacterial infection and/or one or more symptoms associated therewith; (v) the reduction in the duration of a bacterial infection and/or one or more symptoms associated therewith; (vi) the prevention or delay of the generation of a resistant strain or strains of bacteria or reduction of a number of resistant strains of bacteria generated; (vii) the prevention in the recurrence of one or more symptoms associated therewith; (viii) the reduction or elimination in the bacterial cell population (such as reduction in bacterial counts, e.g., in a biological sample of a patient, as measured by CFU/mL or a log reduction by one of the methods known in the art or described herein); (ix) the reduction in hospitalization of a subject; (x) the reduction in hospitalization length; (xi) the increase in the survival of a subject; (xii) the enhancement or improvement of the therapeutic effect of another therapy; (xiii) a reduction in mortality; (xiv) the reduction or elimination in the spread of the bacteria from one subject to another subject, or one organ or tissue to another organ or tissue; (xv) the prevention of an increase in the number of bacteria; (xvi) the prevention of the

development or onset of one or more symptoms associated therewith; (xvii) the reduction in the number of symptoms associated with a bacterial infection; (xviii) the inhibition or reduction in production of a bacterial toxin or toxins associated with a bacterial infection; (xix) the stabilization or reduction of inflammation associated with a bacterial infection; (xx) the reduction in organ failure associated with a bacterial infection or a disease associated therewith; and/or (xxi) improvement in quality of life as assessed by methods well known in the art, e.g., a questionnaire.

[00142] In certain embodiments, administration of the compositions described herein to a subject results in one or more of the following: (i) the induction of the expression of one or more defensin proteins and/or defensin-like proteins; (ii) the induction of the expression of one or more Toll-like receptors; and/or (iii) the induction of the expression of one or more proteins that are beneficial for clearance or reduction of a bacterial infection or one or more symptoms associated therewith.

[00143] In certain embodiments, prevention of a bacterial infection comprises administration of one of the compositions described herein to a subject or a population of subjects to achieve one or more of the following effects: (i) the inhibition of the development or onset of a bacterial infection, or a symptom associated therewith; and/or (ii) the inhibition of the recurrence of a bacterial infection, or a symptom associated therewith.

[00144] In other embodiments, prevention of a bacterial infection comprises administration of one of the compositions described herein to a subject or a population of subjects to prevent a disease associated with a bacterial infection. In specific embodiments, such prevention achieves one or more of the following effects in a subject or a population of subjects: (i) the inhibition of the development or onset of a disease associated with a bacterial infection or a symptom thereof; and/or (ii) the inhibition of the recurrence of a disease associated with a bacterial infection or a symptom associated therewith.

5.4.1 Treatment or Prevention of Bacterial Infection in Wounds

[00145] In certain embodiments, the sNAG nanofiber compositions described herein may be useful for treating a wide variety of bacterially infected wounds affecting any tissue of the body or preventing infection of wounds at risk of becoming infected with bacteria.

[00146] There are two types of wounds, open and closed. Open wounds are classified according to the object that caused the wound. For example, incisions or incised wounds

(including surgical wounds) are caused by a clean, sharp-edged object such as a knife, a razor or a glass splinter. Lacerations are irregular wounds caused by a blunt impact to soft tissue which lies over hard tissue (e.g., laceration of the skin covering the skull) or tearing of skin and other tissues such as caused by childbirth. Abrasions or grazes are superficial wounds in which the topmost layer of the skin (the epidermis) is scraped off. Puncture wounds are caused by an object puncturing the skin, such as a nail or needle. Penetration wounds are caused by an object such as a knife entering the body. Gunshot wounds are caused by a bullet or similar projectile driving into (e.g., entry wound) and/or through the body (e.g., exit wound). In a medical context, all stab wounds and gunshot wounds are considered open wounds. Open wounds also include burn wounds induced by thermal, chemical, or electrical injury. Closed wounds include contusions (more commonly known as a bruise, caused by blunt force trauma that damages tissue under the skin), hematoma (also called a blood tumor, caused by damage to a blood vessel that in turn causes blood to collect under the skin), and crushing injuries (caused by a great or extreme amount of force applied over a long period of time).

[00147] In certain embodiments, the compositions described herein are used to treat a bacterial infected open wound or prevent a bacterial infection in an open wound. In certain embodiments, the compositions described herein may be used to treat or prevent a bacterial infection of a gunshot wound, a puncture wound and/or a penetration wound. In certain embodiments, the compositions described herein may be used to treat or prevent a post-operative bacterial infection, a surgical site bacterial infection, a catheter-related bacterial infection or a hemodialysis-related bacterial infection. In yet another embodiment, the compositions described herein are not used to treat or prevent a bacterial infection in an open wound, a gunshot wound, a puncture wound and/or a penetration wound. In certain embodiments, the compositions described herein are not used to treat or prevent a post-operative bacterial infection, a surgical site bacterial infection, a catheter-related bacterial infection or hemodialysis-related bacterial infection.

[00148] In some embodiments, the wound is a chronic wound. Chronic wound can be any wound that fails to heal properly, including a surgical wound (e.g., a skin graft donor site), a cutaneous ulcer (e.g., a diabetic ulcer, a venous stasis ulcer, a leg ulcer, an arterial insufficiency ulcer, or a pressure ulcer), or a burn wound. In one embodiment, the compositions described herein are used to treat or prevent chronic wound infections (e.g. an infection associated with a

diabetic ulcer, a venous stasis ulcer, a leg ulcer, an arterial insufficiency ulcer, a pressure ulcer, a surgical wound, or a burn). In yet another embodiment, the compositions described herein are not used to treat or prevent chronic wound bacterial infections (e.g. not used to prevent a bacterial infection associated with a diabetic ulcer, a venous stasis ulcer, a leg ulcer, an arterial insufficiency ulcer, a pressure ulcer, a surgical wound, or a burn).

[00149] In certain embodiments, the compositions described herein are used to treat or prevent nosocomial bacterial infections. Of the nosocomial bacterial infections, surgical wound bacterial infections predominate, with statistics showing up to 8% of all surgical patients. The direct cost of these types of infections is approximately 4.5 billion dollars per year. Many of hospital-contracted bacteria developed resistance against antibiotics, and thus non-antibiotic-based treatments are desired. Use of the sNAG compositions described herein in a hospital setting could defray much of the cost and markedly reduce the production of antibiotic resistant species. In yet another embodiment, the compositions described herein are not used to treat or prevent nosocomial bacterial infections, such as surgical bacterial infections.

[00150] In one embodiment, the compositions described herein may be used to treat or prevent bacterial infections in bleeding wounds (e.g., bleeding surface wounds). In one embodiment, the compositions described herein may be used to treat a gunshot wound, a puncture wound, a penetration wound or a surgical wound in order to treat or prevent bacterial infection in such wound. In yet another embodiment, the compositions described herein are not used to treat or prevent bacterial infections in bleeding wounds (e.g., bleeding surface wounds).

[00151] The compositions described herein may be useful for treating or preventing a bacterial infection in cutaneous wounds, such as wounds affecting the epidermal and dermal layers of the skin, as well as injuries to the cornea and epithelia-lined organs, in order to treat or prevent a bacterial infection in such wounds. The wounds may be caused by a wide variety of physical trauma, including cuts, abrasions, burns, chemical exposure, surgical procedures (e.g., surgical incisions, skin grafting). In one embodiment, the compositions described herein may be used for treating corneal and sclera wounds, including wounds which affect the epithelial layer, stromal layer and endothelial layers of the eye in order to treat or prevent a bacterial infection in such wounds. In yet another embodiment, the compositions described herein are not used to treat or prevent bacterial infections in cutaneous wounds.

[00152] In some embodiments, the compositions described herein may be used to treat a wound in a patient diagnosed with a bacterial infection. In certain embodiments, where the compositions described herein are used to treat a bacterial infected wound, a wound is determined to be bacterially infected by a test or an assay for the presence of a bacterial antigen. In one embodiment, a wound culture is performed to detect a bacterial infection in the wound of a patient. In yet other embodiments, a wound is determined to be infected due to the presence of one or more symptoms of bacterial infection.

[00153] In other embodiments, the compositions described herein may be used to treat a wound in a patient when a patient displays one or more of the symptoms of bacterial infection such as: a wound is slow to heal; heat, redness and/or swelling at the site of the wound; tenderness at the site of the wound; drainage of fluid or pus at the site of the wound; and/or fever. Symptoms of wound bacterial infection include but are not limited to localized erythema, localized pain, localized heat, cellulitis, oedema, abscess, discharge which may be viscous, discolored and purulent, delayed of wound healing, discoloration of tissues both within and/or at the wound margins, friable, bleeding granulation tissue, abnormal smell coming from the wound site, unexpected pain and/or tenderness at the site of dressing change, lymphangitis (i.e., a red line originating from the wound and leading to swollen tender lymph glands draining the affected area), and wound breakdown associated with wound pocketing/bridging at base of wound (i.e., a wound develops strips of granulation tissue in the base as opposed to a uniform spread of granulation tissue across the whole of the wound bed). In some embodiments, the compositions described herein prevent the onset or development of one or more of the above-listed symptoms, or reduce duration and/or severity of one or more of these symptoms.

[00154] In one embodiment, the compositions described herein may be used for wound healing and treatment of a wound bacterial infection, or for wound healing and prevention of a wound bacterial infection. In one embodiment, the compositions described herein are used to enhance wound healing while concurrently treating or preventing a wound bacterial infection. Effects of the sNAG nanofiber compositions on wound healing and some of the uses of the sNAG nanofibers in wound healing applications have been described in U.S. Patent Pub. No. 2009/0117175, which is incorporated by reference herein in its entirety (see, e.g., Example 2).

5.4.2 Treatment or Prevention of Other Bacterial Infections

[00155] In certain embodiments, the sNAG nanofiber compositions described herein may be used to treat and/or prevent bacterial infections of the skin, gastrointestinal tract, respiratory tract, urinary tract, reproductive tract, blood, throat, ears, eye, sinus or any other organ or tissue of the body. In another embodiment, the sNAG nanofiber compositions described herein may be used to treat and/or prevent skin conditions, gastrointestinal conditions, respiratory conditions and/or conditions of any other organ or tissue associated with a bacterial infection. In some embodiments, the sNAG nanofiber compositions described herein are applied topically on the skin, mouth, ear, eye, anus or groin areas of a patient to treat or prevent a bacterial infection. In some embodiments, the compositions described herein are used to treat and/or prevent a bacterial infection of an organ or tissue of the body that is not at the site of a wound, and/or is not associated with or caused by a wound.

[00156] In certain embodiments, the sNAG nanofiber compositions described herein are used to treat an existing bacterial infection. For example, such compositions may be used to treat a subject diagnosed with a bacterial infection by a test or an assay, such as one of the tests described herein or known in the art. Alternatively, such compositions may be used to treat a subject displaying one or more symptoms of a bacterial infection or a disease associated with a bacterial infection, such as one or more symptoms of a bacterial infection known to a skilled artisan (e.g., determined by a treating doctor/physician to be a symptom of a bacterial infection) and/or described herein.

[00157] In certain embodiments, the compositions described herein are used to treat a condition associated with an imbalance in bacterial microbiota, or a condition associated with an abnormal or altered bacterial microbiota. For example, such compositions may be used to treat a skin condition in a patient whose skin bacterial microbiota differs from that in control subjects (e.g., subjects with no symptoms of the skin condition). In other examples, such compositions may be used to treat an intestinal condition (or a condition of any other tissue or organ) in a patient whose intestinal bacterial microbiota (or microbiota of the other tissue or organ) differs from that in control subjects (e.g., subjects with no symptoms of the intestinal condition).

[00158] In other embodiments, the compositions described herein may be used to treat any disease known to be associated with or exacerbated by a bacterial infection (e.g., acne). In one

embodiment, the compositions described herein may be used to treat a cystic fibrosis patient infected by *P. aeruginosa*.

[00159] In some embodiments, the compositions described herein are effective against toxins secreted or excreted by bacteria. In one embodiment, the compositions described herein may be used to inhibit/reduce a bacterial toxin (and/or a condition or symptom caused by a bacterial toxin), for example toxins produced by *Bacillus anthracis*, *Clostridium difficile*, *Corynebacterium diphtheriae*, *Pseudomonas aeruginosa*; endotoxins, and/or the cytolsins. Some defensins are able to inhibit bacterial toxins, including those produced by *Bacillus anthracis*, *Clostridium difficile*, *Corynebacterium diphtheriae*, *Pseudomonas aeruginosa*, and the cytolsins (endotoxins produced by Gram-positive bacteria that lyse red blood cells). Given these functions of defensins, activation of pathways resulting in defensin expression and secretion may allow for the antibiotic-independent clearance of bacterial infection, and thus avoid the generation of bacterial resistance.

[00160] In certain embodiments, the compositions described herein may be used to treat and/or prevent one or more bacterial infections of the skin, or diseases of the skin associated with a bacterial infection. In some embodiments, the compositions described herein are used to treat localized skin infections and/or diffuse skin infections. In some embodiments, the compositions described herein are used to treat or prevent skin infections or diseases of the skin associated with bacterial infections affecting the epidermis, dermis, and/or subcutaneous (hypodermis) tissues of the skin. In some of these embodiments, the affected layers of the skin include one or more layers of the epidermis (i.e., stratum basale, stratum spinosum, stratum granulosum, stratum loricatum, and stratum corneum), one or more types of tissues of the dermis (i.e., collagen, elastic tissue, and reticular fibers), one or more layers of the dermis (i.e., the upper, papillary layer and the lower reticular layer); and/or one or more types of tissue of the hypodermis (i.e., fat, elastin and connective tissue). In an embodiment, the compositions described herein are used to treat or prevent bacterial infections on the skin surface. In another embodiment, the compositions described herein are used to treat or prevent a *Staphylococcus* ("Staph") infection of the skin and/or a *Streptococcus* ("Strep") infection of the skin. In yet another embodiment, the compositions described herein are used to treat *Staphylococcus albus* and/or *Staphylococcus aureus* infection of the skin. In some embodiments, the compositions described herein are used to treat or prevent cellulitis, impetigo, folliculitis, erythrasma, carbuncles, furuncles, abscesses,

erysipelas, and/or a cutaneous anthrax. In another embodiment, the compositions described herein are used to treat or prevent cellulitis. Cellulitis affects the deeper dermis and subcutaneous tissues, and usually affects the face, arms, and legs, and almost always occurs due to a break in the skin that leads to a bacterial infection. The symptoms of cellulitis include one or more of: swelling of the skin around the break in the skin, pain, tenderness, outward signs of blistering, red lines between the lymph nodes, fever, and chills. In another embodiment, the composition described herein are used to treat or prevent a bacterial infection of the skin at the hair follicles (e.g., folliculitis). The symptoms of folliculitis include swelling, pustules surrounding the hair, hard nodules, and pain. In another embodiment, the compositions described herein may be used to treat or prevent acne. Appearance of acne is frequently a symptom of a bacterial infection. In one embodiment, the compositions described herein are used to treat or prevent dermatitis associated with or caused by a bacterial infection. In some embodiments, the compositions described herein prevent the onset or development of one or more of the above-listed symptoms, or reduce duration and/or severity of one or more of these symptoms.

[00161] In certain embodiments, the compositions described herein may be used to treat and/or prevent one or more intestinal/digestive bacterial infections or gastrointestinal diseases associated with bacterial infections. The common forms of intestinal bacterial infection include *salmonella*, *shigella*, *E. coli*, *Clostridium*, *Staphylococcus*, *Listeria*, and *Yersinia*. These bacteria cause diarrhea and inflammation of the stomach and intestines, also known as gastroenteritis. Symptoms of an intestinal bacterial infection include but are not limited to abdominal cramps and pain, bloody feces, loss of appetite, nausea sometimes accompanied by vomiting, fever, and diarrhea. In some embodiments, the compositions described herein are used to treat a patient displaying one or more symptoms of food poisoning, which is often associated with a bacterial infection.

[00162] In certain embodiments, the compositions described herein may be used to treat a disease associated with a *Staph* infection (e.g., a *Staphylococcus aureus* infection). In some of these embodiments, the disease is a *Staph* infection of the skin, nose, mouth, and/or genital area. In some embodiments, the disease is pneumonia, meningitis, endocarditis, toxic shock syndrome, and/or septicemia. In an embodiment, the compositions described herein may be used to treat *Staph* bacteria resistant to one or more antibiotics, for example, methicillin resistant *Staph*

aureus ("MRSA"). In certain embodiments, the compositions described herein are administered to a subject diagnosed with a *Staph* infection, or to a subject displaying one or more symptoms of a *Staph* infection (e.g., presence of one or more of: small red bumps, crusty red bumps, pus filled bumps or abscess, boils, styes in the eyes, blisters and/or red scabby skin, such as red scabby skin around the nose and mouth, or symptom/s of a Toxic Shock Syndrome). In some embodiments, the compositions described herein prevent the onset or development of one or more of the above-listed symptoms, or reduce duration and/or severity of one or more of these symptoms.

[00163] In certain embodiments, the compositions described herein may be used to treat or prevent a cold associated with a bacterial infection. For example, the compositions described herein may be used to treat or prevent a cold that persists despite the use of standard pain relief medications. In one embodiment, the compositions described herein are used to treat or prevent a bacterial infection of sinus, ear or throat. The symptoms of such bacterial infections include localized pain and swelling. Bacterial infection in the sinus may lead to nasal discharge and acute pain in parts of the face or forehead. In one embodiment, the compositions described herein are used to treat strep throat (*Streptococcus pyogenes*). In some embodiments, the compositions described herein prevent the onset or development of one or more of the above-listed symptoms, or reduce duration and/or severity of one or more of these symptoms.

[00164] In some embodiments, the compositions described herein may be used to treat or prevent a genital, urinary tract or anal bacterial infection, or a disease of urinary or reproductive tract associated with a bacterial infection. In some of these embodiments, the compositions described herein may be used to treat a sexually transmitted disease associated with a bacterial infection. Symptoms of such infections include but are not limited to painful urination, cloudy discharge, and/or pain during intercourse. In some of these embodiments, the compositions described herein are used to treat or prevent one or more of syphilis, gonorrhea, chlamydia, and trichomoniasis. In one embodiment, the compositions described herein are used to treat Chlamydia. In another embodiment, the described herein are used to treat gonorrhea.

Symptoms of gonorrhea include localized pelvic pain, itching and irritation, painful urination, a thick yellow or green discharge, bleeding between menstrual periods. In one embodiment, the compositions described herein are used to treat or prevent a bacterial vaginosis infection.

Symptoms of bacterial vaginosis include vaginal discharge, odor, vaginal itching, and abdominal

pain. In some embodiments, the compositions described herein prevent the onset or development of one or more of the above-listed symptoms, or reduce duration and/or severity of one or more of these symptoms.

[00165] In other embodiments, the compositions described herein may be used to treat or prevent a respiratory tract infection (e.g., a bacterial infection of the lungs), or a respiratory disease associated with a bacterial infection. In some embodiments, such compositions are used to treat or prevent upper respiratory tract infections. In one embodiment, such compositions are used to treat or prevent tuberculosis. Tuberculosis is caused by *Mycobacterium tuberculosis*, and it is a highly infectious disease that is spread from person to person by sneezing or saliva. Thus, the compositions described herein may be used to treat not only subjects diagnosed with tuberculosis or displaying symptoms of tuberculosis, but also individuals in contact with such subjects (e.g., family members, caretakers or medical personnel). Symptoms of tuberculosis include coughing blood, excessive weight loss, fatigue, loss of appetite and persistent fever. In some embodiments, the compositions described herein prevent the onset or development of one or more of the above-listed symptoms, or reduce duration and/or severity of one or more of these symptoms. In one embodiment, the compositions described herein are used to treat pneumonia and/or a *Streptococcus pneumoniae* infection. In another embodiment, such compositions are used to treat bronchitis. In one embodiment, the compositions described herein are used to treat *Moraxella catarrhalis*, *Streptococcus pneumoniae* and/or *Haemophilus influenza*.

[00166] In some embodiments, the compositions described herein are used to treat or prevent bacterial infections of a mucosal surface (e.g., oral mucosa), or a disease/condition of mucosal surface associated with a bacterial infection. In one embodiment, the compositions described herein are used to treat or prevent bacterial infections of the oral cavity. For example, such compositions may be used to treat or prevent conditions associated with bacterial infections in the mouth such as gingivitis, caries, and/or tooth decay. In one embodiment, the compositions described herein may be used in oral hygiene products.

[00167] In one embodiment, the compositions described herein are used to treat a bacterial infection of the ear, such as middle ear infection, or a disease associated with such infection. In one embodiment, a composition described herein is used to treat otitis media caused by a bacterial infection.

[00168] In some embodiments, the compositions described herein are used to treat or prevent bacterial infections of implanted prosthesis, such as heart valves and catheters. In some embodiments, the compositions described herein are used to treat animal bites, for example cat or dog bite, in order to prevent a bacterial infection.

[00169] The compositions described herein may be used to treat or prevent a variety of diseases associated with a bacterial infection including but not limited to leprosy (Hansen's disease), cholera, anthrax (e.g., cutaneous anthrax, pulmonary anthrax, gastrointestinal anthrax), pertussis, granuloma inguinale, bacterial vaginosis, gonorrhoea, ophthalmia neonatorum, septic arthritis, syphilis, congenital syphilis, whooping cough, mycobacterium avium complex, melioidosis, leptospirosis, tetanus, scarlet fever, strep infections, invasive group A *Streptococcal* disease, *Streptococcal* Toxic shock syndrome, meningococcal disease, bacteremia, strep throat, Typhoid fever type salmonellosis, dysentery, colitis, salmonellosis with gastroenteritis and enterocolitis, bacillary dysentery, amebic dysentery, shigellosis, diphtheria, cutaneous diphtheria, respiratory diphtheria, Legionnaires' disease, tuberculosis, latent tuberculosis, hemophilus influenzae B, typhoid fever, *vibrio parahaemolyticus*, *vibrio vulnificus*, *vibrio*, yersiniosis, Whipple's disease, acute appendicitis, meningitis, encephalitis, impetigo, cellulitis, carbuncle, boil, acne, sepsis, septicemia, pneumonia, mycoplasma pneumonia, meningococcal disease, meningitis, Waterhouse-Friderichsen syndrome, ptomaine food poisoning, Staph food poisoning, Toxic shock syndrome, necrotizing pneumonia, septicemia, acute infective endocarditis, an infection of sweat glands (e.g., *Hidradenitis suppurativa*), a bacterial disease transmitted by a tick (e.g., Rocky Mountain Spotted Fever, Lyme disease), botulism, plague (e.g., bubonic plague, pneumonic plague), tularemia, brucellosis, acute enteritis, nongonococcal urethritis, lymphogranuloma venereum, trachoma, inclusion conjunctivitis of the newborn, psittacosis, pseudomembranous colitis, gas gangrene, acute food poisoning, diarrhea, traveller's diarrhea, diarrhea in infants, hemorrhagic colitis, hemolytic-uremic syndrome, bronchitis, listeriosis, anaerobic cellulitis, peptic ulcer, Pontiac fever, cystitis, endometritis, otitis media, sinusitis, streptococcal pharyngitis, rheumatic fever, erysipelas, puerperal fever, necrotizing fasciitis, nosocomial infections, pseudomonas infection, and/or cat scratch disease.

5.5 Patient Populations

[00170] In certain embodiments, a sNAG nanofiber composition described herein may be administered to a naïve subject, i.e., a subject that does not have a bacterial infection. In one

embodiment, a composition described herein is administered to a naïve subject that is at risk of acquiring a bacterial infection.

[00171] In one embodiment, a sNAG nanofiber composition described herein may be administered to a patient who has been diagnosed with a bacterial infection. In another embodiment, a composition described herein may be administered to a patient who displays one or more symptoms of a bacterial infection.

[00172] In certain embodiments, the compositions described herein are administered to patients diagnosed with a bacterial infection. In certain embodiments, a patient is diagnosed with a bacterial infection prior to administration of a composition described herein. For example, the compositions described herein may be administered to a patient when a bacterial antigen is detected in a biological sample taken from the patient. In one embodiment, a biological sample is obtained from the site or area to be treated by the compositions described herein or an area to which the compositions described herein are to be administered. In one embodiment, a swab is used to collect cells or pus from the site of the suspected infection to detect a bacterial infection. In another embodiment, a fluid is aspirated from the suspected site of an infection (e.g., a wound) to detect a bacterial infection. In yet another embodiment, a tissue biopsy is performed to detect a bacterial infection. In an embodiment where the suspected site of an infection is a wound, a wound culture may be performed to detect a bacterial infection. In another embodiment, the biological sample is obtained from blood, urine, sputum or feces of the patient. In some embodiments, a blood or a urine test may be performed to detect a bacterial infection (e.g., when a bacterial infection is suspected to have spread into the blood or other tissues/organs). In some embodiments, the collected sample (e.g., cells, tissues or fluid) is tested using DNA detection methods such as PCR for presence of one or more types of bacteria. In other embodiments, immunofluorescence analysis, serology, culture (e.g., blood agar culture), or any other test known and/or practiced in the art may be used for laboratory diagnosis of bacterial infection.

[00173] In other specific embodiments, the compositions described herein may be administered to a patient diagnosed with or displaying one or more symptoms of a disease associated with a bacterial infection. In certain embodiments, a patient is diagnosed with a disease associated with a bacterial infection or displays one or more symptoms of a disease associated with a bacterial infection prior to administration of a composition described herein. A disease associated with a bacterial infection may be diagnosed by any method known to a skilled

artisan, including evaluation of the patient's symptoms and/or detection of a bacterial antigen in a biological sample of the patient (e.g., as described above). In one example, the compositions described herein may be administered to a patient diagnosed with a disease associated with a bacterial infection by a treating physician or another medical professional. In another example, a patient may use the compositions described herein upon detection of one or more symptoms of a disease associated with a bacterial infection.

[00174] In certain embodiments, a composition described herein is administered to a patient who has been diagnosed with an infection, e.g., the bacterial infection by *Bacillus anthracis*, *Bordetella pertussis*, *Brerella burgdorferi*, *Brucella abortus*, *Brucella canis*, *Brucella melitensis*, *Brucella suis*, *Campylobacter jejuni*, *Chlamydia psittaci*, *Chlamydia pneumonia*, *Chlamydia trachomatis*, *Clamidaphila psittaci*, *Clostridium botulinum*, *Clostridium difficile*, *Clostridium perfringens*, *Clostridium tetani*, *Corynebacterium diphtheriae*, *Enterococcus faecalis*, *Enterococcus faecium*, *Escherichia coli*, *Francisella tularensis*, *Haemophilus influenzae*, *Helicobacter pylori*, *Legionella pneumophila*, *Leptospira pneumophila*, *Leptospira interrogans*, *Listeria monocytogenes*, *Moraxella catarrhalis*, *Mycobacterium leprae*, *Mycobacterium tuberculosis*, *Mycoplasma pneumoniae*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Pneumocystis jiroveci*, *Rickettsia rickettsii*, *Salmonella typhi*, *Salmonella typhimurium*, *Shigella sonnei*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *Streptococcus agalactiae*, *Streptococcus pneumonia*, *Streptococcus pyogenes*, *Treponema pallidum*, *Vibrio cholerae*, *Yersinia pestis* and/or any other bacterial infection described herein or known in the art. In one embodiment, a composition described herein is administered to a patient who has been diagnosed with the bacterial infection by MRSA or *Pseudomonas aeruginosa*.

[00175] In certain embodiments, a composition described herein is administered to a patient who has been diagnosed with a disease associated with bacterial infection, e.g., leprosy (Hansen's disease), cholera, anthrax (e.g., cutaneous anthrax, pulmonary anthrax, gastrointestinal anthrax), pertussis, granuloma inguinale, bacterial vaginosis, gonorrhea, ophthalmia neonatorum, septic arthritis, syphilis, congenital syphilis, whooping cough, mycobacterium avium complex, melioidosis, leptospirosis, tetanus, scarlet fever, strep infections, invasive group A *Streptococcal* disease, *Streptococcal* Toxic shock syndrome, meningococcal disease, bacteremia, strep throat, Typhoid fever type salmonellosis, dysentery, colitis,

salmonellosis with gastroenteritis and enterocolitis, bacillary dysentery, amebic dysentery, shigellosis, diphtheria, cutaneous diphtheria, respiratory diphtheria, Legionnaires' disease, tuberculosis, latent tuberculosis, hemophilus influenzae B, typhoid fever, vibrio parahaemolyticus, vibrio vulnificus, vibrio, yersiniosis, Whipple's disease, acute appendicitis, meningitis, encephalitis, impetigo, cellulitis, carbuncle, boil, acne, sepsis, septicemia, pneumonia, mycoplasma pneumonia, meningococcal disease, meningitis, Waterhouse-Friderichsen syndrome, ptomaine food poisoning, Staph food poisoning, Toxic shock syndrome, necrotizing pneumonia, septicemia, acute infective endocarditis, an infection of sweat glands (e.g., Hidradenitis suppurativa), a bacterial disease transmitted by a tick (e.g., Rocky Mountain Spotted Fever, Lyme disease), botulism, plague (e.g., bubonic plague, pneumonic plague), tularemia, brucellosis, acute enteritis, nongonococcal urethritis, lymphogranuloma venereum, trachoma, inclusion conjunctivitis of the newborn, psittacosis, pseudomembranous colitis, gas gangrene, acute food poisoning, diarrhea, traveller's diarrhea, diarrhea in infants, hemorrhagic colitis, hemolytic-uremic syndrome, bronchitis, listeriosis, anaerobic cellulitis, peptic ulcer, Pontiac fever, cystitis, endometritis, otitis media, sinusitis, streptococcal pharyngitis, rheumatic fever, erysipelas, puerperal fever, necrotizing fasciitis, nosocomial infections, pseudomonas infection, and/or cat scratch disease.

[00176] In some embodiments, a composition described herein is administered to a patient with a bacterial infection before symptoms of the infection manifest or before symptoms of the infection become severe (e.g., before the patient requires treatment or hospitalization). In some embodiments, a composition described herein is administered to a patient with a disease after symptoms of the disease manifest or after symptoms of the disease become severe (e.g., after the patient requires treatment or hospitalization).

[00177] In some embodiments, a subject to be administered a composition described herein is an animal. In certain embodiments, the animal is a bird. In certain embodiments, the animal is a canine. In certain embodiments, the animal is a feline. In certain embodiments, the animal is a horse. In certain embodiments, the animal is a cow. In certain embodiments, the animal is a mammal, e.g., a horse, swine, mouse, or primate, preferably a human. In some embodiments, the animal is a pet or a farm animal.

[00178] In certain embodiments, a subject to be administered a composition described herein is a human adult. In certain embodiments, a subject to be administered a composition described

herein is a human adult more than 50 years old. In certain embodiments, a subject to be administered a composition described herein is an elderly human subject.

[00179] In certain embodiments, a subject to be administered a composition described herein is a premature human infant. In certain embodiments, a subject to be administered a composition described herein is a human toddler. In certain embodiments, a subject to be administered a composition described herein is a human child. In certain embodiments, a subject to be administered a composition described herein is a human infant. In certain embodiments, a subject to whom a composition described herein is administered is not an infant of less than 6 months old. In a specific embodiment, a subject to be administered described herein is 2 years old or younger.

[00180] In yet other embodiments, a composition described herein may be administered to a patient who is at risk (e.g., at high risk) of developing a bacterial infection. Patients that are at high risk of developing a bacterial infection include but are not limited to the elderly (e.g., human elderly) and immunocompromised. In some embodiments, a composition described herein is administered to a patient at risk of developing a bacterial infection, such as but not limited to an immunosuppressed patient, (e.g., as a result of cancer treatment or a transplantation procedure), a human child, a premature human infant, an elderly human, a person with diabetes, a person diagnosed with cancer, a patient who has been treated with a course of traditional antibiotics, a patient who has undergone a surgery, and/or a patient with a wound. In some embodiments, the compositions described herein may be administered prophylactically to patients who are at risk for developing a bacterial infection, e.g., those with compromised immune systems due to, for example, age, malnourishment, disease, chemotherapy, those who have been treated with a course of traditional antibiotics, or those who have a wound (e.g., an open wound). In other embodiments, a patient at risk of developing a bacterial infection is an HIV/AIDS patient, a cancer patient, a patient who has undergone a transplant procedure, a patient with asthma (e.g., severe asthma), a drug user, a patient with a dermatologic conditions, a patients with an invasive device (e.g., an intravascular catheter), a health care workers or a patient who spends time in a confined facility (e.g., a prison inmate, a soldier, a patient in a long-term healthcare facility such as a nursing home, etc.). A patient to be administered a composition described herein may also be a patient with a chronic obstructive pulmonary disorder (COPD), emphysema, rhinitis, bronchitis, laryngitis, tonsillitis, and/or cystic fibrosis.

[00181] In certain embodiments, a composition described herein is administered to a patient who has not been diagnosed with a viral infection (e.g., HIV/AIDS), a fungal infection, or an yeast infection. In certain embodiments, a composition described herein is administered to a patient who does not belong to one or more of the following patient groups: an immunocompromised patient, a cancer patient, an HIV/AIDS patient, a patient with asthma, a patient who has undergone a transplant procedure, a patient who has undergone a surgery, and/or a patient with a wound. In one embodiment, the patient to be administered a composition described herein does not have a wound (e.g., a chronic wound, or an open wound due, e.g., to a surgery or battlefield trauma).

[00182] In certain embodiments, a subject to be administered a composition described herein is a subject with no or low level of expression of one or more defensin peptides or a mutation/deletion in a gene or genes encoding one or more defensin peptides. In some embodiments, a subject to be administered a composition described herein is a subject with no or low or altered level of expression of one or more α -defensins (e.g., DEFA1, DEFA1B, DEFA3, DEFA4, DEFA5, DEFA6), one or more β -defensins (e.g., DEFB1, DEFB2, DEFB4, DEFB103A, DEFB104A, DEFB105B, DEFB107B, DEFB108B, DEFB110, DEFB112, DEFB114, DEFB118, DEFB119, DEFB123, DEFB124, DEFB125, DEFB126, DEFB127, DEFB128, DEFB129, DEFB131, DEFB136), and/or one or more θ -defensins (e.g., DEFT1P). In some embodiment, a subject to be administered a composition described herein is a subject with no or low or altered level of expression of one or more of DEFA1, DEFA3, DEFA4, DEFA5, DEFB1, DEFB3, DEFB103A, DEFB104A, DEFB108B, DEFB112, DEFB114, DEFB118, DEFB119, DEFB123, DEFB124, DEFB125, DEFB126, DEFB128, DEFB129 and DEFB131. In certain embodiments, a subject to be administered a composition described herein is a subject with no or low or altered level of expression of one or more Toll receptors (e.g., TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10, TLR11, and/or TLR12). In yet other embodiments, a subject to be administered a composition described herein is a subject with no or low or altered level of expression of one or more of IL-1, CEACAM3, SPAG11, SIGIRR (IL-1-like receptor), IRAK1, IRAK2, IRAK4, TBK1, TRAF6 and IKK β . In some embodiments, a subject to be administered a composition described herein is a subject with no or low or altered level of expression of one or more of IRAK2, SIGIRR, TLR1, TLR2, TLR4, TLR7, TLR8, TLR10 and TRAF6. A low level of expression of a gene is a level that is lower

(e.g., more than 1.25 fold, 1.5 fold, 2 fold, 2.5 fold, 3 fold, 3.5 fold, 4 fold, 4.5 fold, 5 fold, 6 fold, 7 fold, 8 fold, 9 fold, 10 fold lower) than the normal level of expression, wherein the normal level of expression is the level of expression is considered normal in the species to which the subject belongs by a skilled artisan and/or the level of expression in the majority of the subjects of the same species. An altered level of expression of a gene is a level that differs (e.g., by more than 20%, 25%, 30%, 50%, 75%, 100%, 150%, 200%, 250%, 300%) from the normal level of expression, wherein the normal level of expression is the level of expression is considered normal in the species to which the subject belongs by a skilled artisan and/or the level of expression in the majority of the subjects of the same species. Wherein the "normal" expression of one or more defensin genes is: (i) the average expression level known to be found in subjects not displaying symptoms or not diagnosed with the disease or infection to be treated; (ii) the average expression level detected in three, five, ten, twenty, twenty-five, fifty or more subjects not displaying symptoms or not diagnosed with the disease or infection to be treated; and/or (iii) the level of expression detected in a patient to be administered a composition described herein before the onset of the disease or infection.

5.6 Modes of Administration of sNAG Nanofiber Compositions

[00183] In certain embodiments, methods are described herein for treating or preventing a bacterial infection or a disease associated with a bacterial infection, wherein a composition comprising the sNAG nanofibers is topically administered to a patient in need of such treatment. In some embodiments, a sNAG nanofiber composition is applied topically to tissue or organ which has an increased risk of a bacterial infection or disease.

[00184] In some embodiments, an effective amount of the sNAG nanofibers and/or a sNAG nanofiber composition is administered to a subject.

[00185] In some embodiments, a composition comprising the sNAG nanofibers is administered topically to the site of the bacterial infection in a patient or to the site affected by a disease associated with bacterial infection. In yet other embodiments, a composition comprising the sNAG nanofibers is administered topically to the site and around the site of the bacterial infection in a patient or to the site affected by a disease associated with bacterial infection. In yet other embodiments, a composition comprising sNAG nanofibers is applied in proximity to the site of the bacterial infection in a patient or in proximity to the site affected by a disease

associated with bacterial infection. In yet another embodiment, a composition comprising the sNAG nanofibers is administered topically to the site at high risk of a bacterial infection.

[00186] The sNAG nanofiber compositions described herein may be administered by any of the many suitable means of topical administration which are well known to those skilled in the art, including but not limited to topically to the skin, topically to any other surface of the body (e.g., mucosal surface), by inhalation, intranasally, vaginally, rectally, buccally, or sublingually. The mode of topical administration may vary depending upon the disease to be treated or prevented. The sNAG nanofiber compositions can be formulated for the various types of topical administration.

[00187] In one embodiment, a composition comprising sNAG nanofibers is applied to the skin of a patient. For example, such composition may be applied topically to the skin of a patient for treating and/or preventing a bacterial infection of the skin or a disease of the skin that is associated with a bacterial infection.

[00188] In another embodiment, a composition described herein may be applied topically to a mucosal surface of a patient. For example, such composition may be applied topically to oral mucosa for treating and/or preventing a bacterial infection of the mouth or gums or a disease of the mouth or gums that is associated with a bacterial infection.

[00189] In some embodiments, a composition comprising sNAG nanofibers is applied to the wound in a patient. For example, such composition may be applied topically directly to site of the wound or in proximity to the site of the wound of a patient for treating and/or preventing a bacterial infection of the wound or a disease associated with a bacterial infection of the wound. In one such embodiment, the wound is a bacterially infected wound, for example, as diagnosed by one of the methods described herein. The wound may be any one of the types of wounds described herein. In yet other embodiments, a composition comprising sNAG nanofibers is not applied to a wound in a patient, or is not applied to a bacterially infected wound in a patient.

[00190] In some embodiments, a composition described herein may be applied topically to a genital, urinal or anal surface/area of a patient. For example, such composition may be applied topically to genital, urinal or anal surface/area for treating and/or preventing genital, urinal or anal bacterial infections or a disease of such tissues that is associated with a bacterial infection.

[00191] The above-listed methods for topical administration may include administration of the sNAG nanofiber in the form of a cream, an ointment, a gel, a liquid solution, a membrane, a film,

a spray, a paste, a powder or any other formulation described herein or known in the art. The sNAG nanofiber may also be applied in a dressing or a bandage, for example to treat localized infections/conditions on the skin of a patient.

[00192] In some embodiments, a composition described herein may be applied as a spray into the oral cavity and/or respiratory system of a patient. For example, such composition may be applied as a spray for treating and/or preventing bacterial infection of the mouth, nose, gums, throat or lungs or a disease/condition of the mouth, nose, gums, throat or lungs that is associated with a bacterial infection. In one such embodiment, the composition may be formulated to be administered as an inhaler.

[00193] In some embodiments, a composition described herein may be applied as a suppository in the rectum, vagina or urethra of a patient. For example, such composition may be applied as a suppository for treating and/or preventing bacterial infection of the digestive tract, urinary tract or reproductive tract or a disease of such tissues that is associated with a bacterial infection.

[00194] In another embodiment, a composition described herein may be applied at the site of a surgical procedure. For example, such composition may be sprayed, applied as a cream, ointment, gel, membrane, or powder, or coated on the surface of the tissue or organ to be subjected to a surgical procedure or that has been subjected to the surgical procedure. In one embodiment, a composition described herein is applied at the site of the surgical incision, at the site of the excised tissue, or at the site of surgical stitches or sutures. Such administration of a composition described herein may prevent a post-surgical infection. For example, a composition described herein may be used during or after a surgical procedure which is known to pose high risk of a bacterial infection. Surgical procedures that are known to pose high risk of a bacterial infection include bowel resection, gastrointestinal surgical procedures, kidney surgery, etc. A composition described herein may be applied at the site of any of the above-listed or other surgical procedures.

[00195] In yet other embodiments, a composition described herein may be coated on a device, for example an oral hygiene product, a catheter, a surgical instrument or another product, to be used in or inserted into a patient, in order to prevent a bacterial infection in a patient.

[00196] In some embodiments, methods contemplated herein include a step that includes detection/diagnosis of a bacterial infection in a patient. In some embodiments,

detection/diagnosis involves a test or assay for one or more bacteria or bacterial antigens in a biological sample of the patient. In other embodiments, diagnosis involves assessing whether the patient has one or more symptoms of a bacterial infection or a disease associated with a bacterial infection.

[00197] The compositions described herein may exhibit sustained release properties and/or may be administered in a formulation resulting in a sustained release of such compositions. In some embodiments, the sNAG nanofibers biodegrade over time as described in Section 5.1, *supra*, and these properties of sNAG nanofibers may lead to or contribute to sustained release of the compositions described herein. In yet other embodiments, the compositions described herein are formulated to display sustained release capabilities using any methods known in the art. The compositions described herein may exhibit sustained release over a time period equal to or more than about 6 hours, 12 hours, 18 hours, 24 hours (1 day), 2 days, 3 days, 5 days, 7 days (1 week), 10 days, 14 days (2 weeks), 3 weeks or 4 weeks after administration of the composition to the patient.

[00198] Contemplated treatment regimes include a single dose or a single application of a sNAG nanofiber composition (e.g., of a cream, a membrane or a dressing), or a regimen of multiple doses or multiple applications of a sNAG nanofiber composition. A dose or an application may be administered hourly, daily, weekly or monthly. For example, a dose of a sNAG nanofiber composition may be administered once a day, twice a day, three times a day, four times a day, five times a day, every 3 hours, every 6 hours, every 12 hours, every 24 hours, every 48 hours, every 72 hours, once a week, 2 times a week, 3 times a week, every other day, once in 2 weeks, once in 3 weeks, once in 4 weeks, or once a month.

[00199] A sNAG nanofiber composition may be administered for a duration equal to or greater than 1 week, 2 weeks, 3 weeks, 1 month, 2 months, 3 months, 4 months, 5 months, 6 months, 9 months, 1 year, 1.5 years, 2 years, 2.5 years, 3 years, 4 years, 5 years, 7 years, 10 years or more. In one such embodiment, a sNAG nanofiber composition does not cause any side effects or causes only mild side effects during the duration of the treatment. In one such embodiment, a sNAG nanofiber composition does not lose its effectiveness or does not cause generation of resistant strains of bacteria in response to the treatment. In another embodiment, a sNAG nanofiber composition does not cause irritation (e.g., moderate or severe irritation) or allergy (e.g., moderate or severe allergy).

[00200] Concentration of the sNAG nanofiber in a composition may vary. In general, an effective amount of the sNAG nanofiber is used. A effective amount may be an amount sufficient to achieve one or more of the effects described herein, for example an amount effective to reduce or eradicate a bacterial infection, or reduce or eradicate one or more symptoms of a bacterial infection. For example, a composition may comprise about 0.2 to 20 mg/cm² of the sNAG nanofibers per dose/application of the composition in a form suitable for topical delivery to a patient. In certain embodiments, a composition described herein comprises about 0.25 to 20 mg/cm², about 0.5 to 20 mg/cm², about 1 to 20 mg/cm², about 1 to 15 mg/cm², about 1 to 12 mg/cm², about 1 to 10 mg/cm², about 1 to 8 mg/cm², about 1 to 5 mg/cm², about 2 to 8 mg/cm², or about 2 to 6 mg/cm² of the sNAG nanofibers per dose/application of the composition in a form suitable for topical delivery to a patient.

5.7 Combination Therapy

[00201] The sNAG nanofiber compositions may be administered in conjunction with other therapies such as substances that boost the immune system, antibacterial agents (e.g., an antibiotic), defensin peptides, defensin-like peptides, pain relief therapy (e.g., an analgesic), fever relief therapy, and/or other agents or drugs known to be effective against or commonly used for treatment and/or prevention of bacterial infections or diseases associated with bacterial infections.

[00202] In some embodiments, a composition described herein is administered in conjunction with an additional anti-bacterial agent, for example an antibiotic. In one such embodiment, a composition described herein may be used to treat a bacterial infection or a disease associated with a bacterial infection in conjunction with a standard therapy commonly used to treat such bacterial infection or such disease. In one embodiment, a composition described herein may be administered to a patient diagnosed with or displaying symptoms of a bacterial infection or a disease associated with a bacterial infection in conjunction with a standard anti-bacterial agent (e.g., an antibiotic) known to be effective against such bacterial infection or such disease.

[00203] In certain embodiments, a composition described herein is administered in conjunction with an antibiotic of one of the following classes of antibiotics: macrolides (e.g., erythromycin, azithromycin), aminoglycosides (e.g., amikacin, gentamicin, neomycin, streptomycin), cephalosporins (e.g., cefadroxil, cefaclor, cefotaxime, cefepime), fluoroquinolones (e.g., ciprofloxacin, levofloxacin), penicillins (e.g., penicillin, ampicillin,

amoxicillin), tetracyclines (e.g., tetracycline, doxycycline), and carbapenems (e.g., meropenem, imipenem). In some embodiments, a composition described herein is administered in conjunction with an agent (e.g., an antibiotic) effective to treat or prevent or commonly used to treat or prevent an *S. aureus* infection, MRSA infection, a *Pseudomonas* infection, or a *C. difficile* infection.

[00204] In a specific embodiment, a composition described herein is administered in conjunction with one or more of vancomycin, sulfa drug (e.g., co-trimoxazole/trimethoprim-sulfamethoxazole), tetracycline (e.g., doxycycline, minocycline), clindamycin, oxazolidinones (e.g., linezolid), daptomycin, teicoplanin, quinupristin/dalfopristin (synercid), tigecycline, alicin, bacitracin, nitrofurantoin, hydrogen peroxide, novobiocin, netilmicin, methylglyoxal, and bee defensin-1. A composition described herein may also be administered in conjunction with a dressing comprising one or more of hydrogen peroxide, tobramycin, chlorhexidine gluconate, chlorhexidine gluconate, levofloxacin, and silver. In one embodiment, a composition described herein is administered with one or more of the listed agents to treat or prevent a *S. aureus* infection, and particularly, an MRSA infection.

[00205] In some embodiments, the compositions described herein are administered before (e.g., 1 minute, 15 minutes, 30 minutes, 1 hour, 2 hours, 3 hours, 6 hours, 12 hours, 24 hours or more before, or any time period in between), simultaneously with, or after (e.g., 1 minute, 15 minutes, 30 minutes, 1 hour, 2 hours, 3 hours, 6 hours, 12 hours, 24 hours or more after, or any time period in between) administration of another therapy. For example, such compositions maybe administered before, simultaneously with or after administration of an anti-bacterial agent (e.g., an antibiotic).

[00206] In some of these embodiments, the compositions described herein may be administered to a patient to treat or prevent a bacterial infection or a disease associated with bacterial infection after the patient has undergone a course of treatment of the bacterial infection with another anti-bacterial agent (e.g., an antibiotic). In some embodiments, the compositions described herein may be administered to a patient who has developed resistance to one or more anti-bacterial agents (e.g., an antibiotic). In one embodiment, the compositions described herein may be administered to a patient who has undergone a course of treatment with an antibiotic (e.g., an antibiotic standardly used for the treatment of such bacterial infection) and developed resistance to such antibiotic.

[00207] However, in certain embodiments, a sNAG nanofiber composition is administered alone. In one such embodiment, a sNAG nanofiber composition is not administered with any other therapies, for example, it is not administered with an immunomodulator, an antibacterial agent (e.g., an antibiotic), a defensin peptide, a defensin-like peptide, a pain relief therapy (e.g., an analgesic), or a fever relief therapy. In one embodiment, a sNAG nanofiber composition is not administered in conjunction with an antibiotic. In certain embodiments, sNAG nanofiber compositions are not administered in conjunction with an anti-viral agent, an anti-fungal agent or an anti-yeast agent.

5.8 Kits

[00208] A pharmaceutical pack or kit which comprises any of the above-described sNAG compositions is also contemplated. The pack or kit may comprise one or more containers filled with one or more ingredients comprising the compositions described herein. The composition is preferably contained within a sealed, water proof, sterile package which facilitates removal of the composition without contamination. Materials from which containers may be made include aluminum foil, plastic, or another conventional material that is easily sterilized. The kit can contain material for a single administration or multiple administrations of the composition, preferably wherein the material for each administration is provided in a separate, waterproof, sterile package.

[00209] In another embodiment, a container having dual compartments is provided. A first compartment contains any of the above-described sNAG compositions, while the second compartment contains another active agent such as another anti-bacterial agent. In the field or the clinic, the composition in the first compartment can be readily combined with the agent in the second compartment for subsequent administration to a patient.

[00210] Additionally, a kit designed for emergency or military use can also contain disposable pre-sterilized instruments, such as scissors, scalpel, clamp, tourniquet, elastic or inelastic bandages, or the like.

[00211] Optionally associated with such kit or pack can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration. For example, a kit can comprise a notice regarding FDA approval and/or instructions for use.

[00212] The kits encompassed herein can be used in the above applications and methods.

6. EXAMPLES

6.1 Example 1: sNAG Nanofibers From a Marine Diatom Promote Wound Healing and Defensin Expression via an Akt1/Ets1-Dependent Pathway

[00213] This example demonstrates that sNAG nanofibers promote cutaneous wound healing and expression of defensins, and that the Akt1—>Ets1 pathway plays a central role in the regulation of cutaneous wound healing by sNAG nanofibers.

6.1.1 Materials and Methods

[00214] sNAG/Taliderm nanofibers are produced and supplied by Marine Polymer Technologies and formed into suitable patches for wound treatment. Wildtype C57 Black and Akt1 null mice were housed at the Medical University of South Carolina animal facilities. Wildtype and Akt1 null mice, ages ranging from eight to 12 weeks, were anesthetized with 50% pure oxygen and 50% isoflurane gas. Immediately before wounding, Nair Hair Removal Lotion was applied to their dorsum to remove any unwanted hair. A dorsal 4mm circular area of skin was removed using an excision biopsy punch. Taliderm was placed onto each wound at day 0 or wounds were left untreated. At days 1,3,5, and 7 the wounds were photographed, measured, and excised using an 8 mm biopsy punch to ensure complete removal of the wound and surrounding skin. Wildtype and Akt1 null wounds with and without Taliderm treatment were embedded in paraffin in preparation for H&E and immunofluorescent staining.

[00215] Paraffin-embedded sections were sectioned and placed on microscope slides for staining. Slides were washed with xylenes to remove paraffin and rehydrated through a series of graded alcohols. The sections were then incubated in 0.1% Triton x100 for permeabilization. Sections were incubated in a boiling Antigen Retrieval solution. 1% Animal serum was used for blocking before incubating in the primary goat antibody, β -defensin 3 1:400 dilution. The sections were then incubated in the primary antibody overnight at 4° in a humidity chamber. An immunofluorescent secondary Donkey α -goat 488 antibody 1:200 dilution was used, followed by nuclear staining with TOPRO-3. Images were captured using confocal microscopy.

[00216] Hematoxylin and eosin staining was used to visualize basic structures such as the epidermis, dermis, muscle, and blood vessels and to determine the orientation and approximate location in the wound. H&E staining was also used to begin to identify which cell types are stimulated by Taliderm in an Akt1-independent manner.

[00217] Other materials and methods are described in figure descriptions and in the results section below, and performed in accordance with the methods known in the art.

6.1.2 Results

[00218] *sNAG Nanofibers stimulate Akt1 activation, an upstream regulator of Ets1.* Figure 1A shows a Western blot analysis of phospho-Akt in response to NAG and sNAG stimulation of serum starved EC. Figure 1B shows RT-PCR analysis of EC infected either with scrambled control or Akt1 shRNA lentiviruses and assessed for expression of Ets1 and S26 as a loading control. Figure 1C illustrates a signal transduction pathway transducing a signal from sNAG nanofibers to Akt1, Ets1 and Defensins.

[00219] *Delayed wound healing in Akt1 null animals is partially rescued by Taliderm (sNAG) treatment.* Figure 2A shows representative images of wounded WT and AKT1 null mice with and without treatment of Taliderm. Figure 2B shows H&E staining of representative mouse skin sections from day 3 wounds. H&E staining of wildtype and Akt1 wound excisions indicate a Taliderm dependent increase in keratinocyte proliferation and migration. The dashed lines indicate the area of keratinocyte proliferation across the wound margin. In both the wildtype and Akt1 treated wounds there is an evident increase in reepithelialization across the wound margin compared to the wildtype and Akt1 control. This indicates that Taliderm increases keratinocyte recruitment independent of the Akt1 pathway. Although Taliderm induces a complete reepithelialization of the epidermis across the wound margin, there is still substantial lack of revascularization in the underlying tissue compared to the wildtype. This is evident by substantial hemorrhaging and infiltration of red blood cells in the Akt1 animals.

[00220] *sNAG nanofibers stimulate cytokine and defensin expression in primary endothelial cells.* Figure 3A shows immunohistochemistry of EC treated with or without sNAG using an antibody directed against α -defensin. Figure 3B presents ELISA showing that nanofiber treatment of EC results in the secretion of α -defensins 1-3.

[00221] *sNAG nanofibers stimulate defensin expression in primary endothelial cells in an Akt1 dependent manner.* Figures 4A and 4B show quantitative RT-PCR analyses of serum starved EC treated with or without sNAG, with or without PD98059 (MAPK inhibitor), Wortmannin (PI3K inhibitor) or infected with a scrambled control or Akt1 shRNA lentiviruses and assessed for expression of the genes indicated.

[00222] *sNAG nanofibers stimulate β -defensin 3 expression in mouse keratinocytes.* Figure 5A shows immunofluorescent staining with β -defensin 3 and Involucrin antibodies of paraffin embedded mouse cutaneous wound sections from WT and Akt1 null animals on Day 3. A cutaneous wound healing model was developed in both WT and Akt1 null mice to assess the effects of Taliderm *in vivo*. These findings show that β -defensin 3 expression increases in Taliderm treated animals in an Akt1-dependent manner. The ability of Taliderm to increase defensin expression in a healing wound has important implications for treating and controlling wound infection. Figure 5B shows quantification of β -defensin 3 immunofluorescent staining using NIHImageJ software. Figure 5C shows immunofluorescent staining of WT and Akt1 null treated and untreated keratinocytes with β -Defensin 3 and TOPRO-3. Notice the increase in green β -Defensin 3 staining in WT and Akt1 Taliderm treated wounds. The immunofluorescent labeling of wound sections illustrates that Taliderm treated wounds show an increase in β -defensin 3 expression in an Akt1 dependent manner. Although the Akt1 treated wounds show a reasonable increase in β -defensin 3, the wildtype treated wounds illustrate a more remarkable increase. This indicates that β -defensin 3 expression is not only increased by application of the nanofiber, but is at least partially dependent on the Akt1 pathway. β -defensin 3 expression seems limited to the keratinocytes indicating this expression is keratinocyte specific.

[00223] *Akt1 dependent transcription factor binding sites.* Figure 6 shows schematic of Akt1 dependent transcription factor binding sites. Using Genomatix software, 500 bp upstream of the transcription start site was analyzed for conserved sites on the mRNA of DEF1, 4, and 5.

6.1.3 Conclusions

[00224] The provided data show that sNAG nanofiber stimulation of Ets1 results from the activation of Akt1 by these nanofibers. Nanofiber treatment resulted in marked increases in the expression of genes involved in cellular recruitment, such as IL-1 (a known Ets1 target), VEGF and several defensins (β 3, α 1, α 4, and α 5), small anti-microbial peptides recently shown to act as chemoattractants. Both pharmacological inhibition of the PI3K/Akt1 pathway and Akt1 knockdown using shRNAs resulted in decreased expression of these chemotactic factors. Akt1 null mice exhibited a delayed wound healing phenotype that is partially rescued by Taliderm nanofibers. Taliderm treated wounds also showed an increase in defensin expression that is Akt1 dependent.

[00225] The increase of β -defensin 3 expression and keratinocyte proliferation in Taliderm treated wounds demonstrates the beneficial use of Taliderm as an effective wound healing product. Taliderm acts to increase anti-microbial peptide expression in keratinocytes in an Akt1 dependent manner suggesting the essential role of Akt1 in the function of sNAG nanofibers. This correlates with the results from other studies in the laboratory (Buff, Muise-Helmericks, unpublished) that inhibition of the PI3K/Akt1 pathway and Akt1 knockdown using shRNAs results in decreased expression of these chemotactic factors.

[00226] Although the increased expression of β -defensin 3 is Akt1-dependent, H&E staining of 8 mm wound excisions (Figure 2B) indicated that Taliderm acts independent of Akt1 in wound reepithelialization. Even though the new keratinocytes span the entire wound margin, the underlying tissue did not demonstrate the same stimulation in vascular growth. This indicates the that absence of Akt1 is responsible for leaky blood vessels and the large amount of floating red blood cells in the dermis. This suggests that Taliderm is dependent on the Akt1 pathway for an increase in vascularization.

[00227] In summary, (i) sNAG nanofibers (Taliderm) increase wound healing in part by stimulating angiogenesis; (ii) sNAG nanofibers treatment of endothelial cells activate an Akt1/Ets1 dependent pathway leading to changes in cell motility and cytokine secretion; (iii) Taliderm treated wounds show increased expression of β -defensin 3 in an AKT1 dependent manner; (iv) treatment of Akt1 null animals with Taliderm partially rescues the phenotype, leading to markedly increased keratinocyte proliferation/migration; and (v) bioinformatics analysis indicates that ETS1 is likely involved in the sNAG activated pathway leading to increased wound healing and cytokine secretion.

[00228] Taken together these findings suggest a central role of the Akt1 \rightarrow Ets1 pathway in the regulation of cutaneous wound healing by sNAG nanofibers and support the use of these nanofibers as a novel and effective method for enhancing wound healing.

6.2 Example 2: sNAG Nanofibers Increase Defensin Expression, Increase Kinetics of Wound Closure, and Have an Indirect Defensin-Dependent Anti-Bacterial Effect.

[00229] This example demonstrates that sNAG nanofibers have a potent anti-bacterial effect against *Staphylococcus aureus* in vivo, which is indirect and defensin-dependent. This example also shows that sNAG nanofibers induce expression of defensins in vitro in keratinocytes and

endothelial cells and *in vivo* in cutaneous wounds, in an Akt-1 dependent manner, and increase the kinetics of wound closure.

6.2.1 Materials and Methods

[00230] *Tissue Culture, Pharmacological Inhibition, ELISA:* Human umbilical cord vein EC (Lonza) were maintained at 37° with 5% CO₂ in endothelial basal medium 2 (Lonza). Endothelial basal medium 2 (EBM2) was supplemented with EC growth medium 2 SingleQuots as described by Lonza procedures and 1% penicillin/streptomycin (Invitrogen). Serum starvation was performed at 80-90% confluence in EBM2 supplemented with 0.1% fetal calf serum (Valley Biomedical) for 24 hours followed by stimulation with highly purified pGlcNAc (50µg/ml) nanofibers (sNAG) in sterile water (provided by Marine Polymer Technologies, Inc., Danvers, Mass., USA). The pGlcNAc diatom-derived nanofibers used in this study are short biodegradable fibers derived from a longer form (NAG), and have an average length of 4-7µm and a polymer molecular weight of approximately 60,000Da. For inhibition using PD098059 (50µM) or wortmannin (100nM), cells were pre-treated for 45 minutes prior to 3 hour stimulation with sNAG (50µg/ml).

[00231] *Statistical Analysis:* Each quantitative experiment was performed at least in triplicate at least three independent times. All statistical analyses were performed using Microsoft Excel to calculate means, standard deviations and student t-test.

[00232] *Lentiviral Infection:* Mission shRNA lentiviral constructs directed against Akt1 were purchased from Sigma/Aldrich. A scrambled pLKO.1 shRNA vector was purchased from Addgene. Lentiviruses were propagated in 293T cells, maintained in DMEM supplemented as above. Lentiviral production was performed using psPAX2 and pMD2.G packaging vectors purchased from Addgene using the protocol for producing lentiviral particles from Addgene. For infection of target cells, 7.5 X 10³ cells were plated on 100 mm² plates and allowed to incubate overnight. The next day, cells were transduced using a final concentration of 1 µg/ml polybrene and either scrambled control or Akt1 shRNA lentiviruses. After transduction, endothelial cells were serum starved overnight and stimulated with sNAG (50g/ml) for 3 hours. All infections were monitored for appropriate knockdown by RT-PCR.

[00233] *RT-PCR:* For semi-quantitative RT-PCR, RNA was extracted with RNeasy (QIAGEN) following manufacturer's instructions. cDNA was synthesized from 2 µg total RNA with a Superscript First Strand Synthesis Kit (Invitrogen), using Oligo(dT) following the

manufacturer's instructions. PCR reactions contained equal amounts of cDNA and 1.25 µM of the appropriate primer pair (Sigma- Proligo, St. Louis, MO, USA). All primer sequences used in these analyses are as follows:

Akt1 F	5' GAGGCCGTCAGCCACAGTCTG 3'
Akt1 R	5' ATGAGCGACGTGGCTATTGTG 3'
β-Defensin3 F	5' GTGGGGTGAAGCCTAGCAG 3'
β-Defensin 3 R	5' TTTCTTCTTCGGCAGCATT 3'
α-Defensin1 F	5' CACTCCAAGCAAGAGCTGAT 3'
α-Defensin1 R	5' TCCCTGGTAGATGCAGGTTTC 3'
S26 F	5' CTCCGGTCCGTGCCCTCCAAG 3'
S26 R	5' CAGAGAATAGCCTGTCTTCAG 3'

[#0234] Cycling conditions were: 94°C for 5 min; 30-35 cycles of 94°C for 1 min, 55-65°C (based on primer T_m) for 1 min, 72°C for 1 min; 72°C for 7 min and cooled to 4°C. Cycle number was empirically determined to be within the linear range of the assay for each primer pair used. All semi-quantitative RT-PCR was performed with the ribosomal protein subunit S26 primers as internal controls. Products were visualized on a BioRad Molecular Imaging System (Hercules, CA, USA). Real time PCR was performed using a Brilliant CYBR green QPCR kit in combination with an Mx3000P Real-Time PCR system both purchased from Stratagene. Primers detecting the ribosomal subunit S26 were used as internal controls.

[#0235] *Excisional Wound Healing Model:* Wild Type C57BL/6 and Akt1-/- [43] were used in all experiments. The Akt1 null animals were created using an insertional mutagenesis strategy at the translational start site that blocks expression of the entire protein. Wounding was performed on anesthetized adult male mice between 8-12 weeks old. Two full thickness cutaneous wounds were created using a 4mm biopsy punch (Miltex), to create two identical wounds on each flank. Mice were anesthetized using an O₂/Isoflurane vaporizing anesthesia machine (VetEquip, Inc.). Isoflurane was used at 4% for induction, 2% for surgery. Prior to surgery hair was removed by depilation and the area was washed and sterilized using 70% ethanol. Wounds were either treated with sNAG membrane moistened with distilled water or left untreated. On days 3 and 5 animals were euthanized and entire wounds were harvested including the surrounding skin using an 8 mm biopsy punch (Miltex). Wounds were fixed in 4% paraformaldehyde overnight at 4°, embedded in paraffin, and sectioned for analysis.

[00236] *Hematoxylin and Eosin Staining (H&E):* All H&E staining was performed in the Histology Core Facility at the Medical University of South Carolina, Department of Regenerative Medicine and Cell Biology. Briefly, sections were cleared in xylene, rehydrated through a series of graded alcohols, placed in Hematoxylin followed by acid alcohol. Samples were then placed in ammonia water, rinsed in ethanol and exposed to Eosin before dehydrating through graded alcohols and clearing in xylene. Sections were mounted using Cytoseal-XYL (Richard-Allan Scientific). H&E sections were visualized using an Olympus BX40 microscope (4x objective lens, 0.13) and captured using an Olympus Camera (Model DP25) and DP2-BSW acquisition software.

[00237] *Bacterial Inoculation, Tissue Gram Staining, Colony Forming Unit Quantitation:* Male mice between 8-12 weeks were wounded as described above. Single colonies of *Staphylococcus aureus* (ATCC 25923) were picked and cultured overnight at 37° and adjusted to an absorbance of OD₆₀₀= 0.53. One mL of *S. aureus* was spun at 10,000rpm, re-suspended in sterile PBS, and 15µl was used to inoculate each wound. sNAG membranes were applied to the treated group thirty minutes post inoculation. Mice were euthanized on day 3 and 5 post wounding and wounds were harvested using an 8 mm biopsy punch. One wound per animal was fixed overnight in 4% paraformaldehyde at 4°C and the other wound was cultured and plated on LB media without antibiotic for bacterial quantitation (see below). Wounds for tissue gram staining were embedded in paraffin and sectioned. Sections were cleared in xylene and rehydrated through a series of alcohol and were stained using a tissue gram stain (Sigma-Aldrich) by procedures described by the manufacturer.

[00238] For culturing, wound sections were placed in 0.5ml bacterial media and incubated for 30 min at 37oC while shaking. Colony forming units (CFU) were quantitated using a dilution series plated overnight at 37°C. Number of colonies per plate/per dilution were counted and CFU/ml were calculated.

[00239] To determine CFU/ml from sNAG treated bacterial cultures, *S. aureus* cultures in solution were treated with varying concentrations of sNAG (10µl and 20 µl of 10.8 mg/ml sNAG) for three hours. Cultures were then plated overnight at 37° and CFU/ml were determined.

[00240] *β-defensin 3 Peptide Application:* Three test concentrations (1.0µM, 2.5µM, 5.0µM) of biologically active human β-defensin 3 peptide (Peptide Institute, Inc.) were tested for their effect on bacterial growth in the infected wound healing model described above. Each

concentration negatively affected bacterial growth so the lowest concentration was chosen for analyses. After each wound was infected with *S. aureus*, 10ul of peptide was applied. After three days, wounds were harvested, embedded for sectioning and gram staining, or cultured for CFU/ml quantitation as described above.

[00241] *β -defensin 3 Antibody Blockade:* Wild Type male mice were wounded and infected with 15ul of *S. aureus* as described above. After inoculation, one wound was treated with 0.2ug/mL of β -defensin 3 antibody (Santa Cruz) while the other was treated with 0.2ug/mL of normal goat IgG control antibody (Santa Cruz). sNAG membranes were applied to all mice after antibody treatment on day 0. Antibody was applied every 24 hours. Mice were euthanized on day 3 and wounds were harvested using an 8 mm biopsy punch. Wounds were fixed overnight in 4% paraformaldehyde at 4°C, embedded in paraffin, sectioned, and analyzed using tissue gram stain. CFU/ml quantitation was performed from wounds harvested on day 3 as described above.

[00242] *Immunofluorescence, Microscopy:* Paraffin embedded tissue sections were rehydrated through xylene and a series of graded alcohols. Sections were treated with 0.01% Triton-X100 and subjected to antigen retrieval using antigen unmasking solution (Vector Laboratories) in a pressure cooker for 5min and allowed to cool. Skin sections were labeled with β -defensin 3 goat polyclonal antibody (Santa Cruz), involucrin rabbit polyclonal antibody (Santa Cruz), and TO-PRO 3-iodide (Molecular Probes). Sections were incubated in primary antibody overnight at 4° and appropriate secondary immunofluorescent antibodies (Invitrogen) for 1 hour at room temperature. Control sections for each antibody were stained without primary antibody. Tissue sections were visualized using an Olympus FluroView laser scanning confocal microscope (Model IX70) and captured at ambient temperature using an Olympus camera (Model FV5-ZM) and Fluoview 5.0 acquisition software. All tissue sections were imaged using 60x oil immersion lens (Olympus Immersion Oil)

[00243] HUVECs were either serum starved or treated with sNAG for 5 hours in culture and stained with antibodies directed against α -defensin 5 (FITC), β -defensin 3 (Texas Red), or TOPRO 3 (Blue). Images were taken using immunofluorescent microscopy. Cell culture defensin expression was visualized using a Zeiss Axiovert 100M confocal microscope and was captured at ambient temperature, using water as the medium, using LSM 510 camera (Zeiss Fluor 63xW/1.2A objective).

[00244] *Western Blot Analysis:* Endothelial cells were serum starved prior to stimulation with sNAG (50µl/ml) for a given time course. Cells were then lysed and subjected to Western blot analysis. The antibodies used for Western blot analysis are as follows: anti-p85 subunit of PI3K and phosphospecific Akt antibody (Cell Signalling Technologies).

6.2.2 Results

6.2.2.1. Keratinocytes and Endothelial Cells Express and Secrete Defensins When Stimulated With sNAG

[00245] This example demonstrates that sNAG treatment modulates the expression of defensins, small anti-microbial peptides that are part of the innate immune response.

[00246] To investigate the affect of sNAG treatment on defensin expression *in vitro*, primary human umbilical vein endothelial cells in culture were used. Endothelial cells express both α-type and β-type defensins when stimulated with sNAG. As shown in Figure 7A endothelial cells treated with sNAG show an up-regulation of β-defensin 3 and α-defensin 1 mRNA expression within 1 hour of stimulation. Similar up-regulation of α-defensin 4 and 5 by sNAG treatment was also observed (data not shown). Custom gene arrays containing over 25 different defensin genes were used to confirm the expression of the α-type defensins in primary endothelial cells and the β-type defensins in keratinocytes. sNAG stimulation of endothelial cells was shown to increase the expression specifically of α-defensins 1, 4 and 5 and β-defensin 3. Additionally, sNAG stimulation of human keratinocytes increased expression of β-defensin like genes, several of which are listed in Table I. These findings suggest that at least three α-defensin genes and β-defensin 3 are expressed in primary endothelial cells and multiple β-defensin genes are expressed in primary keratinocytes in response to sNAG stimulation.

Table I: Gene array analysis reveals numerous defensin genes upregulated by sNAG

HUVEC	Gene Name	Fold Change	Keratinocyte	Gene Name	Fold Change
	α-defensin 1	+1.36		β-defensin 1	+1.4
	α-defensin 4	+2.74		β-defensin 126	+1.73
	α-defensin 5	+2.46		β-defensin 105B	+2.55
	β-defensin 1	+2.19		β-defensin 123	+1.65
	β-defensin 4	+3.06		β-defensin 129	+1.46

[00247] To test whether the sNAG-dependent defensin expression also occurred on the protein level, sNAG stimulated endothelial cells were subjected to immunofluorescence using antibodies directed against both α and β defensins. As shown in Figure 7B, both β -defensin 3 and α -defensin 5 are up-regulated upon sNAG stimulation in this cell type. However, stimulation of primary human keratinocytes (HaCat) with sNAG did not cause increased expression of α -defensin but does cause an increase in the expression of β -defensin 3 (Figure 7C). Taken together, these experiments suggest that sNAG stimulation results in an up-regulation of defensin peptides in both primary keratinocytes and primary endothelial cells.

6.2.2.2. *sNAG-Dependent Defensin Expression Requires Akt1*

[00248] Previously published data show that sNAG stimulation of primary endothelial cells results in increased integrin activation, Ets1 expression and MAP kinase activation. (Vourmakis, J.N., et al., 2008, *J Vasc Res*, 45(3):222-32.) Findings position Akt1 upstream of Ets1 in endothelial cells and in *Drosophila*. (Lavenburg, K.R., et al., 2003, *FASEB J*, 17(15): 2278-80.) To begin to determine the signaling pathway responsible for the expression of defensins, endothelial cells were serum starved and pre-treated with pharmacological inhibitors directed against PI3K (wortmannin) or MAP kinase (PD098059) prior to sNAG stimulation. Quantitative real time PCR analysis shows that α -defensin 1 mRNA levels are greatly diminished after inhibition of either the PI3K/Akt pathway or the MAP kinase pathway (Figure 8A). RT-PCR analysis of β -defensin 3 also shows that levels are decreased by the inhibition of these pathways as well (Figure 8B). sNAG treatment of endothelial cells for a short time course leads to phosphorylation of Akt1, a standard indicator of its activation (Figure 8C). To confirm that Akt1 is indeed required for defensin expression, lentiviral delivery of shRNA directed against Akt1 was used. Quantitative RT-PCR of serum starved endothelial cells infected with scrambled (SCR) control or Akt1 shRNA followed with sNAG treatment confirms that Akt1 expression is required for sNAG-dependent α -defensin expression (Figure 8D). Since β -defensins are known to be expressed in epithelial cells, lentiviral delivery of shRNA directed against Akt1 was used in human keratinocytes (HaCat). sNAG treatment of serum starved keratinocytes infected with scrambled (SCR) control leads to a significant increase in β -defensin 3 expression that is abrogated by Akt1 knockdown (Figure 8E). These results illustrate that sNAG treatment activates Akt1 in endothelial cells and strongly suggest that sNAG-dependent defensin expression requires Akt1 in both endothelial cells and keratinocytes.

6.2.2.3. sNAG Treatment of Cutaneous Wounds Increase Defensin Expression

In Vivo

[00249] To confirm the dependence of Akt1 for the expression of defensins *in vivo*, wild type and Akt1 null animals were used in an excisional wound healing model. Although most mammalian leukocytes express α -defensins (human, rabbit, rat, and hamster), mouse leukocytes do not express α -defensins. Therefore, β -defensin expression in these mouse models was focused on. Treatment of cutaneous wounds with a dried form of sNAG, a thin biodegradable membrane, for three days results in a statistically significant increase in β -defensin 3 expression in keratinocytes of wild type animals (Figure 9A). Involucrin (Watt, F.M., 1983, J Invest Dermatol. 81(1 Suppl):100s-3s) staining (red) was used to mark the keratinocyte cell layers and show that the expression of β -defensin 3 is confined to the epidermal layer. To assess if sNAG-dependent defensin expression is dependent on Akt1, a similar assay was performed using an Akt1 null animal model. Wounds from Akt1 null mice treated with sNAG membranes show a markedly reduced induction of β -defensin 3 expression (Figure 9A). To better visualize the epidermal layers that are expressing β -defensin 3, Figure 9B shows a representative image of a sNAG treated wild type wound harvested on day 3. sNAG treatment of cutaneous wounds induced β -defensin 3 expression mainly in the suprabasal layers of skin (Figure 9B). Quantitative analyses shown in Figure 9C shows an approximate 5-fold increase in β -defensin 3 expression in sNAG treated wild type animals and that Akt1 is required for this increase.

6.2.2.4. sNAG Treatment Increases the Kinetics of Wound Closure in WT Animals

[00250] Previous results have shown an increased kinetics of wound closure in diabetic mouse models in response to sNAG treatment. sNAGs were tested for a similar effect in wild type animals. Excisional wounds were created in wild type animals which were either treated with the membrane form of sNAG or left untreated. Tissue sections were taken at 1, 3 and 5 days post wounding and subjected to H&E staining. As shown in Figure 10, sNAG treatment of wild type wounds results in complete closure, as visualized by the solid line, at day 3 post wounding. This occurs two days earlier than in the control wounds. Akt1 null animals display a delay in wound closure; these animals do not fully close the wound until 7 days post wounding. The delay in wound closure in the Akt1 null animals is not rescued by sNAG treatment (data not shown). These findings suggest that sNAG not only induces defensin expression but also increases wound healing kinetics in wild type mice and may be a novel and effective therapeutic.

6.2.2.5. sNAG Is an Effective Antimicrobial Against *S. aureus*

[00251] Defensin peptides are known to possess antimicrobial properties that are active against gram-positive and gram-negative bacteria. Since treatment of endothelial cells with sNAG increases defensin expression (both α - and β -type) and treatment of cutaneous wounds with sNAG dramatically increases β -defensin 3 expression *in vivo*, the antimicrobial efficacy of sNAG treatment in bacterially infected wounds was assessed.

[00252] To determine if sNAG decreases bacterial load in cutaneous wounds, wild type and Akt1 null animals were subjected to cutaneous wound healing, followed by infection with *Staphylococcus aureus*. Infected wounds were either treated with sNAG or left untreated for 3 and 5 days post infection. As shown by the tissue gram staining in Figures 11A and 11B, wild type animals treated with sNAG show a significant reduction in gram positive staining by day 5 post wounding as compared with untreated wounds. In contrast, gram stained tissue derived from untreated wounds in Akt1 null animals at 5 days post wounding show an accumulation of neutrophils which stain gram positive (Figure 11B), indicating a potential lack of bacterial clearance in these animals that is not rescued by sNAG treatment. These findings suggest that Akt1 null animals have a defect in immune clearance mechanisms which is not rescued by sNAG treatment.

[00253] To quantitate sNAG-specific bacterial changes in colony forming units (CFU), infected wounds from both wild type and Akt1 null mice either sNAG treated or untreated were harvested and cultured. As shown in Figure 11C, at 5 days post wounding bacterial number is markedly reduced (10-fold) in wild type animals treated with sNAG. However, although the number of bacteria detected in the Akt1 null animals is reduced in comparison to wild type, sNAG treatment had a little effect on absolute bacterial number in the Akt1 null animals. At 3 days post-infection (Figure 11D), there is a similar 10-fold decrease in CFU in sNAG treated wild type mice as compared to untreated controls. The sNAG treated Akt1 null animals show a 2-fold decrease in CFU as compared to untreated Akt1 null animals. In general, the Akt1 null animals have a lower bacterial load per wound which may be reflective of an Akt1-dependent effect on other processes in addition to defensin expression. These findings suggest that sNAG treatment results in a marked reduction in bacterial load in infected cutaneous wounds in wild type mice but not in Akt1 null mice, suggesting the possibility that defensins are mediating the anti-bacterial response.

[00254] To show that the antibacterial effect of sNAG treatment is not due to a direct effect of the nanofibers on bacterial growth or on their survival, *S. aureus* bacterial cultures were treated in solution with different amounts of sNAG, for 3 hours and colony forming units were determined. As shown in Figure 11E, sNAG treatment had no direct effect on the growth of *S. aureus*, indicating that sNAG is not directly inhibiting bacterial growth and may then be working via the up-regulation of defensins.

6.3.2.6. Application of Defensin Peptide Mimics the sNAG Antibacterial Effect

[00255] To determine whether addition of defensin peptide can block bacterial infection similarly to that shown for sNAG treatment, wild type mice were wounded and inoculated with *S. aureus* as described above and then treated with biologically active human β -defensin 3 peptide ($1.0\mu\text{m}$) for three days. Tissue biopsies were stained using a tissue gram stain and CFU was quantitated. Figure 11 F-G shows the results of these experiments. Infected mice treated with β -defensin 3 peptide have a decreased bacterial load, an approximate 7.5 fold decrease in viable bacteria (Figure 11G), similar to that shown in wild type mice treated with sNAG.

[00256] One of the mechanisms by which defensin expression is induced is through stimulation by bacterial LPS, possibly through the activation of Toll like receptors. (Selsted, M.E. and A.J. Ouellette, 2005, Nat Immunol. 6(6):551-7.) To test whether bacterial infection alone is able to induce β -defensin expression within the time periods tested, expression of β -defensin was assessed in infected wounds from wild type animals after three days post wounding. As shown in Figure 12A, bacterial infection alone does not induce the expression of β -defensin within 3 days of infection, as is shown with sNAG treatment. However, in wild type animals, sNAG treatment of infected wounds causes approximate 3- to 5-fold increase in the expression of β -defensin within a similar time period (Figure 12B). These findings suggest that sNAG treatment rapidly induces the expression of defensin expression resulting in marked bacterial clearance in *S. aureus* infected wounds.

6.3.2.7. Antibodies Directed Against β -Defensin 3 Block the Antibacterial Effect of sNAG

[00257] Since defensins are secreted proteins, the inventors hypothesized that antibodies directed against β -defensin 3 may be able to block the antibacterial activities. To test this hypothesis, wounds were created, infected with *S. aureus* and treated with sNAG as described above. The wounds were either treated with a β -defensin 3 antibody or an isotype control; one

application each day for three days. Wound sections were obtained and stained for gram positive bacteria. As shown in Figure 13A, sections derived from wounds treated with β -defensin antibody have more gram positive bacteria than those treated with isotype control antibodies. Each section shown was derived from the wound area directly under the scab. Quantitation of CFU in these wounds shows that neutralization of β -defensin 3 prior to sNAG treatment in *S. aureus* infected wounds results in a significant increase in bacteria. Animals that were treated with an IgG isotype control show an approximate 5-fold reduction in viable bacteria (Figure 13B). Taken together, these results suggest that sNAG treatment not only results in the increased kinetics of wound healing but also promotes an endogenous anti-bacterial response and supports the use of this nanofiber as novel therapy to enhance wound healing while concurrently decreasing wound infection.

6.2.3 Conclusions

[00258] The findings presented here demonstrate that a marine diatom derived nanofiber, sNAG, may be used as a novel and effective method to enhance wound healing while concurrently decreasing wound infection. The data demonstrates that this FDA approved material, which is presently used for hemostasis, stimulates the expression of both α -type and β -type defensins in primary endothelial cells and an up-regulation of the β -type in primary keratinocytes.

[00259] Defensins are an essential component of the innate immune system. These peptides possess anti-microbial properties that are active against gram-positive and negative bacteria, fungi, and many viruses. Defensins are small (3-4 kDa), cysteine-rich cationic peptides found in mammals, insects, and plants that are classified into different families (α , β , and θ) based on their pattern of disulfide bonding. α -defensins are thought to be specific to neutrophils, are found in very high concentrations (comprising approximately 5-7% of the total cellular protein) (Ganz, T., and R.L. Lehrer, 1994, Curr Opin Immunol. 6(4):584-9), and are secreted during anti-microbial responses (Ganz, T., 1987, Infect Immun. 55(3):568-71). It has also been shown that rabbit alveolar macrophages possess α -defensins in levels comparable to rabbit neutrophils. (Ganz, T., et al., 1989, J Immunol. 143(4):1358-65.) β -defensins are found in epithelial cell types such as keratinocytes, mucosal epithelial cells (Harder, J., et al., 1997, Nature 387(6636):861; and Harder, J., et al., 2001, J Biol Chem. 276(8):5707-13), oral cavity tissues and salivary secretions (Mathews, M., et al., 1999, Infect Immun. 67(6):2740-5), and kidney where they can be up-

regulated in response to infections or inflammatory stimuli (Ganz, T. and R.I. Lehrer, 1994, *Curr Opin Immunol.* 6(4):584-9). Human β -defensin 1 (hDEFB1) is one of the most important antimicrobial peptides in epithelial tissues. Defensin expression and secretion could be extremely important for creating wound therapeutics. The anti-microbial action by defensins is considered part of innate immunity and is non-specific and broad spectrum. Therefore acquired bacterial resistance, as seen with the overuse of antibiotics, is not an issue.

[#0260] The data presented here also demonstrate that both *in vitro* and *in vivo* Akt1 is required for defensin expression. sNAG treatment decreases *Staph aureus* infection of cutaneous wounds in wild type control animals but not in similarly treated Akt1 null animals. It is also important to note that sNAG stimulation of wild type cutaneous wounds results in an increased kinetics of wound closure. Antibody blockade of β -defensin results in a reduction in the sNAG-antibacterial activity. Taken together these findings suggest a central role for Akt1 in the regulation of defensin expression that is responsible for the clearance of bacterial infection and that sNAG treatment activates these pathways in wild type animals.

[#0261] The data that suggests that sNAG treatment of infected wounds could drastically decrease bacterial load in patients, at least in part, by the induction of defensin expression. *Staphylococcus aureus* is a bacterium frequently found colonizing the skin and in the nose. It is still a common cause of nosocomial infections, often causing postsurgical wound infections. *S. aureus* infections in hospitals have plagued healthcare workers for years and the widespread usage of antibiotics for treatment has lead to antibiotic resistant strains. The data presented herein shows that treatment of Staph infected wounds with sNAG dramatically decreased the bacterial load. For example, the lack of dark purple gram staining in the treated WT mice in Figures 11A and 11B indicates that the *S. aureus* infection has been cleared from these wounds. Both the *in vitro* and *in vivo* data provides strong evidence for the use of Taliderm/sNAG in the treatment of wounds to decrease bacterial infection and therefore enhance wound healing.

[#0262] Control experiments indicate that the antibacterial effect of sNAG is not due to a direct interaction of the material with the bacteria but is due to downstream affects such as the regulation of defensins by Akt1 activation. It is widely accepted that defensins are important players in innate immunity and function in antimicrobial activities. Most of the evidence for their function is the direct killing of bacteria by *in vitro* mixing experiments with purified defensin peptides (Selsted, M.E. and A.J. Ouellette, 2005, *Nat Immunol.* 6(6):551-7) or in

similar experiments as shown in Figure 11 with direct application of the purified active peptide. The data here show that an induction of defensin expression in wild type animals using a topical application of sNAG results in an antibacterial response. It has recently been shown that transgenic mouse models expressing the human defensin 5 gene are resistant to *S. typhimurium*, an infection that results in death of wild-type animals (Salzman, N.H., et al., 2003, *Nature* 422(6931):522-6) again suggesting the importance of defensins in the regulation of the antimicrobial response.

[80263] It has been accepted that the α -subtype of defensins are specifically expressed in neutrophils, whereas the β -type defensins are epithelial in origin. β -type defensin expression induced in response to sNAG in human keratinocytes both in culture and in the cutaneous wound healing model was detected. The *in vivo* data illustrates that β -defensin 3 is mainly expressed in the suprabasal layers after treatment with sNAG. This is consistent with previous data which localized human β -defensin 2 to the spinous and granular layers of the skin. (Oren, A., et al., 2003, *Exp Mol Pathol.* 74(2):180-2.) The skin is in constant contact with injury and infection and functions not only as a mechanical barrier but also maintains the ability to mount an active defense against infection. The expression of β - defensin in the outer layers of skin supports their role in cutaneous innate immunity. However, the data show that sNAG specifically stimulates the expression of three different α -defensins (1, 4 and 5) in endothelial cells. This is shown by RT-PCR, gene array analysis, immunofluorescence and ELISA (data not shown). The interaction between endothelial cells and leukocytes in tissue repair is one of the initial and most important steps in wound healing. The process of extravasation of leukocytes from the vasculature is initiated by chemotactic factors, therefore; it is interesting that α -defensins are induced by sNAG and may contribute to the necessary neutrophil/endothelial cellular interactions. More recently, it has come to light that defensins exhibit biological activities beyond the inhibition of microbial cells, including their contribution to the adaptive immune response by exhibiting chemotactic activity on dendritic (Hubert, P., et al., 2007, *FASEB J.* 21(11):2765-75) and T cells, monocytes, and macrophages (Garcia, J.R., et al., 2001, *Cell Tissue Res.* 306(2):257-64) and keratinocytes (Niyonsaba, F., et al., 2007, *J Invest Dermatol.* 127(3):594-604). Previous work shows that human beta defensins 1 and 2 have the ability to chemoattract immature dendritic cells and T cells through the CC-chemokine receptor 6 (CCR6) (Yang, D., et al., 1999, *Science* 286(5439):525-8), and that human beta defensin 2 can

chemoattract TNF α treated neutrophils via the CCR6 receptor (Niyonsaba, F., H. Ogawa, and I. Nagacka, 2004, *Immunology* 111(3):273-81). Human β -defensin 2 and 3 have also been shown to induce chemotaxis by interacting with CCR2, a receptor expressed on macrophages, monocytes, and neutrophils. (Rohrl, J., et al., 2010, *J Immunol*, 2010.) Interestingly, the data show that sNAG treatment induces both α and β -defensin expression in endothelial cells. Taken together, the recent data suggest that defensins may mediate wound healing not only by their antimicrobial properties, but also by being chemotactic for other cell types necessary for proper healing. However, application of β -defensin 3 alone did not result in an increase in wound closure (data not shown) implying that topical application of a single defensin does not sustain the cellular interactions required for increased chemo attraction, cellular recruitment and wound closure.

[00264] The *in vivo* data using both wild type and Akt1 knockout animals confirms the requirement for Akt1 in sNAG-induced β -defensin 3 expression. Since mouse leukocytes do not express α -defensins like most other mammalian leukocytes (Ganz, T., 2004, *C R Biol*, 327(6):539-49) *in vivo* α -defensin staining of infiltrating immune cells was not possible. Treatment of airway epithelial cells *in vitro* with alpha defensins 1-3 causes a dose and time-dependent increased cell migration that requires activation of PI3K and MAPK pathways. (Aarbiou, J., et al., 2004, *Am J Respir Cell Mol Biol*, 30(2):193-201.) sNAG stimulation of endothelial cells has been shown to result in the activation of MAPK (Vournakis, J.N., et al., 2008, *J Vasc Res*, 45(3):222-32) and in data presented here, pharmacological inhibition of MEK also inhibits the expression of the defensins *in vitro*. These findings suggest that both pathways impinge on the regulation of defensin expression by sNAG, however, Akt1 ablation results in a marked reduction of its expression both *in vitro* and *in vivo*. In myeloid cells, β -defensin 1 expression is controlled at the level of transcription, in part, by the Ets-family member PU.1. (Yaneya, M., et al., 2006, *J Immunol*, 176(11):6906-17; and Ma, Y., Q. Su, and P. Tempst, 1998, *J Biol Chem*, 273(15):8727-40.) PU.1 is a downstream target of Akt1 in the B-cell lineage. (Rieske, P. and J.M. Pongubala, 2001, *J Biol Chem*, 276(11):8460-8.) In primary endothelial cells it has been shown that Akt1 is upstream of Ets1 both *in vitro* and *in vivo* during *Drosophila* tracheal development. (Lavenburg, K.R., et al., 2003, *FASEB J*, 17(15):2278-80.) sNAG stimulation of endothelial cells results in increased expression of Ets1 (probably through Akt1)

which is required for the migration of endothelial cells. (Vourmakis, J.N., et al., 2008, *J Vasc Res.* 45(3):222-32.)

[00265] Thus far, sNAG treatment has resulted in a series of downstream activities; hemostasis, cell migration, cell proliferation, increased wound closure, and as described here, stimulation of the innate immune response resulting in anti-bacterial functions.

[00266] Given the dramatic increase of diabetic patients within the population who present with chronic wounds and complications due to wound infection, new clinical treatments are in high demand. Here, marine derived pGlcNAc nanofibers are described that not only increase the kinetics of wound healing but act to stimulate innate immunity thus providing anti-bacterial activity. The obvious importance of these observations is the application to nosocomial infections. Of the nosocomial infections, surgical wound infections predominate; with statistics showing up to 8% of all surgical patients. The direct cost of these types of infections is approximately 4.5 billion dollars per year. Given that defensins are part of the innate immune system, activation of these pathways will preclude the generation of resistant organisms as well as allow for the antibiotic-independent clearance of bacterial infection. Use of sNAG in a hospital setting would defray much of the cost and markedly reduce the production of antibiotic resistant species. Taken together, these findings suggest that these marine derived pGlcNAc nanofibers will be highly beneficial in the clinical arena.

6.3 Example 3: sNAG is an Effective Antimicrobial Against *Pseudomonas aeruginosa*

[00267] This example demonstrates that sNAG nanofibers have an anti-bacterial effect against *Pseudomonas aeruginosa* *in vivo*.

[00268] *Materials and Methods:* Wild Type C57Bl/6 male mice between 8-12 weeks old were wounded created using a 4mm biopsy punch (Miltex), to create two identical wounds on each flank. Mice were anesthetized using an O₂/Isoflurane vaporizing anesthesia machine (VetEquip, Inc.). Isoflurane was used at 4% for induction; 2% for surgery. Prior to surgery hair was removed by *Pseudomonas aeruginosa* were picked and cultured overnight at 37° and adjusted to an absorbance of OD₆₀₀= 0.53. Each wound was inoculated with 1.5x10⁸ cfu/wound of *P. aeruginosa*. After 30 minutes post inoculation, wounds were either treated with sNAG membrane moistened with distilled water (test group, n=6) or left untreated (control group, n=6). On day 3 animals were euthanized and entire wounds were harvested including the surrounding skin using an 8 mm biopsy punch (Miltex). One wound per animal was fixed overnight in 4%

paraformaldehyde at 4°C, embedded in paraffin, and sectioned for analysis, and the other wound was cultured and plated on LB media without antibiotic for bacterial quantitation. For culturing, wound sections were placed in 0.5ml bacterial media and incubated for 30 min at 37°C while shaking. Colony forming units (CFU) were quantitated using a dilution series plated overnight at 37°C. Number of colonies per plate/per dilution were counted and CFU/ml were calculated.

[00269] *Results:* The efficacy of sNAG treatment of wounds infected with gram negative bacteria was assessed. As shown in Figure 14, at 3 days post infection bacterial number is markedly reduced (more than 2 fold) in animals treated with sNAG in comparison to untreated animals. These findings suggest that sNAG treatment results in a marked reduction in bacterial load of gram negative bacteria, and specifically *P. aeruginosa*, in infected cutaneous wounds (in addition to reduction in bacterial load of gram positive bacteria shown in Example 2).

6.4 Example 4: sNAG Nanofibers Upregulate Expression of a Number of Defensins and Toll Receptor Genes.

[00270] This example demonstrates that a number of defensins and Toll-like receptors are up-regulated by sNAG treatment of human endothelial cells.

[00271] *Materials and Methods:* Human Chip probes were printed on epoxy slides. HUVEC cells were cultured as described in section 6.2, and treated with sNAG nanofibers ("sNAG") for 5 hours. RNA was extracted with RNASol (Telttest, Inc.) following manufacturer's instructions, amplified using Amino Allyl MessageAMP™ II aRNA amplification kit (Applied Biosystems), and labeled. The slides were prepared for hybridization with aRNA by soaking in blocking solution (Sigma Tris-buffered saline pH8.0, in 1000ml dH₂O, 1% BSAw/v, NaN₃ to 0.05%) at RT O/N, then rinsed and dried. Samples containing labeled target aRNA from sNAG-treated cells were hybridized with the slides (65ul/slide; denatured at 95°C for 5 min; hybridized for 48 hours at 37°C in 0.1% SDS and 5 X SSC and 1% BSA), rinsed and dried. The slides were scanned and hybridization detected using Perkin-Elmer Scan Array equipment and ScanArray Express software V3.0, updated. To identify up-regulated genes, microarray data was analyzed using Agilent GeneSpring GX v.11 Bioinformation Data Analysis.

[00272] Genes of interest analyzed: IL-1, CEACAM3, SPAG11, defensins ("DEFA"=α-defensin, and "DEFB"=β-defensin); Toll-like receptors ("TLR"), SIGIRR (Single Ig IL-1-related receptor), and TRAF6 (TNF receptor associated factor 6). Positive controls: 1433Z (Tyrosine-3-monohydrogenase/tryptophan 5 monohydrogenase actitton protein); GAPD

(glyceraldehydes-3-phosphate dehydrogenase); RPL13A (Ribosomal protein L13a); UBC (Ubiquitin C); ACTB (Actin B).

[60273] *Results:* Results of the microarray gene chip analyses and Q-PCR validation of microarray results are presented in Tables II-VI below. Using a custom gene chip it was determined that a number of defensins and Toll-like receptors are up-regulated by sNAG treatment of human endothelial cells.

[60274] Toll-like receptors (TLRs) are highly conserved receptors that recognize specific molecular patterns of bacterial components leading to activation of innate immunity. Interestingly, *Drosophila* lack an adaptive immune system but are still resistant to microbial infections. (Imler, J.L. and J.A. Hoffmann, 2000, *Curr Opin Microbiol*, 3(1):16-22.) This host defense is the result of an innate immune system that provides protection by synthesizing the antimicrobial peptides dToll and 18-wheeler which are induced by TLRs. (Lemaire, B., et al., 1996, *Cell* 86(6):973-83; and Williams, M.J., et al., 1997, *EMBO J*, 16(20):6120-30.) Recent work has also linked human defensin expression to TLR activation. Human β -defensin 2 was shown to be induced in airway epithelial cells in a TLR-2 dependent manner. (Hertz, C.J., et al., 2003, *J Immunol*, 171(12): p. 6820-6.) Toll-like receptor 4 has been shown to mediate human β -defensin 2 inductions in response to *Chlamydia pneumoniae* in monocytes. (Romano Carratelli, C., et al., 2009, *PEMS Immunol Med Microbiol*, 57(2):116-24.) Importantly, the PI3K/Akt pathway is a key component in TLR signal transduction, controlling cellular responses to pathogens. (Weichhart, T. and M.D. Saemann, 2008, *Ann Rheum Dis*, 67 Suppl 3:iii70-4.) Since it is known that stimulation of TLRs can lead to increased defensin synthesis, this work suggests the potential for sNAG as a stimulator of innate immunity and bacterial clearance via the activation of Akt1.

Table II: List of some genes up-regulated in response to sNAG stimulation.

Gene	Function
IL-1	Pro-inflammatory cytokine involved in immune defence
CEACAM3	Cell adhesion molecule which directs phagocytosis of several bacterial species
SPAG11	β -defensin-3 like molecule that exhibits antimicrobial properties
Defensins	A series of defensins that exhibit antimicrobial activity
TLRs	Toll-like receptors: Important for stimulation of cellular responses toward infection

GENE	LIGAND/FUNCTION	FOLD INDUCTION
TLR1	Triacyl lipopeptides from bacteria and mycobacteria	7.6

TLR4	LPS, viral proteins, Hsp60 (Chlamydia)	5.064
TLR7	synthetic compounds	3.271
TLR8	synthetic compounds	2.067
TRAF6	Downstream signalling modulator	6.167
SIGRR	IL-1 receptor related TLR modulator	5.895

Table III: Defensin Microarray Gene Expression
(HUVEC Response to sNAG 10ug/ml 5 hours)

Gene Name [Oligo ID]	HUVEC_18s_48h37C normalized(Fold)
D107A_HUMAN [H300005354]	4.2 (2.6 to 5.2)
DEFA4 [H200000646]	4.2 (3.243 to 4.946)
DEFA5 [H2000005803]	4.8 (3.664 to 6.123)
DEFB1 [H200004191]	2.7 (1.7 to 3.7)
DEFB103A [H300008014]	9.8 (7.4 to 12.6)
DEFB118 [H200017001]	2.7 (1.502 to 4.779)
DEFB119 [H300002796]	6.2 (4.68 to 8.04)
DEFB123 [H300009262]	8.9 (7.791 to 11.1)
DEFB124 [H300001942]	3.8 (1.6 to 5.1)
DEFB128 [H200012496]	9.2 (8.286 to 10)
DEFB129 [H300005026]	5.2 (4.338 to 6.277)
ACTB_HUMAN [H300006234]	6.8 (6.603 to 7.284)
GAPD [H200007839]	16.9 (12.81 to 21.13)
RPL13A [opHsV04TC000041]	9.4 (7.311 to 12.01)
UBC [H200014214]	7.2 (6.789 to 9.979)
1433Z_HUMAN [opHsV04TC000038]	0.6 (0.4 to 0.844)

**Table IV: DEFB3 Microarray Gene Validation
(AB Prism 7000; sNAG (10ug/ml), HUVEC for 5 h)**

TaqMan Relative qPCR Fold Change Calculations (ΔΔCt method)

Sample	DEFB3	1433z	$\Delta Ct = DEFB3 - 1433z$	$\Delta\Delta Ct = \Delta Ct_{treated} - \Delta Ct_{untreated}$	Fold difference in DEFB3 relative to untreated
untreated	37.41±0.74	14.71±0.26	22.7±0.78	0.00±0.78	1.4 (1.22-1.7)
treated	40.30±1.0	17.84±0.07	22.46±1.0	-0.24±1.0	1.8 (1.24-2.36)

Table V: Toll-Like Receptors Microarray Gene Expression

Gene Name [Oligo ID]	Fold Change
SIGIRR [opHsV0400002471]	5.895 (3.916 to 7.926)
TLR1 [H3000000701]	7.613 (3.756 to 11.33)
TLR4 [H2000007406]	5.084 (1.085 to 10.66)
TLR7 [H3000008345]	3.271 (1.938 to 3.938)
TLR7 [H3000008395]	3.3 (1.5 to 3.7)
TLR8 [H2000169151]	2.067 (1.8 to 2.2)
TRAF6 [H2000104651]	6.167 (5.3 to 7)

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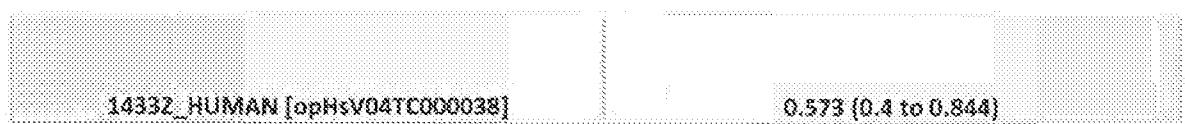


Table VI: Real Time Q-PCR Gene Validation of TLK3 & 4
(HUVEC, 10ug/ml sNAG for 5 h)

Sample	Target C _r ave	Reference (3433) C _r ave	$\Delta C_r = \frac{\text{Target}C_r - 3433rC_r}{\text{Reference}}$	$\Delta\Delta Ct = \Delta Ct_{\text{base}} + \frac{(S_{\text{target}} - S_{\text{reference}})}{S_{\text{reference}}}$		Fold up: $\Delta Ct \pm \Delta Ct \text{ sd}$	Fold down: $\Delta Ct \pm \Delta Ct \text{ sd}$	Fold ave
				$\Delta Ct \pm \Delta Ct \text{ sd}$	$\Delta Ct \pm \Delta Ct \text{ sd}$			
TLK3								
untreated	31.12	2.2	37.84	0.34	23.28	3.25	0	2.37
treated	28.54	0.37	37.53	0.2	35.33	0.42	0.42	0.83
TLK4								
untreated	26.97	0.46	37.84	0.34	9.13	0.56	0	0.56
treated	25.04	0.38	37.53	0.2	7.53	0.43	-2.62	0.43

6.5 Example 5: sNAG and Long Fiber NAG Differ in Their Gene Expression Profiles.

[00275] This example demonstrates that sNAG nanofibers differ from long p-GlcNAc fibers in their effect on gene expression, and specifically in their effect on expression of some of the defensins and Toll-like receptors.

[00276] *Materials and Methods:* Human Defensin Chip probes (concentration: 20uM, quantity 18-20, solvent: SSC based spotting buffer) were printed on epoxy slides using standard techniques. HUVEC and HaCat cells were cultured as described in section 6.2, and treated with either long fibers ("LNAG") or sNAG nanofibers ("sNAG"), for 2 hours or 20 hours. RNA was extracted with RNASol (Teltest, Inc.) following manufacturer's instructions, and amplified using Amino Allyl MessageAMP™ II aRNA amplification kit (Applied Biosystems). During RNA amplification, aRNA from cells treated with LNAG and aRNA from cells treated with sNAG was differentially labeled with Cy3 or Cy5 fluorescent dyes. The slides were prepared for hybridization with aRNA by soaking in blocking solution (Sigma Tris-buffered saline pH8.0, in 1000ml dH₂O, 1%BSAw/v, NaN₃ to 0.05%) at RT O/N, then rinsed and dried. Samples containing equal amounts of differentially labeled target aRNA from LNAG and sNAG-treated cells were mixed, hybridized with the slides (65ul/slide; denatured at 95°C for 5 min; hybridized for 48 hours at 37°C in 0.1% SDS and 5 X SSC and 1% BSA), rinsed and dried. The following exemplary graphs in Table VII illustrate experimental set up.

TABLE VII:
Labeling of aRNA

Name	aRNA ng/u (100ul)	260/280 nm	260/280 label (ul)	dye used	labeled conc. pmol/uL	260/280 aRNA (20uL)	total labeled aRNA (20uL)
HaCat_e14d3 ctr	897.42	2.09	23.29	cY3	851.58	1.34	17031.6
HaCat_e14d3 LNAG100	1339.08	2.07	14.24	cY5	687.01	1.87	13740.2
HaCat_e14d3 SNAG100	1515.62	2.05	13.20	cY5	519.15	1.93	10383
HUVEC_e18d4 ctr	1656.37	2.05	12.07	cY3	529.11	1.88	19577.07
HUVEC_e18d4 LNAG100	1078.63	2.07	18.54	cY5	760.26	1.9	15205.2
HUVEC_e18d4 SNAG100	1447.87	2.06	13.81	cY5	617.57	1.84	12351.4

Labeled aRNA Hybridization

	Total aRNA used (uL)	aRNA conc. (ng/uL)	Total vol. (uL)	10% 20% 50%	DNA vol. (uL)	DNA vol. (uL)	10% 20% 50%	DNA vol. (uL)	10% 20% 50%
Actr									
Actr e18d4 {Mix 1}	800	851.58	0.9	2	50	125.9	200	D1038	D1034
Actr e18d4 {Mix 2}	800	687.01	1.2	0	0				
HaCat									
Actr	800	851.58	0.9	2	50	125.5	200	D1037	D1033
SNAG100	800	519.15	1.5	0	0				
HUVEC									
Vctr	800	529.11	1.3	2	50	125.4	200	D1036	D1032
LNAG100 {Mix 3}	800	760.26	1.1						
SNAG100 {Mix 4}	800	529.11	1.3	2	50	125.2	200	D1035	D1031

[00277] The slides were scanned and hybridization detected using Perkin-Elmer Scan Array equipment and ScanArray Express software V3.0, updated. For each slide, Cy5, Cy3 and composite fluorescence was visualized. To identify up-regulated and down-regulated genes microarray data was analyzed using Agilent GeneSpring GX v.11 Bioinformation Data Analysis. Genes of interest analyzed: DEFA1, DEFA3, DEFA4, DEFAS, DEFA6, DEFB1, DEFB013A, DEFB104A, DEFB105B, DEFB108B, DEFB112, DEFB114, DEFB118, DEFB119, DEFB123, DEFB124, DEFB125, DEFB126, DEFB127, DEFB128, DEFB129, DEFB131, and DEFB4 ("DEFA"= α -defensin, and "DEFB"= β -defensin); TLR1, TLR10, TL2, TLR3, TLR4, TLR5, TLR6, TLR7 and TLR8 ("TLR"=Toll receptor); SIGIRR (Single Ig IL-1-related receptor); IRAK2 (IL-1 receptor-associated kinase 1); TRAF6 (TNF receptor associated factor 6); D106A (β -defensin 106), D107A (β -defensin 107). Negative controls: three random sequences (1, 2, 3). Positive controls: 1433Z (Tyrosine-3-monohydrogenase/tryptophan 5 monohydrogenase actin protein); GAPD (glyceraldehydes-3-phosphate dehydrogenase); RPL13A (Ribosomal protein L13a); UBC (Ubiquitin C); ACTB (Actin B).

[00278] *Results:* Results of the microarray gene chip analyses are presented in Tables VIII and IX below. Table VIII shows gene expression in human umbilical vein endothelial cells ("HUVEC") after 2h or 24 h exposure to either LNAG fibers or sNAG nanofibers. Table IX shows gene expression in human keratinocyte cell line (HaCat) after 2h or 24 h exposure to either LNAG fibers or sNAG nanofibers. The results demonstrate that gene expression profile induced by long poly-N-acetylglucosamine fibers ("LNAG") differs from the gene expression profile induced by sNAG nanofibers ("sNAG"). Specifically, LNAG and sNAG differ in their effect on expression of defensin genes and Toll receptor genes.

Table VIII: Microarray Defensin Gene Expression in Human Umbilical Vein Endothelial Cells (HUVEC), Fold Change

Name	[2h, sNAG]	[2h, sNAG]	Name	[20h, sNAG]	[20h, sNAG]
1433Z_HUMAN	0.039	0.329	1433Z_HUMAN	-0.046	-0.180
ACTB_HUMAN	-0.140	0.032	ACTB_HUMAN	0.874	-0.413
D106A_HUMAN	-1.376	-0.195	D106A_HUMAN	1.107	0.522
D107A_HUMAN	1.825	1.431	D107A_HUMAN	-1.007	0.372
DEF A1	0.407	-1.107	DEF A1	-0.333	0.384
DEF A3	0.000	0.528	DEF A3	1.195	-2.335
DEF A4	-1.007	-0.123	DEF A4	0.496	2.636
DEF A5	-0.863	0.451	DEF A5	-0.287	-0.476
DEF B6	1.969	0.805	DEF B6	0.333	-1.402
DEF B1	0.315	1.441	DEF B1	1.933	0.413
DEF B103A	1.426	1.486	DEF B103A	0.628	1.348
DEF B104A	1.296	2.260	DEF B104A	1.543	0.344
DEF B105B	0.616	0.667	DEF B105B	0.723	-0.162
DEF B108B	2.210	0.441	DEF B108B	0.351	1.895
DEF B112	0.000	-0.528	DEF B112	-0.862	1.107
DEF B114	0.000	0.667	DEF B114	-0.862	1.799
DEF B118	-0.142	0.631	DEF B118	0.456	0.577
DEF B119	0.137	1.472	DEF B119	0.808	-1.530
DEF B123	1.664	1.814	DEF B123	0.390	-0.375
DEF B124	1.242	1.533	DEF B124	1.113	1.357
DEF B125	1.169	1.969	DEF B125	1.269	-2.053
DEF B126	-0.064	0.801	DEF B126	1.818	0.385
DEF B127	1.723	0.000	DEF B127	0.000	1.085
DEF B128	1.602	-0.528	DEF B128	0.805	2.238
DEF B129	1.528	0.407	DEF B129	1.936	-0.005
DEF B131	-0.333	0.636	DEF B131	-0.723	-0.608
DEF B4	0.406	0.567	DEF B4	0.401	-0.190
GAPD	0.420	0.602	GAPD	0.616	0.324
IRAK2	-0.035	1.106	IRAK2	1.084	0.984
RPL13A	0.671	1.329	RPL13A	0.789	0.208
SIGIRR	0.358	1.481	SIGIRR	1.870	-0.050
TLR1	-0.194	1.089	TLR1	0.196	-0.631
TLR10	0.000	-0.333	TLR10	-0.528	0.644
TLR2	0.653	2.078	TLR2	1.848	4.494
TLR3	-0.528	-0.333	TLR3	-1.484	-1.361
TLR4	0.613	2.073	TLR4	2.616	0.634
TLR5	1.723	1.181	TLR5	0.723	-0.417

TLR6	1.333	0.528		TLR6	0.246	-0.482
TLR7	1.839	1.274		TLR7	-0.160	0.199
TLR8	-0.033	0.843		TLR8	-0.371	1.219
TRAF6	1.569	0.472		TRAF6	0.731	3.266
UBC	-0.285	0.072		UBC	-0.009	-0.265

Table IX: Microarray Defensin Gene Expression in Human Keratinocyte Cell Line (HaCat), Fold Change

Name	2h, LNAG	2h, sNAG		Name	20h, LNAG	20h, sNAG
I433Z	0.255	-0.282		I433Z	0.000	0.205
GAPD	0.041	-0.191		GAPD	0.000	0.378
RPL13A	-0.532	0.698		RPL13A	0.000	-1.187
UBC	0.136	-0.065		UBC	0.834	-0.023
ACTB	0.130	0.447		ACTB	0.333	0.988
Negative Control	0.000	0.000		Negative Control	0.000	0.000
Negative Control	0.000	0.000		Negative Control	0.000	0.000
Negative Control	0.000	0.000		Negative Control	0.000	0.000
DEFB1	-0.647	1.390		DEFB1	-0.333	-0.426
DEFB126	0.348	1.737		DEFB126	1.000	0.744
DEFB129	0.382	1.464		DEFB129	-0.528	-0.931

6.6 Example 6: Effect of Irradiation on sNAG Membranes

[00279] *Method of Preparation of sNAG Membrane.* The sNAG membrane is derived from microalgal pGlcNAc fibers produced as previously described (see Vourouskis *et al.* U.S. Patent Nos. 5,623,064; and 5,624,679, the content of each of which is incorporated herein by reference in its entirety). Briefly, microalgae were cultured in unique bioreactor conditions using a defined growth media. Following the harvest of microalgae from high-density cultures, fibers were isolated via a stepwise separation and purification process resulting in batches of pure fibers suspended in water for injections (wfi). Fibers were formulated into patches by concentration

and oven drying, and were packaged and sterilized by gamma-irradiation. Fiber dimensions average 20-50nm x 1-2nm x ~100 μ m. Batches of fibers were individually quality controlled using chemical and physical test parameters, and each batch met strict purity criteria prior to release. Final batches were required to be substantially free of proteins, metal ions, and other components. The fibers were then shortened by irradiation to produce sNAG membranes. Briefly, the starting material contained 60 g of pGlcNAc slurry at a concentration of 1 mg/mL. The concentration of the pGlcNAc slurry was confirmed by filtering 5 mL into a 0.2 μ m filter. 15 L of pGlcNAc slurry containing 15 g pGlcNAc was filtered until formation of a wet cake. The wet cake was then transferred into a foil pouch, which is a gamma radiation compatible container, and subjected to 200 kGy gamma radiation. Other irradiation conditions were tested for their effects on pGlcNAc compositions, as reflected in Figure 15A.

[00280] *Effect of Irradiation on pGlcNAc Membranes.* While irradiation reduces the molecular weight of pGlcNAc, irradiation did not disturb the microstructure of the fibers. pGlcNAc was irradiated under different conditions: as a dry, lyophilized material; as a dry membrane; as a concentrated slurry (30:70 weight by volume); and as a dilute slurry (5 mg/ml). A suitable molecular weight reduction (to a molecular weight of 500,000-1,000,000 daltons) was achieved at an irradiation dose of 1,000 kgy for dry polymer, and 200 kgy for wet polymer (Figure 15A).

[00281] The chemical and physical structure of the fibers was maintained throughout irradiation as verified by infrared (IR) spectrum (Figure 15B), elemental assay, and scanning electron microscopes (SEMs) analysis. Microscopic observation of irradiated fibers showed a decrease in the particle length (Figures 15C and 15D). The majority of the fibers are less than about 15 μ m in length, with an average length of about 4 μ m.

6.7 Example 7: sNAG Nanofibers and Long Form p-GlcNAc Fibers Differ in Their Effects on Metabolic Rate and Serum Deprivation of Umbilical Cord Vein Endothelial Cells

[00282] *Materials and Methods.* Pooled, multiple-donor human umbilical cord vein endothelial cells (EC) (Cambrex) were maintained at 37°C with 5% CO₂ in endothelial basal medium 2 (Cambrex) supplemented with EC growth medium 2 SingleQuots as described by Cambrex procedures. Serum starvation was performed at 80-90% confluence in RPMI-1640 supplemented with 0.1% fetal calf serum (Gibco BRL) for 24 h followed by stimulation with

VEGF 165 (20 ng/ml, R&D Systems) or with highly purified pGlcNAc nanofibers or sNAG nanofibers in sterile water (provided by Marine Polymer Technologies, Inc., Danvers, Mass., USA) with the amounts indicated in the figure descriptions. For cellular proliferation/viability assessment, 2 different assays were used: trypan blue exclusion by direct cell counts using a hemacytometer and an MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide] assay in procedures described by the manufacturer (Promega).

[00283] *Results—pGlcNAc:*

[00284] *pGlcNAc did not affect metabolic rate.* As shown in Figure 16, pGlcNAc did not result in a higher metabolic rate as measured by MTT assays, indicating that this polymeric material was not causing marked increases in cellular proliferation.

[00285] *pGlcNAc Protected EC from Cell Death Induced by Serum Starvation.* To test if pGlcNAc fibers had a direct effect on EC, serum-starved EC cells were treated with VEGF or with different concentrations of pGlcNAc fibers. As shown in Figure 17 at 48 h and 72 h after serum starvation, as compared with the total number of cells plated (control), there was about 2-fold reduction in the number of cells after 48 h or 72 h. At 48 h, this decrease in cell number was rescued by the addition of VEGF or by the addition of pGlcNAc fibers at either 50 or 100 µg/ml. At 72 h, the decrease in cell number was rescued by the addition of VEGF or largely rescued by the addition of pGlcNAc fibers at 100 µg/ml. These results indicated that like VEGF, pGlcNAc fiber treatment prevented cell death induced by serum deprivation.

[00286] *Results—sNAG:*

[00287] *sNAG Induced Marked Increase in Metabolic Rate.* As measured by MTT assays, sNAG at 50, 100 or 200 µg/ml resulted in a higher metabolic rate of EC than VEGF (Figure 18).

[00288] *sNAG did not protect EC from cell death induced by serum deprivation.* To test if sNAG fibers had a direct effect on EC, serum-starved EC cells were treated with VEGF or with different concentrations of sNAG fibers. As shown in Figure 19, at 48 h after serum starvation, as compared with the total number of cells plated (control), there was about 2-fold reduction in the number of cells. This decrease in cell number was rescued by the addition of VEGF but not by the addition of sNAG fibers at 50, 100 or 200 µg/ml. These results indicated that not like VEGF, sNAG fiber treatment did not prevent cell death induced by serum deprivation.

[00289] *Conclusion:* The above results demonstrate that sNAG, unlike long form pGlcNAc, increases the metabolic rate of serum-starved EC in a MTT assay and does not rescue apoptosis of serum-starved EC in a trypan blue exclusion test.

6.8 Example 8. Preclinical Testing of sNAG

6.8.1 Test Article

[00290] A test article comprising sNAG produced as previously described in Section 6.2.1 *supra*, was utilized. The test article was supplied sterile by Marine Polymer Technologies, Inc.

6.8.2 Biocompatibility Testing – L929 MEM Elusion Test – ISO 10993-5

[00291] Biocompatibility of the test article was tested in mouse fibroblast L929 mammalian cells. No biological reactivity (Grade 0) was observed in the L929 cells at 48 hours, post exposure to the test article. The observed cellular response obtained from the positive control article (Grade 4) and negative control article (Grade 0) confirmed the suitability of the test system. Based on the criteria of the protocol, the test article is considered non-toxic and meets the requirements of the Elution Test, International Organization for Standardization (ISO) 10993-5 guidelines. See Table X below.

Table X:

Time	Test Article			REACTIVITY GRADES								
				Controls								
	Medium			Negative			Positive					
	A	B	C	A	B	C	A	B	C	A	B	C
0 Hours	0	0	0	0	0	0	0	0	0	0	0	0
24 Hours	0	0	0	0	0	0	0	0	0	3	3	3
48 Hours	0	0	0	0	0	0	0	0	0	4	4	4

Grade	Reactivity	Description of Reactivity Zone
0	None	Discrete intracytoplasmic granules; no cell lysis
1	Slight	Not more than 20% of the cells are rounded, loosely attached, and without intracytoplasmic granules; occasional lysed cells are present
2	Mild	Not more than 50% of the cells are rounded and devoid of intracytoplasmic granules; no extensive cell lysis and empty areas between cells
3	Moderate	Not more than 70% of the cell layers contains rounded cells or are lysed
4	Severe	Nearly complete destruction of the cell layers

6.8.3 Intramuscular Implantation Test – ISO – 4 Week Implantation

6.8.3.1. Materials and Methods

[00292] To evaluate the potential of the test article to induce local toxic effects, the Intramuscular Implantation Test – ISO – 4 Week Implantation (“Intramuscular Implantation Test”) was used. Briefly, the test article was implanted in the paravertebral muscle tissue of New Zealand White rabbits for a period of 4 weeks. The test article was then evaluated separately using two control articles: positive control Surgicel (Johnson and Johnson, NJ) and negative control High Density Polyethylene (Negative Control Plastic).

[00293] *Preparation of Test and Control Articles.* The test article measured approximately 1 mm to in width and 10 mm in length. The two control articles were prepared. The positive control, Surgicel (C1), measured approximately 1 mm in width by 10 mm in length and was received sterile. Negative Control Plastic (C2), measured approximately 1 mm in width by 10 mm in length and was sterilized by dipping in 70% ethanol.

[00294] *Pre-Dose Procedure.* Each animal was weighed prior to implantation. On the day of the test, the dorsal sides of the animals were clipped free of fur and loose hair was removed by means of a vacuum. Each animal was appropriately anesthetized. Prior to implantation, the area was swabbed with a surgical preparation solution.

[00295] *Dose Administration.* Four test article strips were surgically implanted into each of the paravertebral muscles of each rabbit, approximately 2.5 cm from the midline and parallel to the spinal column and approximately 2.5 cm from each other. The test article strips were implanted on one side of the spine. In a similar fashion, positive control article strips (Surgicel) were implanted in the contralateral muscle of each animal. Two negative control strips (Negative Control Plastic) were implanted caudal (toward the tail) to the test article and to C1 control implant sites on either side of the spine (total of four strips). A total of at least eight test article strips and eight of each control article strips are required for evaluation.

[00296] *Post-Dose Procedures.* The animals were maintained for a period of 4 weeks. The animals were observed daily for this period to ensure proper healing of the implant sites and for clinical signs of toxicity. Observations included all clinical manifestations. At the end of the observation period, the animals were weighed. Each animal was sacrificed by an injectable barbiturate. Sufficient time was allowed to elapse for the tissue to be cut without bleeding.

[00297] *Gross Observations.* The paravertebral muscles in which the test or control articles were implanted were excised *in toto* from each animal. The muscle tissue was removed by carefully slicing around the implant sites with a scalpel and lifting out the tissue. The excised implant tissues were examined grossly, but without using excessive invasive procedures that might have disrupted the integrity of this tissue for histopathological evaluation. The tissues were placed in properly labeled containers containing 10% neutral buffered formalin.

[00298] *Histopathology.* Following fixation in formalin, each of the implant sites was excised from the larger mass of tissue. The implant site, containing the implanted material, was examined macroscopically. Each site was examined for signs of inflammation, encapsulation, hemorrhaging, necrosis, and discoloration using the following scale:

0 = Normal

1 = Mild

2 = Moderate

3 = Marked

After macroscopic observation, the implant material was left in-situ and a slice of tissue containing the implant site was processed. Histologic slides of hematoxylin and eosin stained sections were prepared by Toxikon. The slides were evaluated and graded by light microscopic examination.

[00299] *Pathological Assessment of the Effects of the Implant.* The following categories of biological reaction were assessed by microscopic observation for each implant site:

1. Inflammatory Responses:

- a. Polymorphonuclear leukocytes
- b. Lymphocytes
- c. Eosinophils
- d. Plasma cells
- e. Macrophages
- f. Giant cells
- g. Necrosis
- h. Degeneration

2. Healing Responses:

- a. Fibrosis
- b. Fatty Infiltrate

[00300] Each category of response was graded using the following scale:

0 = Normal

0.5 = Very Slight

1 = Mild

2 = Moderate

3 = Marked

[00301] The relative size of the involved area was scored by assessing the width of the area from the implant/tissue interface to unaffected areas which have the characteristics of normal tissue and normal vascularity. Relative size of the involved area was scored using the following scale:

0 = 0 mm, No site

0.5 = up to 0.5 mm, Very slight

1 = 0.6 - 1.0 mm, Mild

2 = 1.1 - 2.0 mm, Moderate

3 = > 2.0 mm, Marked

[00302] The Intramuscular Implantation Test was conducted based upon the following references:

1. ISO 10993-6, 1994, Biological Evaluation of Medical Devices – Part 6: Tests for Local Effects After Implantation.
2. ISO 10993-12, 2002, Biological Evaluation of Medical Devices – Part 12: Sample Preparation and Reference Materials.
3. ASTM F981-04, 2004, Standard Practice for Assessment of Compatibility of Biomaterials for Surgical Implants with Respect to Effect of Materials on Muscle and Bone.
4. ASTM F763-04, 2004, Standard Practice for Short Term Screening of Implant Materials.
5. ISO/IEC 17025, 2005, General Requirements for the Competence of Testing and Calibration Laboratories.

[00303] The results of the Intramuscular Implantation Test were evaluated based upon the following criteria:

1. Calculated Rating: For each implanted site, a total score is determined. The average score of the test sites for each animal is compared to the average score of the control sites for that animal. The average difference between test and control sites for all animals is calculated and the initial Bioreactivity Rating is assigned as follows:

0 - 1.5 No Reaction*

> 1.5 - 3.5 Mild Reaction

> 3.5 - 6.0 Moderate Reaction

> 6.0 Marked Reaction

* A negative calculation is reported as zero (0).

2. Modification of the Rating: The pathology observer reviews the calculated level of bioreactivity. Based on the observation of all factors (e.g., relative size, pattern of response, inflammatory vs. resolution), the pathology observer may revise the Bioreactivity Rating. Justification for the modification to the rating is presented in the narrative report (A descriptive

narrative report regarding the biocompatibility of the test material is provided by the pathology observer).

6.8.3.2. Results

[00304] The results indicated that the test article was non-reactive when implanted for 4 weeks (Bioreactivity Rating of 0.2) when compared to positive control Surgicel; and non-reactive (Bioreactivity Rating of 0.0) when compared to negative control High Density Polyethylene (Negative Control Plastic).

[00305] *Clinical observation.* **Table XI** below shows results of the macroscopic evaluation of the test article and control implant sites indicated no significant signs of inflammation, encapsulation, hemorrhage, necrosis, or discoloration at the 4 week time period. Some test sites and the majority of the positive control, Surgicel, were not seen macroscopically and serial sections were submitted for microscopic evaluation.

Table XI:

**Macroscopic Observations
4 Week Implantation**

Animal No.: 600939															
Test Site	T1	T2	T3	T4	Test Area	C3-1	C3-2	C3-3	C3-4	C3-A	C3-B	C3-C	C3-D	C3-E	Control C3-Acc
Subcapsular	S	NPF	S	NPF	S	NPF	NPF	NPF	NPF	N/A	S	S	S	S	S
Peritoneal	S	NPF	S	NPF	S	NPF	NPF	NPF	NPF	N/A	S	S	S	S	S
Hemorrhage	S	NPF	S	NPF	S	NPF	NPF	NPF	NPF	N/A	S	S	S	S	S
Necrosis	S	NPF	S	NPF	S	NPF	NPF	NPF	NPF	N/A	S	S	S	S	S
Encapsulation	S	NPF	S	NPF	S	NPF	NPF	NPF	NPF	N/A	S	S	S	S	S
Test	S	N/A	S	N/A	S	N/A	N/A	N/A	N/A	S	S	S	S	S	S

Animal No.: 600961															
Test Site	T1	T2	T3	T4	Test Area	C3-1	C3-2	C3-3	C3-4	C3-A	C3-B	C3-C	C3-D	C3-E	Control C3-Acc
Subcapsular	NPF	NPF	NPF	NPF	N/A	NPF	NPF	NPF	NPF	N/A	S	S	S	S	S
Peritoneal	NPF	NPF	NPF	NPF	N/A	NPF	NPF	NPF	NPF	N/A	S	S	S	S	S
Hemorrhage	NPF	NPF	NPF	NPF	N/A	NPF	NPF	NPF	NPF	N/A	S	S	S	S	S
Necrosis	NPF	NPF	NPF	NPF	N/A	NPF	NPF	NPF	NPF	N/A	S	S	S	S	S
Encapsulation	NPF	NPF	NPF	NPF	N/A	NPF	NPF	NPF	NPF	N/A	S	S	S	S	S
Test	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	S	S	S	S	S

Animal No.: 600968															
Test Site	T1	T2	T3	T4	Test Area	C3-1	C3-2	C3-3	C3-4	C3-A	C3-B	C3-C	C3-D	C3-E	Control C3-Acc
Subcapsular	NPF	NPF	NPF	NPF	N/A	NPF	NPF	NPF	NPF	N/A	S	S	S	S	S
Peritoneal	NPF	NPF	NPF	NPF	N/A	NPF	NPF	NPF	NPF	N/A	S	S	S	S	S
Hemorrhage	NPF	NPF	NPF	NPF	N/A	NPF	NPF	NPF	NPF	N/A	S	S	S	S	S
Necrosis	NPF	NPF	NPF	NPF	N/A	NPF	NPF	NPF	NPF	N/A	S	S	S	S	S
Encapsulation	NPF	NPF	NPF	NPF	N/A	NPF	NPF	NPF	NPF	N/A	S	S	S	S	S
Test	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	S	S	S	S	S

T = test site (representative sections were submitted for macroscopic assessment)

C1 = Surgicel (Close to the source of the material, representative sections were submitted for macroscopic assessment)

C2 = Negative Control High Density Polyethylene (Negative Control Plastic)

Grading Scale:

0 = no reaction

2 = moderate reaction

NPF = No Site Found

1 = mild reaction

3 = marked reaction

N/A = Not Applicable

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[00306] Implantation Site Observations (Microscopic). **Table XII** below shows results of the microscopic evaluation of the test article implant sites indicated no significant signs of inflammation, fibrosis, hemorrhage, necrosis, or degeneration as compared to each of the control article sites. The Bioreactivity Rating for the 4 week time period (average of three animals) was 0.2, (C1 – Surgicel) and 0.0 (C2 – Negative Control Plastic) indicating no reaction as compared to either of the control implant sites. The pathologist noted there was a moderate polymorphic and histiocytic (macrophages) infiltrate around the *in situ* test article that was not unexpected given the nature of the test material.

Table XII:

**Microscopic Observations
4 Week Implantation**

Animal No.: 60039

Categories	Test Sites**			Control Sites							
	T1	T2	T3	C1-1	C1-2	C1-3	C1-4	C2-1	C2-2	C2-3	C2-4
Foreign Body	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
% of Test Site Areas	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
* Polymorphs	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
* Lymphocytes	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
* Eosinophils	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
* Plasma Cells	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
* Macrophages	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
* Giant Cells	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
* Degeneration	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
* Necrosis	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
* Fibrosis	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
* Fatty Tissue	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	1.000	1.5	2.0	2.5	1.5	1.5	1.8	1.2	1.5	1.5	1.2

T = Test Site

C1 = Surgicel

C2 = Negative Control High Density Polyethylene (Negative Control Plastic)

Animal Test Scores (Average)** = 2.0

Animal C1 Score (Average**) = 1.5

Animal C2 Score (Average**) = 1.4

Animal Score (Average Test Score - Average C1 Score) = 0.5

Animal Score (Average Test Score - Average C2 Score) = 0.6

** Used in calculation of Bioreactivity Rating.

*** No site fixed in TA.

Table XII:

Microscopic Observations (Cont.)
4 Week Implants

Animal No.: 63981		Test Sites**								Control Sites**										
Categories		T1	T2	T4	C1-1	C1-3	C1-4	C2-1	C2-3	C2-4	Total	T1	T2	T4	C1-1	C1-3	C1-4	C2-1	C2-3	C2-4
Reaction		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.5	1.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	
Fibrous Debris		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.5	1.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	
Ext. Area of Increased Score		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.5	1.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	
Inflammation		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.5	1.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	
Lymphocytes		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.5	1.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	
* Macrophages		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.5	1.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	
* Neutrophils		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.5	1.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	
* Plasma Cells		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.5	1.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	
* Mastocytes		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.5	1.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	
* Tissue Cells		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.5	1.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	
* Degeneration		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.5	1.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	
* Necrosis		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.5	1.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	
* Fibrotic		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.5	1.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	
* Total Inflamm.		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.5	1.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	
		Total	1.5	1.0	1.0	1.5	1.5	1.5	1.5	1.5	1.5	1.0	1.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0

** Test Site

C1 = Surgical

C2 = Negative Control High Density Polyethylene (Negative Control Plastic)

Animal Test Score (Average**) = 1.5

Animal C1 Score (Average**) = 1.0

Animal C2 Score (Average**) = 1.5

Animal Score (Average Test Score - Average C1 Score) = -0.4

Animal Score (Average Test Score - Average C2 Score) = -0.7

* Used in calculation of Biocompatibility Rating.

** No site found in T2, C1-3, and C2-4.

Table XII:

Microscopic Observations (Cont.)
4 Weeks Post-implantation

Animal No.: 60968		Test Sites				Control Sites**					
Category	Reaction	T1	T2	T3	T4	C1	C2	C3	C4	C5	C6
Epithelial Cells	++	++	++	++	++	++	++	++	++	++	++
Cell Type of Epithelial Cells	++	++	++	++	++	++	++	++	++	++	++
Basal Layer	++	++	++	++	++	++	++	++	++	++	++
Endothelial Cells	++	++	++	++	++	++	++	++	++	++	++
Fibroblasts	++	++	++	++	++	++	++	++	++	++	++
Connective Tissue	++	++	++	++	++	++	++	++	++	++	++
Plasma Cells	++	++	++	++	++	++	++	++	++	++	++
Macrophages	++	++	++	++	++	++	++	++	++	++	++
Granular Cells	++	++	++	++	++	++	++	++	++	++	++
Degeneration	++	++	++	++	++	++	++	++	++	++	++
Necrosis	++	++	++	++	++	++	++	++	++	++	++
Edema	++	++	++	++	++	++	++	++	++	++	++
Scar Tissue	++	++	++	++	++	++	++	++	++	++	++
Total	3.8	3.8	3.8	3.8	3.8	3.8	3.8	3.8	3.8	3.8	3.8

* = Test Site

C1 = Surgicell

C2 = Negative Control High Density Polyethylene (Negative Control Panel)

Adjusted Test Score (Average) = 3.8

Adjusted C1 Score (Average) = 3.8

Adjusted C2 Score (Average) = 3.8

Animal Score (Average Test Score - Average C1 Score) = 0.0

Animal Score (Average Test Score - Average C2 Score) = 0.0

* Used as calculation of Bioreactivity Rating.

** No site found at C3-4.

	C1	C2
Adjusted Score 60968 =	0.3	0.8
Adjusted Score 60961 =	0.4	0.7
Adjusted Score 60962 =	0.3	0.1
Bioreactivity Rating =	0.3 ** No Reaction	
Bioreactivity Rating =	0.4 ** No Reaction	

6.8.4 Intracutaneous Injection Test – ISO 10993-10

[00307] USP 0.9% Sodium Chloride for Injection (NaCl) and Cottonseed Oil (CSO) extracts of the test article were evaluated for their potential to produce irritation after intracutaneous injection in New Zealand White rabbits. The test article sites did not show a significantly greater biological reaction than the sites injected with the control article. Based on the criteria of the protocol, the test article is considered a negligible irritant and meets the requirements of the ISO 10993-10 guidelines. Results are shown below in **Table XIII**.

Table XIII:

Intracutaneous Test Skin Reaction Scores

NaCl Extract

Animal #	Vehicle	Time	Site Numbers Scoring (ER/ED)									
			T-1	T-2	T-3	T-4	T-5	C-1	C-2	C-3	C-4	C-5
61917	NaCl	0 hours ^a	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		24 hours	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		48 hours	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		72 hours	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
61919	NaCl	0 hours ^a	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		24 hours	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		48 hours	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		72 hours	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Total			0.0					0.0				

^a Immediately after injection, not used for the evaluation criteria.Overall Mean Score^b for Test Article = 0.0Overall Mean Score^b for Control Article = 0.0

Difference between Test Article and Control Article Overall Mean Score = 0.0 - 0.0 = 0.0

CSO Extract

Animal #	Vehicle	Time	Site Numbers Scoring (ER/ED)									
			T-1	T-2	T-3	T-4	T-5	C-1	C-2	C-3	C-4	C-5
61917	CSO	0 hours ^a	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		24 hours	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		48 hours	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		72 hours	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
61919	CSO	0 hours ^a	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		24 hours	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		48 hours	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		72 hours	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Total			0.0					0.0				

^a Immediately after injection, not used for the evaluation criteria.Overall Mean Score^b for Test Article = 0.0Overall Mean Score^b for Control Article = 0.0

Difference between Test Article and Control Article Overall Mean Score = 0.0 - 0.0 = 0.0

ER = Erythema; ED = Edema; T = Test Sites; C = Control Sites

^b Overall Mean Score = Total erythema plus edema scores divided by 12
(2 animals x 3 scoring periods x 2 scoring categories)

6.8.5 Kligman Maximization Test – ISO 10993-10

[00308] UPS 0.9% Sodium Chloride for Injection (NaCl) and Cottonseed Oil (CSO) extracts of the test article elicited no intradermal reaction in Hartley guinea pigs at the challenge (0% sensitization), following an induction phase. Therefore, as defined by the scoring system of Kligman, this is a Grade I reaction and the test article is classified as having weak allergenic potential. Based on the criteria of the protocol, a Grade I sensitization rate is not considered significant and the test article meets the requirements of the ISO 10993-10 guidelines. Results are shown below in Table XIV.

Table XIV:

Skin Examination Data

Group	Animal #	Sex	Scores			Percent Animals Sensitized	Allergenic Potential
			Day 25	Day 26	Day 27		
Test Article (NaCl Extract)	1	Male	0	0	0		
	2	Male	0	0	0		
	3	Male	0	0	0		
	4	Male	0	0	0		
	5	Male	0	0	0		
	6	Female	0	0	0	0%	Weak
	7	Female	0	0	0		
	8	Female	0	0	0		
	9	Female	0	0	0		
	10	Female	0	0	0		
Test Article (CSO Extract)	11	Male	0	0	0		
	12	Male	0	0	0		
	13	Male	0	0	0		
	14	Male	0	0	0		
	15	Male	0	0	0		
	16	Female	0	0	0	0%	Weak
	17	Female	0	0	0		
	18	Female	0	0	0		
	19	Female	0	0	0		
	20	Female	0	0	0		
Negative Control (NaCl)	21	Male	0	0	0		
	22	Male	0	0	0		
	23	Female	0	0	0	0%	Weak
	24	Female	0	0	0		
	25	Female	0	0	0		
Negative Control (CSO)	26	Male	0	0	0		
	27	Male	0	0	0		
	28	Female	0	0	0	0%	Weak
	29	Female	0	0	0		
	30	Female	0	0	0		
Positive Control (DNCB)	31	Male	3	4	0		
	32	Male	3	3	0		
	33	Female	3	2	0	100%	Extreme
	34	Female	3	2	0		
	35	Female	3	2	0		

Sensitization Rate (%)	Grade	Class
0-8	I	Weak
9-28	II	Mild
29-64	III	Moderate
65-89	IV	Strong
91-100	V	Extreme

The test results are interpreted based upon the percentage sensitization observed.

7. INCORPORATION BY REFERENCE

[00309] All publications, patents and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference. Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the

teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

[00310] In the claims which follow and in the preceding description of the invention, except where the context requires otherwise due to express language or necessary implication, the word "comprise" or variations such as "comprises" or "comprising" is used in an inclusive sense, i.e. to specify the presence of the stated feature but not to preclude the presence or addition of further features in various embodiments of the invention.

[00311] It is to be understood that, if any prior art publication is referred to herein, such reference does not constitute an admission that the publication forms a part of the common general knowledge in the art, in Australia or any other country.

The claims defining the invention are as follows:

1. A method for treating a disease or a condition associated with a bacterial imbalance in a subject in need thereof, comprising topically administering a composition comprising shortened fibers of poly- β -1 \rightarrow 4-N-acetylglucosamine (“sNAG nanofibers”) to a subject, wherein the sNAG nanofibers are less than 10 μm in length, wherein the sNAG nanofibers comprise 70% or more than 70% of N-acetylglucosamine monosaccharides, and wherein the sNAG nanofibers do not have an effect, or substantially have no effect, on bacterial growth or survival of *Staphylococcus aureus* bacterial cultures *in vitro*.
2. A method for treating a disease or a condition associated with a bacterial imbalance in a subject in need thereof, comprising topically administering a composition comprising shortened fibers of poly- β -1 \rightarrow 4-N-acetylglucosamine (“sNAG nanofibers”) to a subject, wherein the sNAG nanofibers are from about 1 μm to less than 10 μm in length, wherein the sNAG nanofibers comprise 70% or more than 70% of the N-acetylglucosamine monosaccharides, and wherein the sNAG nanofibers do not have an effect, or substantially have no effect, on bacterial growth or survival of *Staphylococcus aureus* bacterial cultures *in vitro*.
3. Use of a composition comprising shortened fibers of poly- β -1 \rightarrow 4-N-acetylglucosamine (“sNAG nanofibers”) in the manufacture of a medicament for treating a disease or a condition associated with a bacterial imbalance in a subject in need thereof, said treating comprising topically administering the medicament to the subject, wherein the sNAG nanofibers are less than 10 μm in length, wherein the sNAG nanofibers comprise 70% or more than 70% of N-acetylglucosamine monosaccharides, and wherein the sNAG nanofibers do not have an effect, or substantially have no effect, on bacterial growth or survival of *Staphylococcus aureus* bacterial cultures *in vitro*.
4. Use of a composition comprising shortened fibers of poly- β -1 \rightarrow 4-N-acetylglucosamine (“sNAG nanofibers”) in the manufacture of a medicament for treating a disease or a condition associated with a bacterial imbalance in a subject in need thereof, said treating comprising topically administering the medicament to the subject, wherein the sNAG nanofibers are from about 1 μm to less than 10 μm in length, wherein the sNAG nanofibers

comprise 70% or more than 70% of the N-acetylglucosamine monosaccharides, and wherein the sNAG nanofibers do not have an effect, or substantially have no effect, on bacterial growth or survival of *Staphylococcus aureus* bacterial cultures *in vitro*.

5. The method or use of any one of claims 1 to 4, wherein the sNAG nanofibers have the microstructure of non-irradiated poly- β -1 \rightarrow 4-N-acetylglucosamine fibers that are about 100 μm in length.

6. The method or use of any one of claims 1 to 4, wherein the sNAG nanofibers have the microstructure of non-irradiated microalgal poly- β -1 \rightarrow 4-N-acetylglucosamine fibers.

7. The method or use of any one of claims 1 to 4, wherein the infrared spectrum of the sNAG nanofibers is equivalent or substantially equivalent to that of non-irradiated poly- β -1 \rightarrow 4-N-acetylglucosamine fibers that are: (i) about 100 μm in length, and/or (ii) derived from microalgae.

8. The method or use of any one of claims 1 to 4, wherein the chemical and physical structure of the sNAG nanofibers is the same or substantially the same as the chemical and physical structure of non-irradiated poly- β -1 \rightarrow 4-N-acetylglucosamine fibers as determined by infrared spectrum, elemental assay and scanning electron microscopic analyses, wherein the non-irradiated poly- β -1 \rightarrow 4-N-acetylglucosamine fibers are: (i) about 100 μm in length, and/or (ii) derived from microalgae.

9. The method or use of any one of claims 1 to 8, wherein the subject is a subject diagnosed with the disease or condition, or displaying one or more symptoms of the disease or condition.

10. The method or use of any one of claims 1 to 9, wherein the bacterial imbalance is an imbalance in bacterial microbiota.

11. The method or use of any one of claims 1 to 9, wherein the bacterial imbalance is an abnormal or altered bacterial microbiota.

12. The method or use of any one of claims 1 to 11, wherein disease or condition is an intestinal condition.

13. The method or use of claim 12, wherein the subject has an intestinal bacterial microbiota that differs from intestinal bacterial microbiota in a subject or subjects with no symptoms of the intestinal condition.

14. The method or use of any one of claims 1 to 11, wherein disease or condition is a skin condition.

15. The method or use of claim 14, wherein the subject has a skin bacterial microbiota that differs from skin bacterial microbiota in a subject or subjects with no symptoms of the skin condition.

16. The method or use of any one of claims 1 to 15, wherein the sNAG nanofibers are in an amount effective to achieve one or more of the following: (i) reduce the severity of the disease or condition, or one or more symptoms thereof, (ii) reduce the duration of the disease or condition, one or more symptoms thereof, and (iii) prevent the progression of the disease or condition, or one or more symptoms thereof.

17. The method of any one of claims 1 to 15, wherein the sNAG nanofibers are in an amount effective to achieve one or more of the following: (i) reduce or eliminate a bacterial cell population, and (ii) prevent an increase in the number of bacteria in a bacterial cell population.

18. The method or use of any one of claims 1 to 17, wherein the bacterial imbalance is associated with a bacteria resistant to one or more antibiotics.

19. The method or use of any one of claims 1 to 18, wherein the subject is a human.

20. The method or use of any one of claims 1 to 19, wherein the sNAG nanofibers fibers are less than $10 \mu\text{m}$ in average length.

21. The method or use of any one of claims 1 to 19, wherein the sNAG nanofibers fibers are less than 8 μm in average length.

22. The method or use of any one of claims 1 to 19, wherein the sNAG nanofibers fibers are between 4 μm and 7 μm in average length.

23. The method or use of any one of claims 1 to 22, wherein the majority of the sNAG nanofibers fibers are at least 1 μm in length.

24. The method or use of any one of claims 1 to 23, wherein the length of the fibers is determined by scanning electron microscopic (SEM) analysis.

25. The method or use of any one of claims 1 to 24, wherein the poly- β -1 \rightarrow 4-N-acetylglucosamine is a microalgal poly- β -1 \rightarrow 4-N-acetylglucosamine.

26. The method or use of any one of claims 1 to 24, wherein the poly- β -1 \rightarrow 4-N-acetylglucosamine is not a crustacean poly- β -1 \rightarrow 4-N-acetylglucosamine.

27. The method or use of any one of claims 1 to 26, wherein the sNAG nanofibers comprise more than 80% of the N-acetylglucosamine monosaccharides.

28. The method or use of any one of claims 1 to 26, wherein the sNAG nanofibers comprise more than 90% of the N-acetylglucosamine monosaccharides.

29. The method or use of any one of claims 1 to 26, wherein the sNAG nanofibers comprise 100% of the N-acetylglucosamine monosaccharides.

30. The method or use of any one of claims 1 to 29, wherein the sNAG nanofibers are non-reactive when tested in an elution test, an intramuscular implantation test, an intracutaneous test, and/or a systemic test.

31. The method or use of any one of claims 1 to 30, wherein the composition does not comprise an additional anti-bacterial agent.

32. The method or use of any one of claims 1 to 31, wherein the composition is not administered in conjunction with an anti-bacterial agent.

33. The method or use of any one of claims 1 to 32, wherein the composition is not administered with an immunomodulator.

34. The method or use of any one of claims 1 to 33, wherein the composition does not comprise an additional therapy which is encapsulated, immobilized or formulated in the sNAG nanofibers.

35. The method or use of any one of claims 1 to 34, wherein the composition does not comprise an additional active ingredient.

36. The method or use of any one of claims 1 to 35, wherein the composition is not administered in conjunction with another therapy.

37. The method or use of any one of claims 1 to 36, wherein the composition is formulated as a cream, a gel, a liquid, a suspension, an ointment, a membrane, a powder, a spray, or a suppository.

38. The method or use of claim 12, wherein the composition is formulated as a suppository.

39. The method or use of any one of claims 1 to 38, wherein the sNAG nanofibers have been produced by irradiation of the poly- β -1 \rightarrow 4-N-acetylglucosamine fibers.

40. The method or use of claim 39, wherein (i) the poly-N-acetylglucosamine fibers have been irradiated in the form of dry fibers, a dry fiber membrane or a dry lyophilized material at 500-2,000 kgy, or (ii) the poly-N-acetylglucosamine fibers have been irradiated in the form of a suspension, a slurry or a wet cake at 100-500 kgy.

41. The method or use of claim 39, wherein (i) the poly-N-acetylglucosamine fibers have been irradiated by gamma irradiation in the form of dry fibers, a dry fiber membrane or a dry lyophilized material at 750-1,250 kgy, or (ii) the poly-N-acetylglucosamine fibers have been irradiated by gamma irradiation in the form of a suspension, a slurry or a wet cake at 150-250 kgy.

42. The method or use of any one of claims 39 to 41, wherein the irradiation is gamma irradiation.

43. The method or use of any one of claims 1 to 42, wherein the composition comprises 0.2 to 20 mg/cm² of the sNAG nanofibers per dose or application of the composition.

44. The method or use of any one of claims 1 to 43, wherein the composition increases metabolic rate of serum-starved human umbilical cord vein endothelial cells in a MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, and/or does not rescue apoptosis of serum-starved human umbilical cord vein endothelial cells in a trypan blue exclusion test.

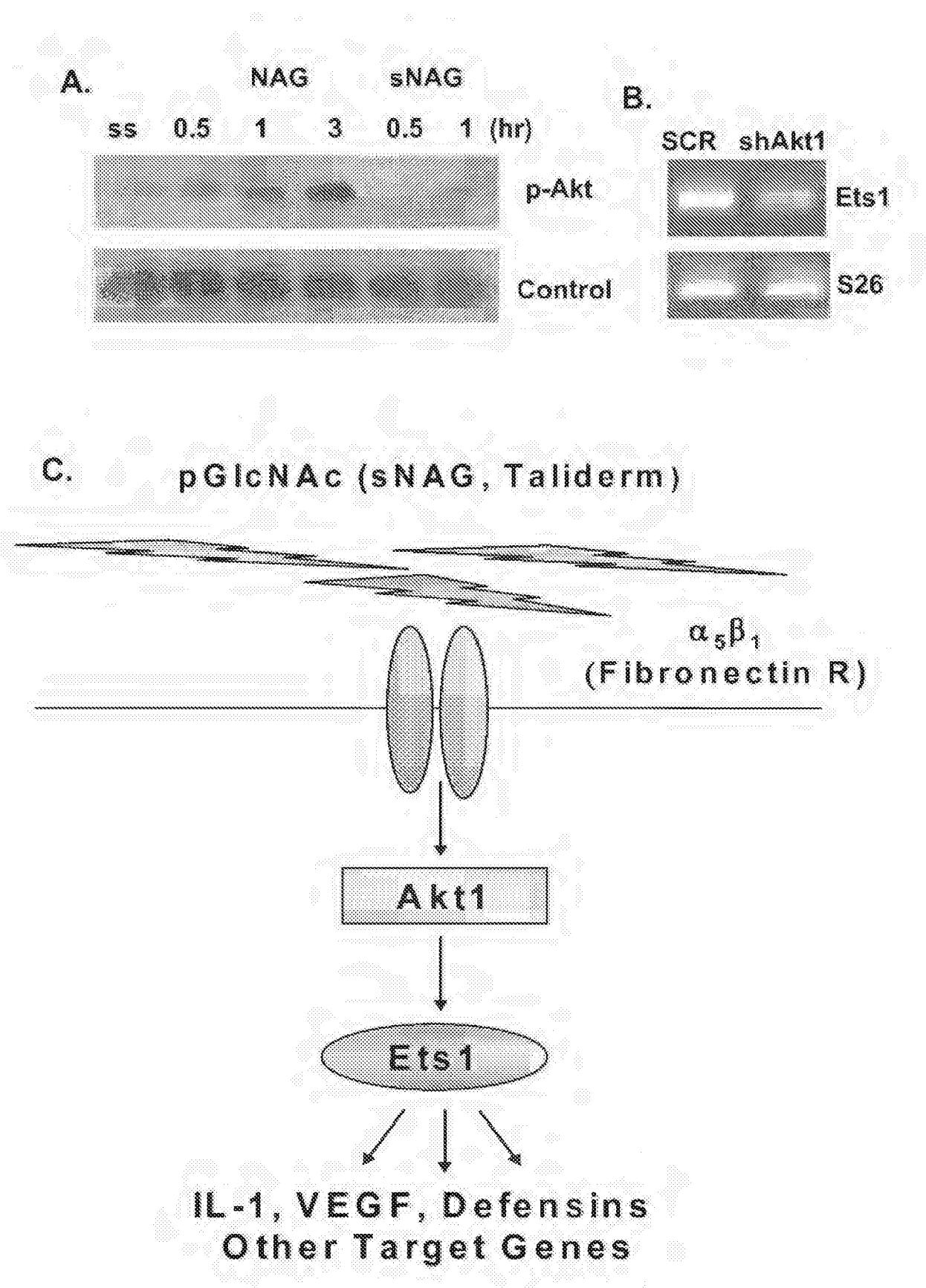


Fig. 1

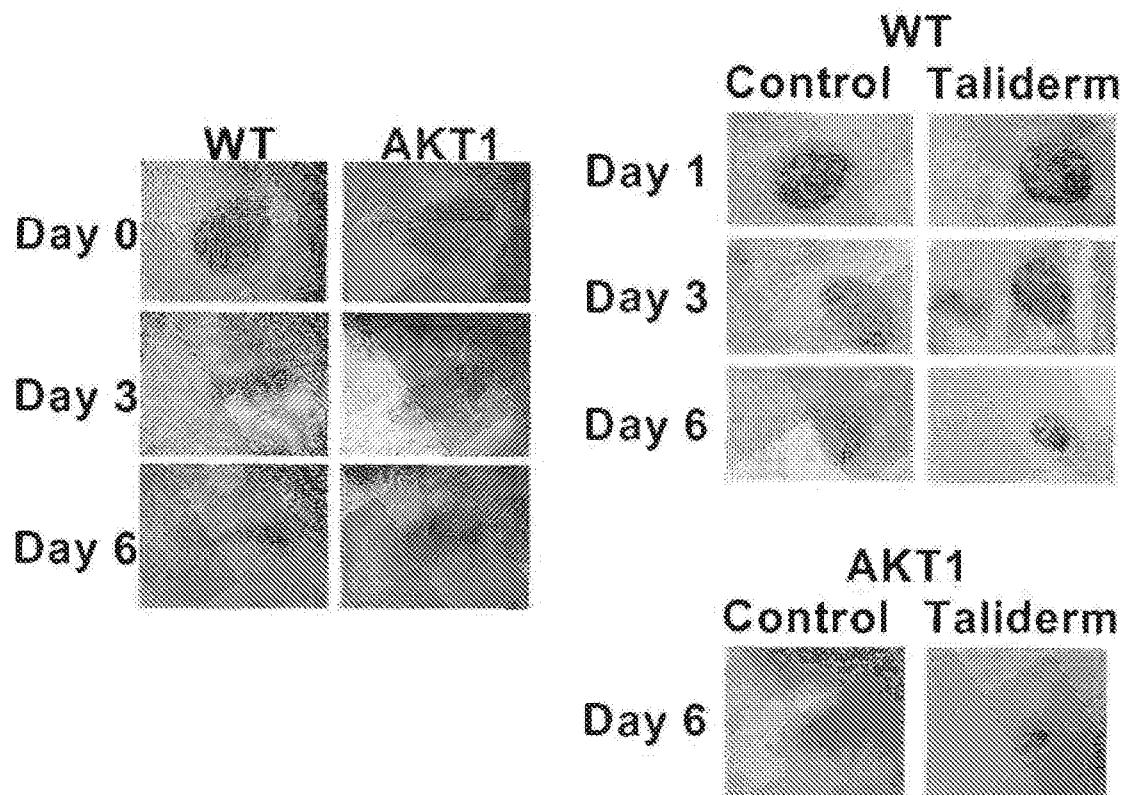


Fig. 2A

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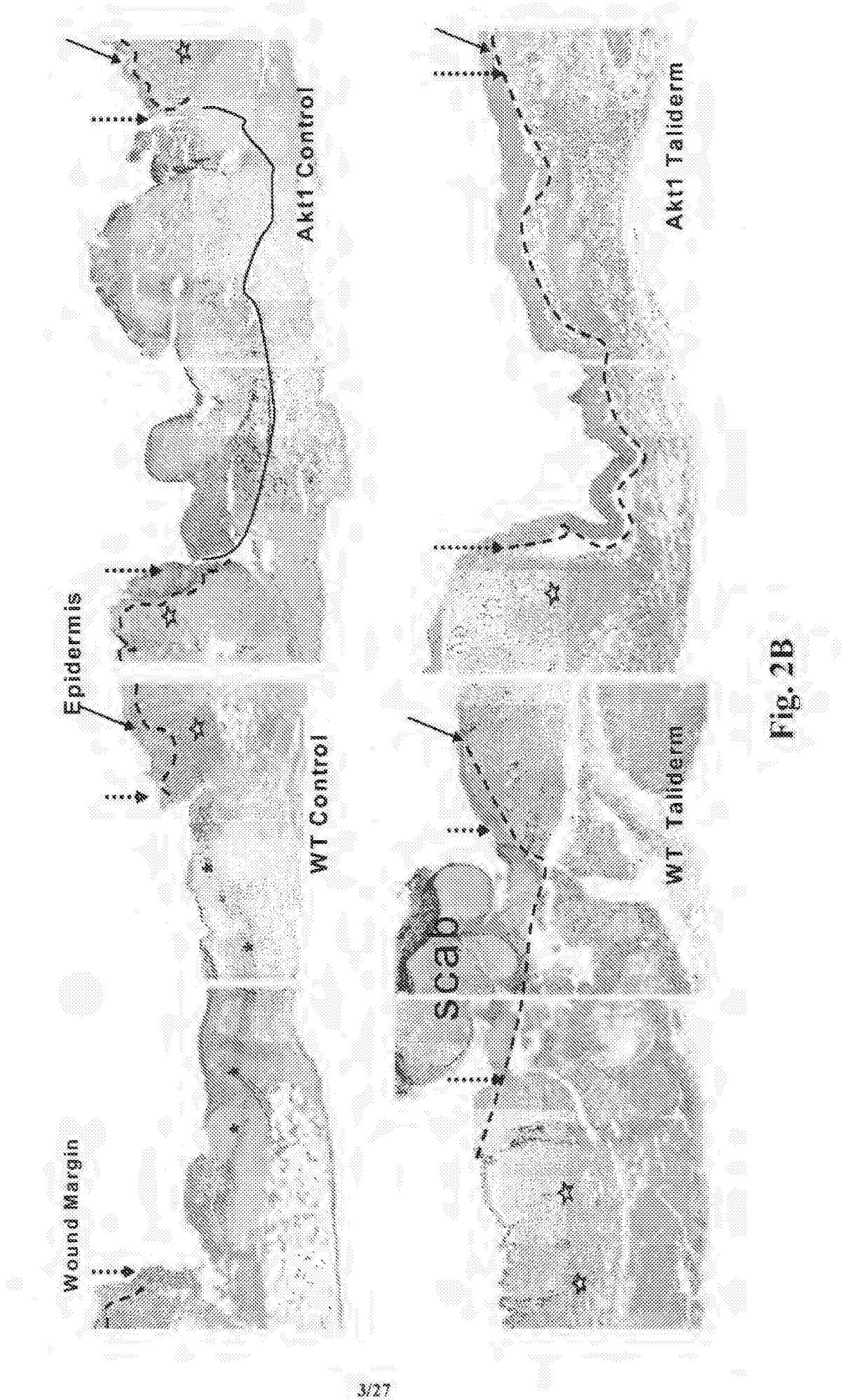
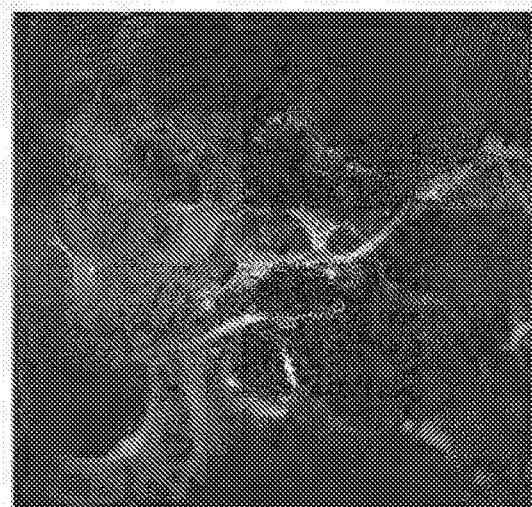


Fig. 2B

A.



Control (no treatment)



sNAG (10 μ g/ml)

B.

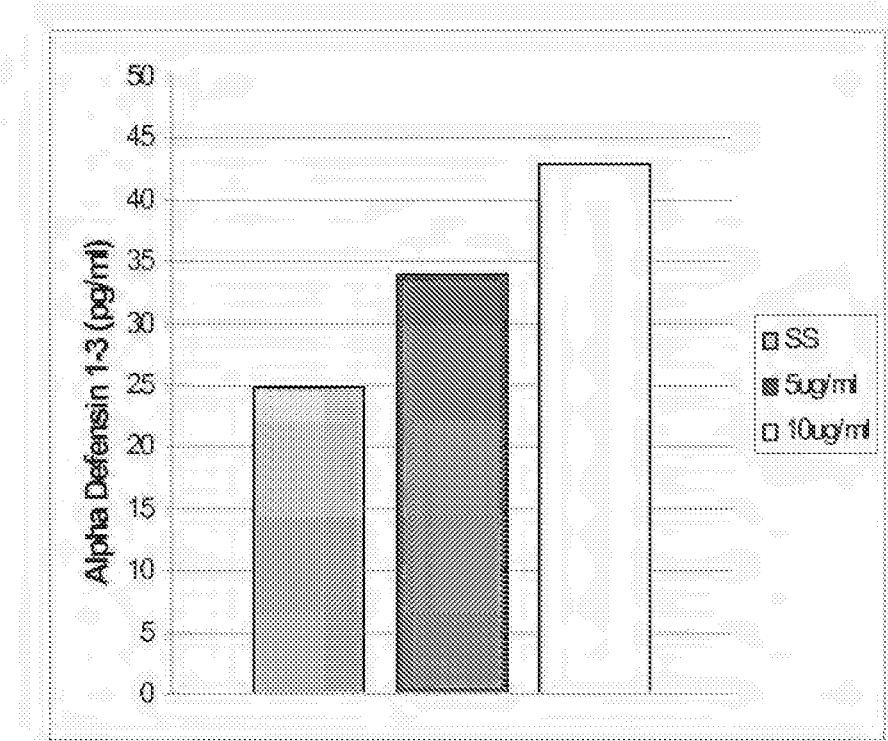
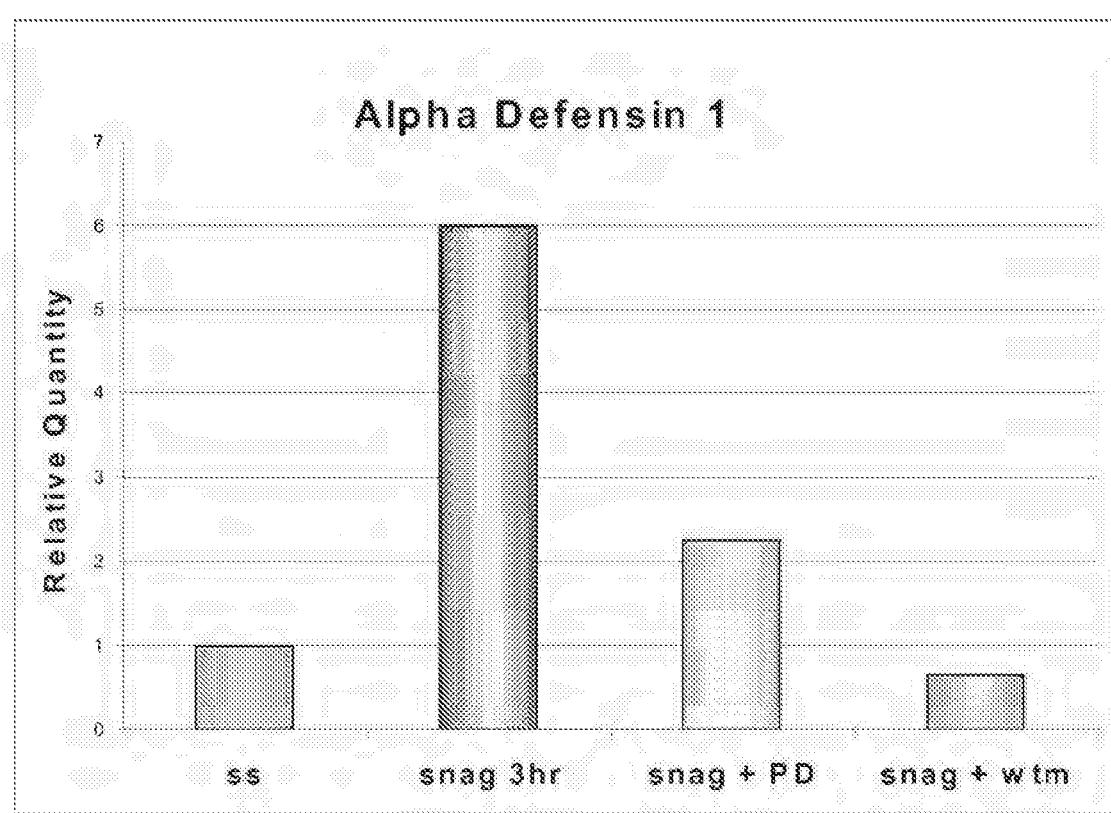


Fig. 3

A.



B.

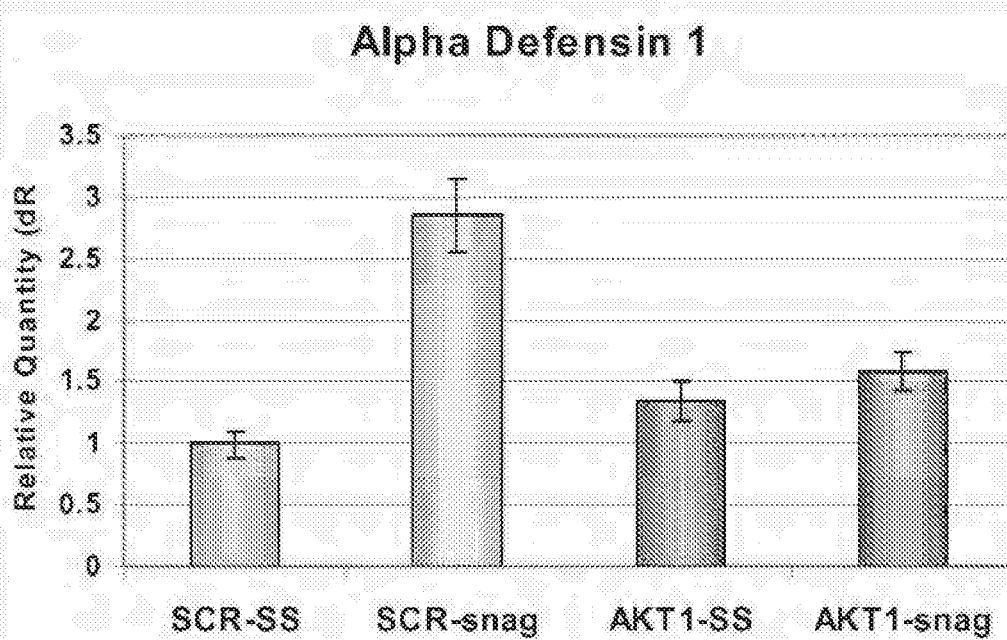


Fig. 4

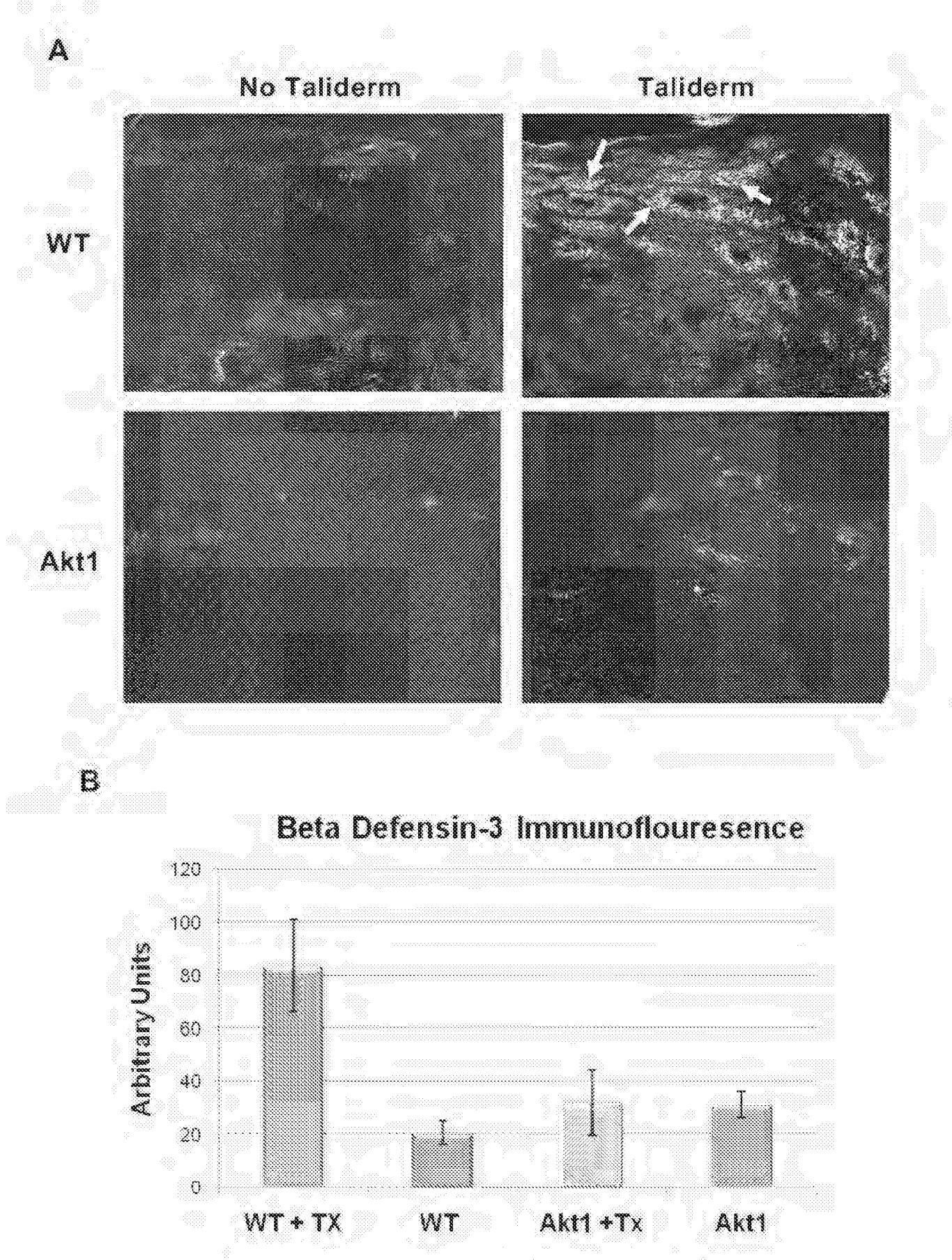


Fig. 5A – 5B

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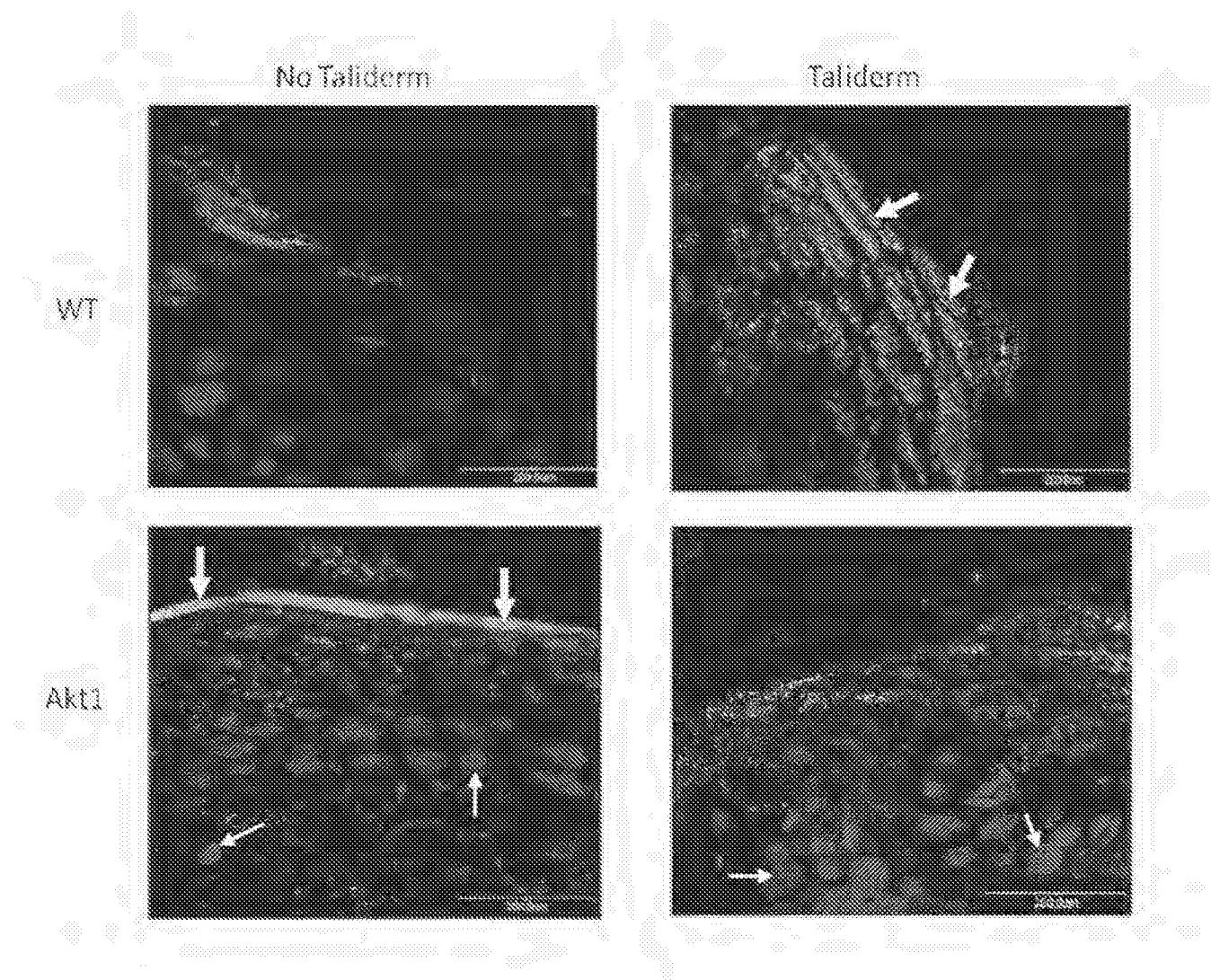


Fig. 5C

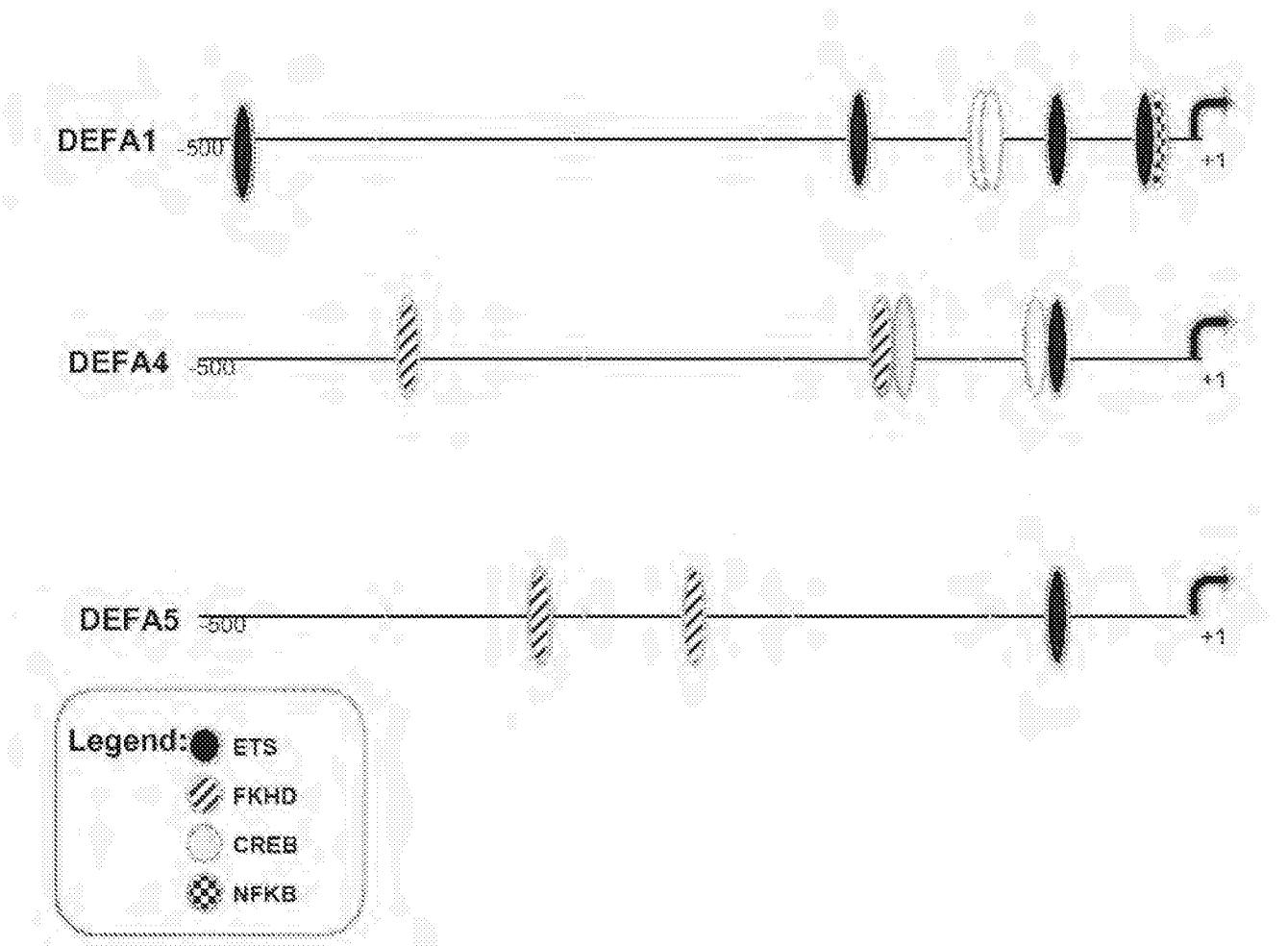


Fig. 6

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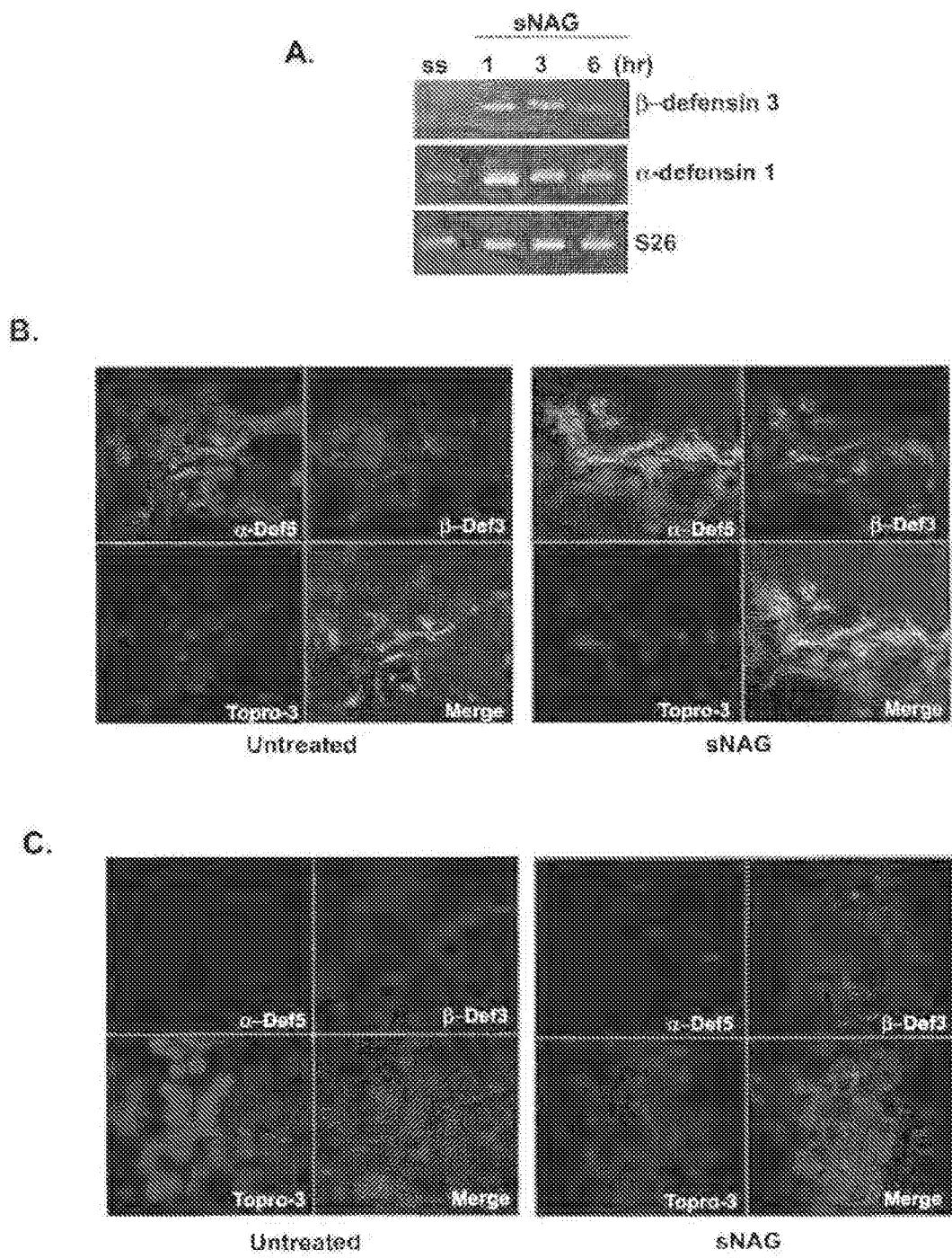


Fig. 7

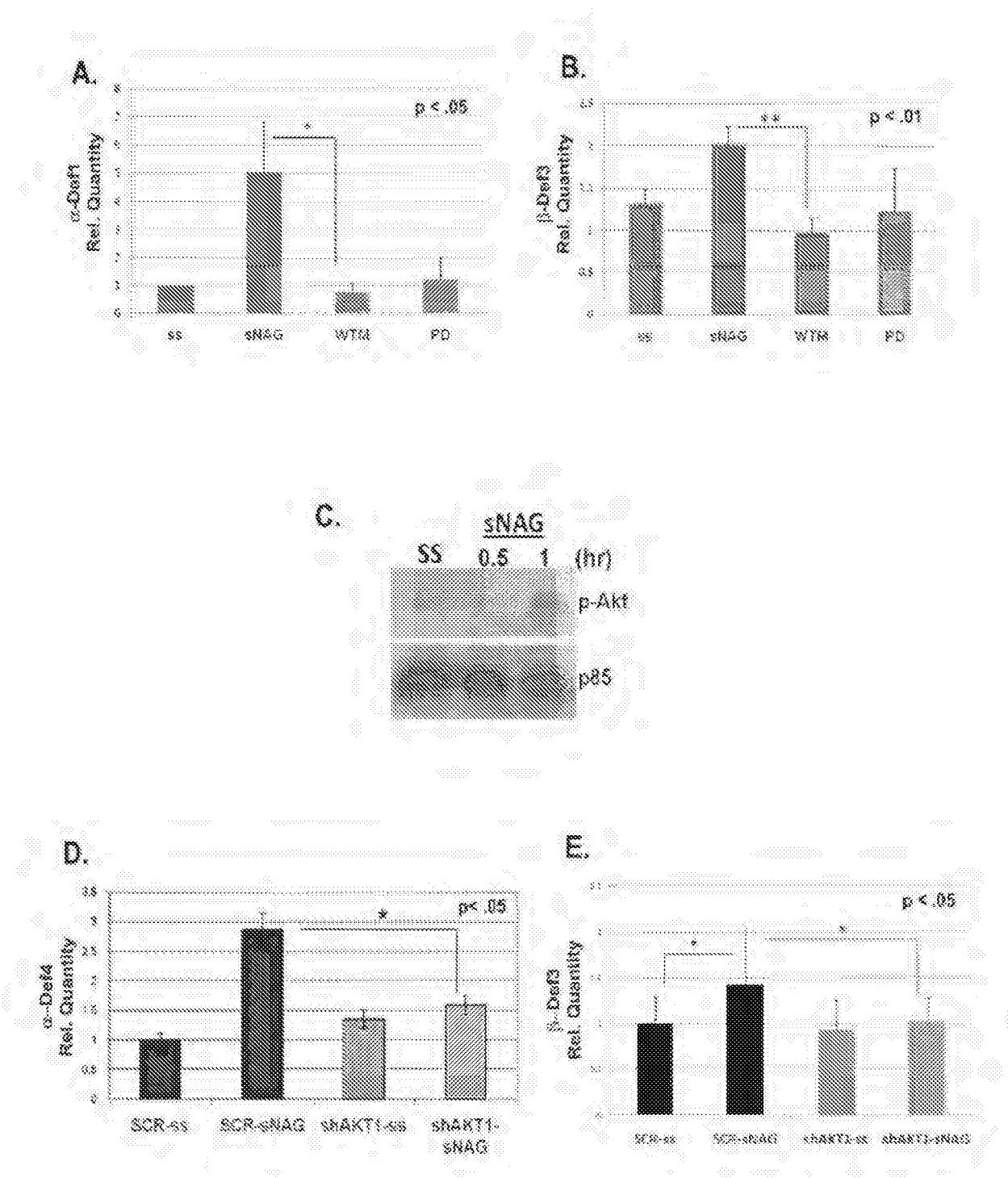


Fig. 8

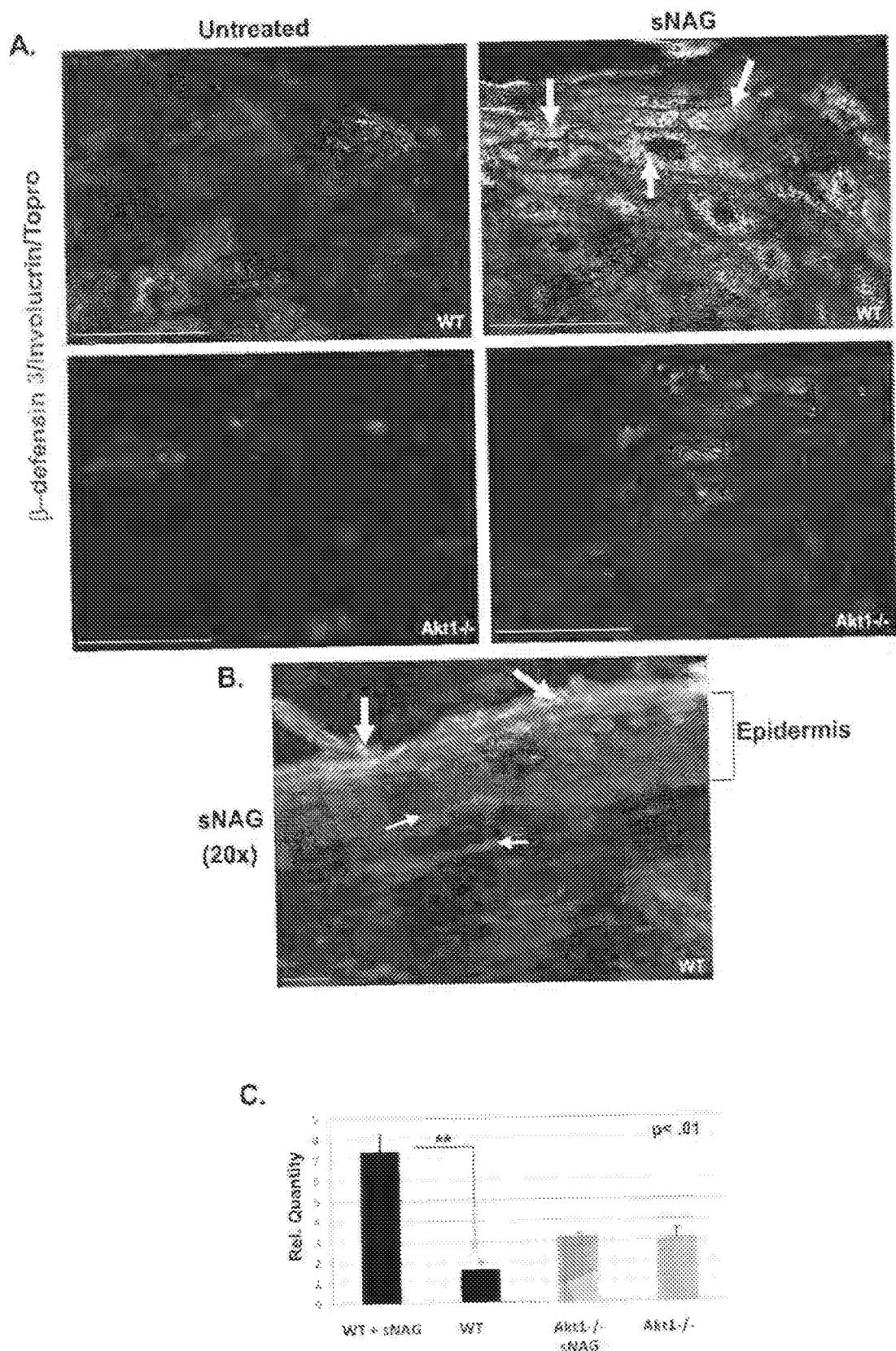


Fig. 9

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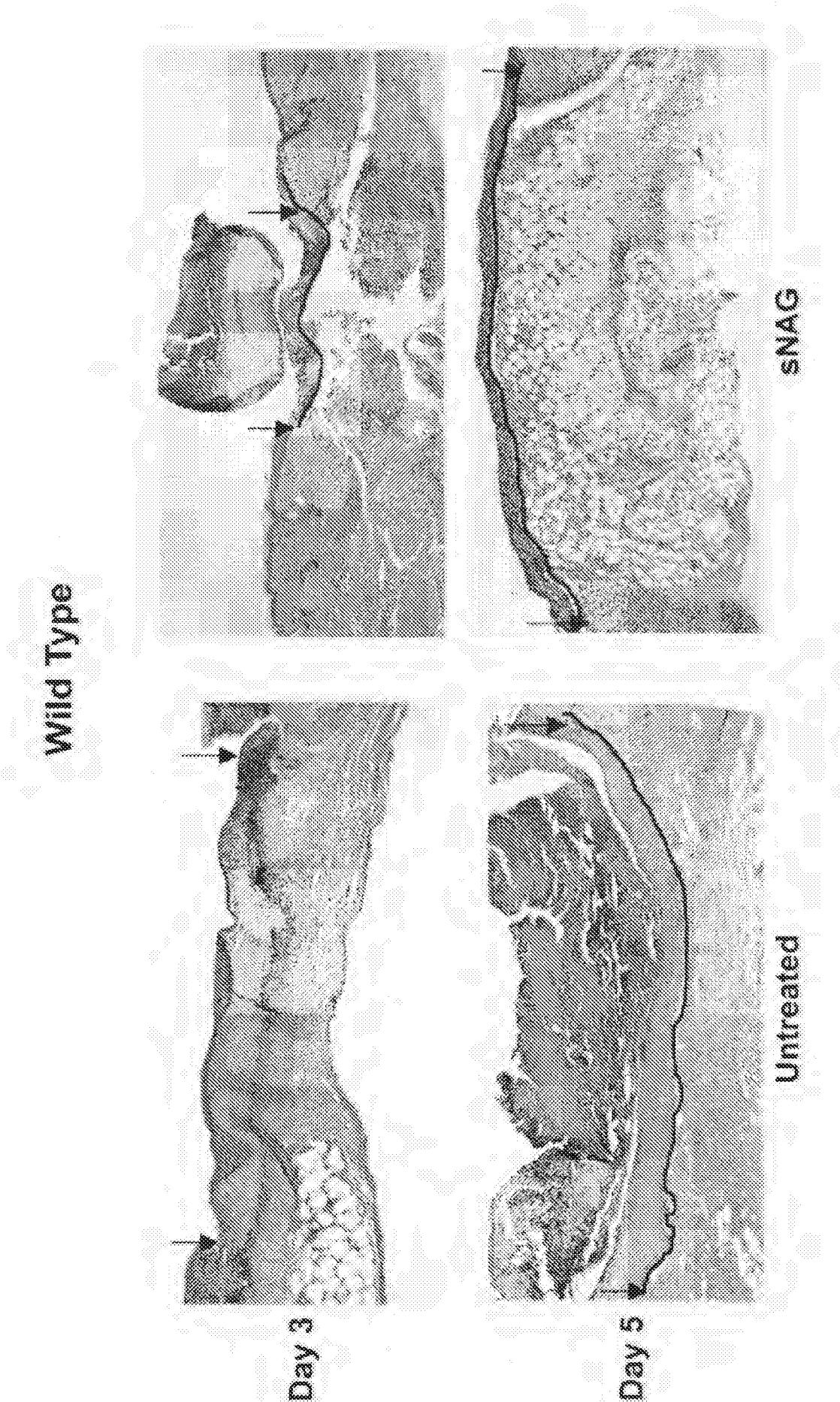


Fig. 10

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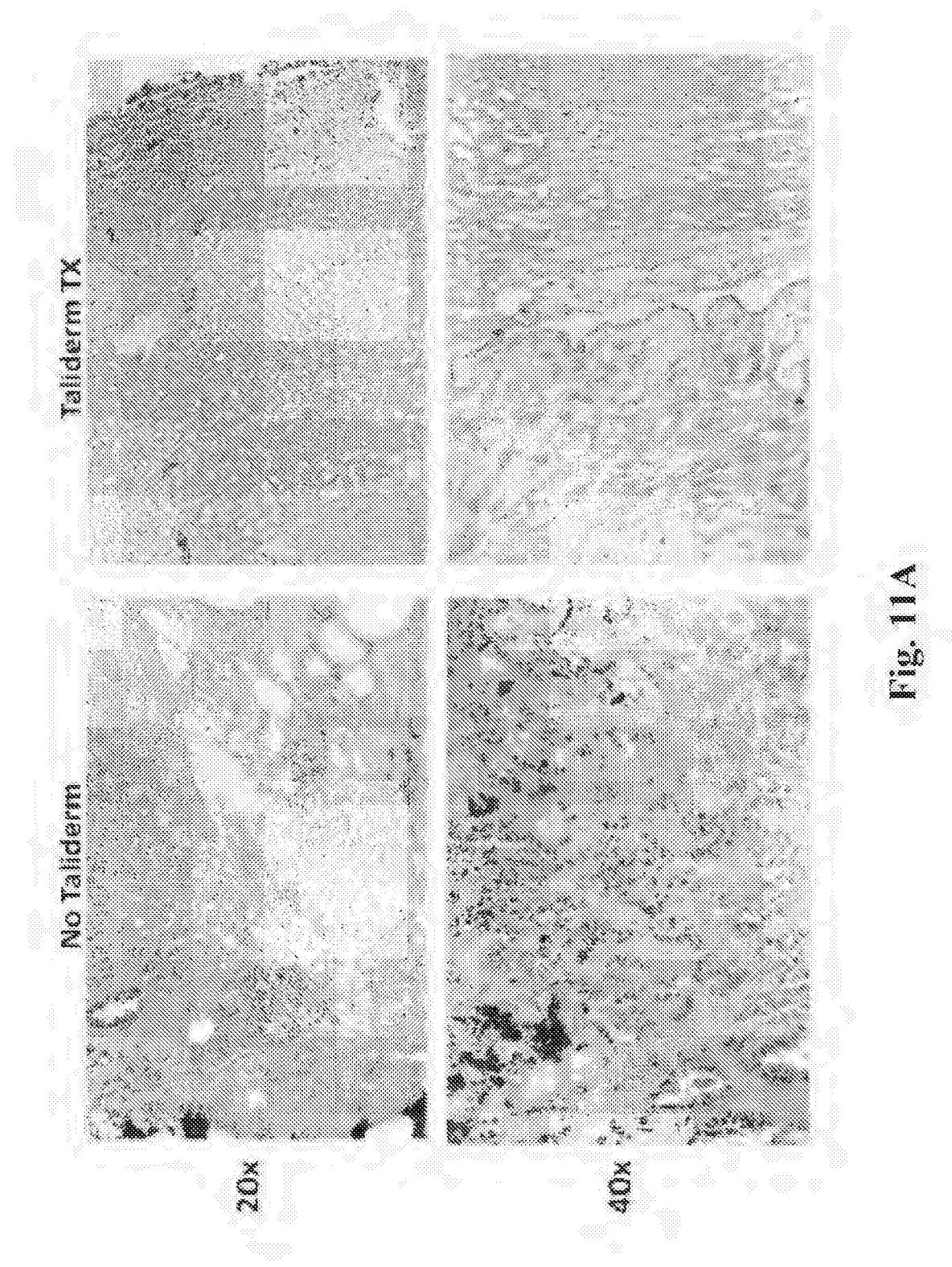


Fig. 11A

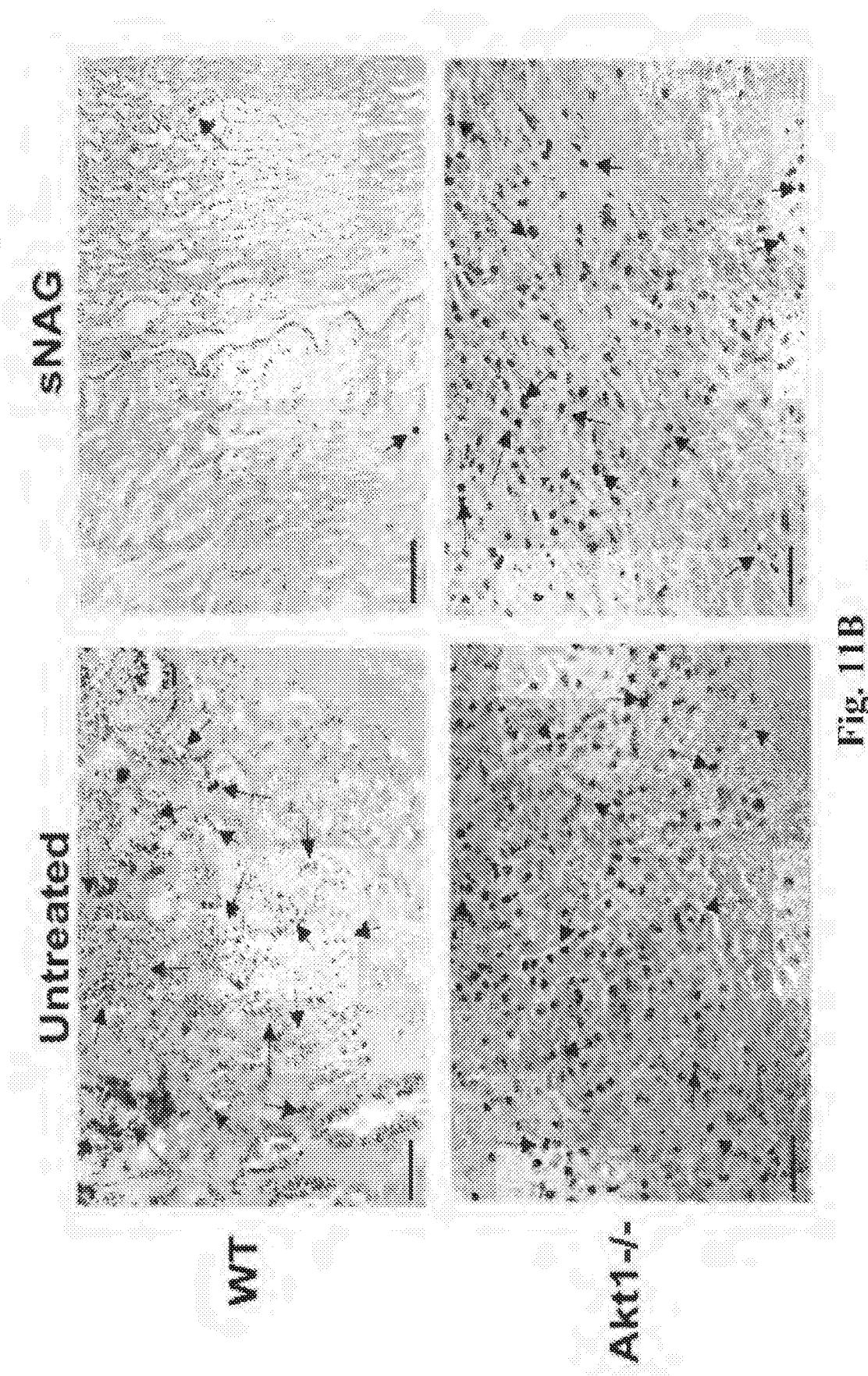


Fig. 11B

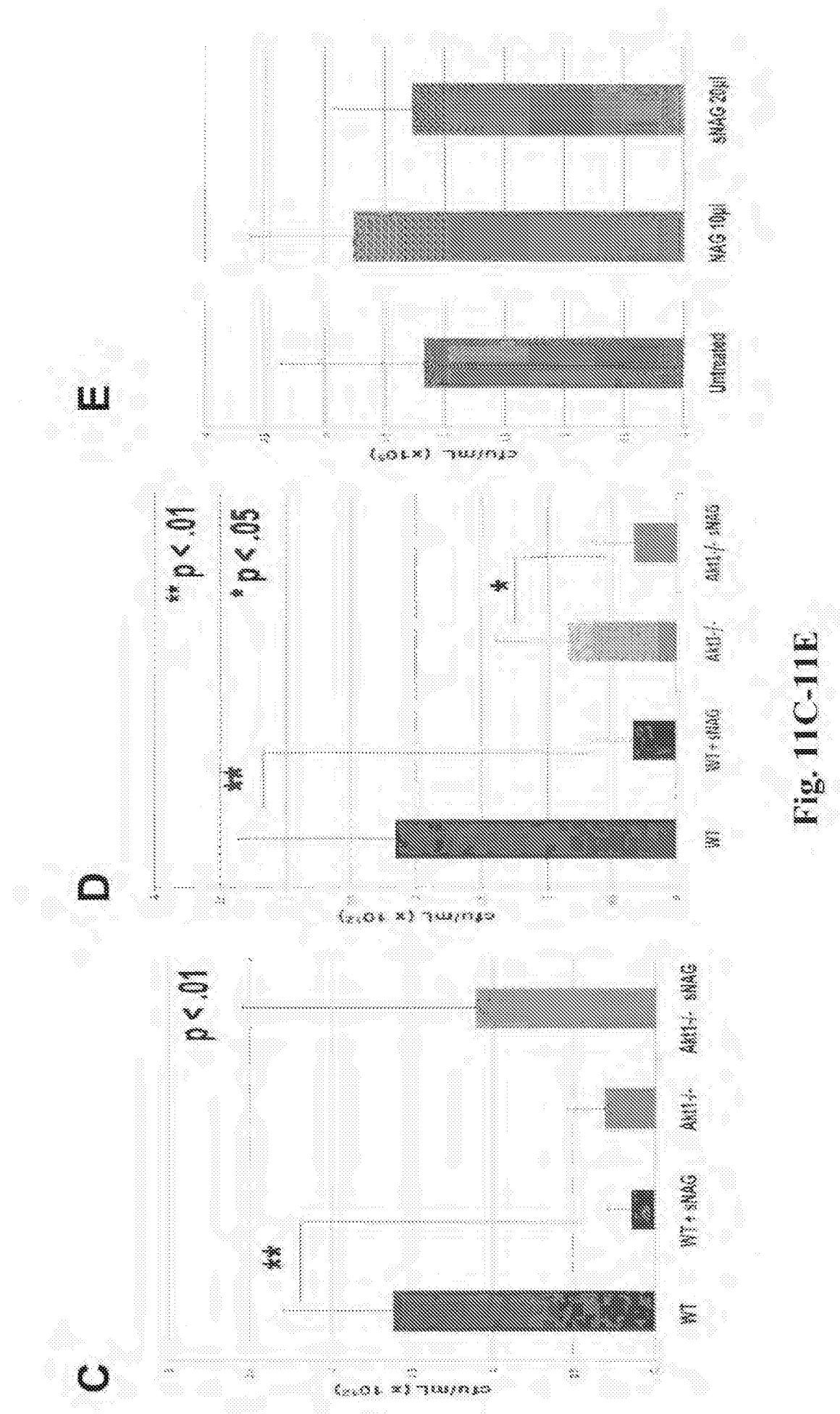


Fig. 11C-11E

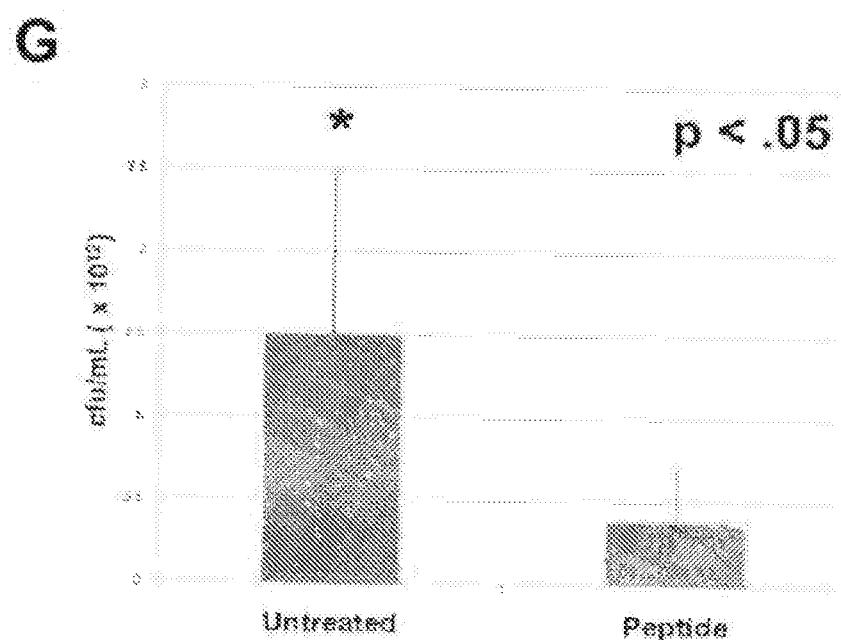
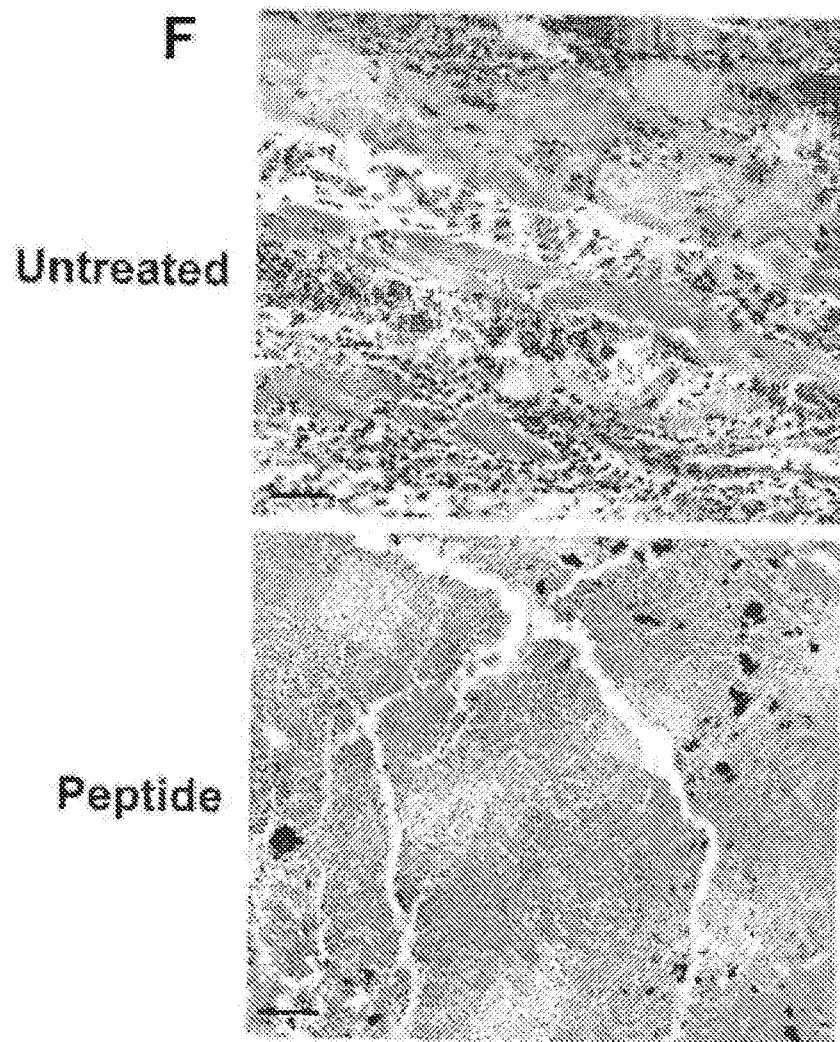


Fig. II F-II G

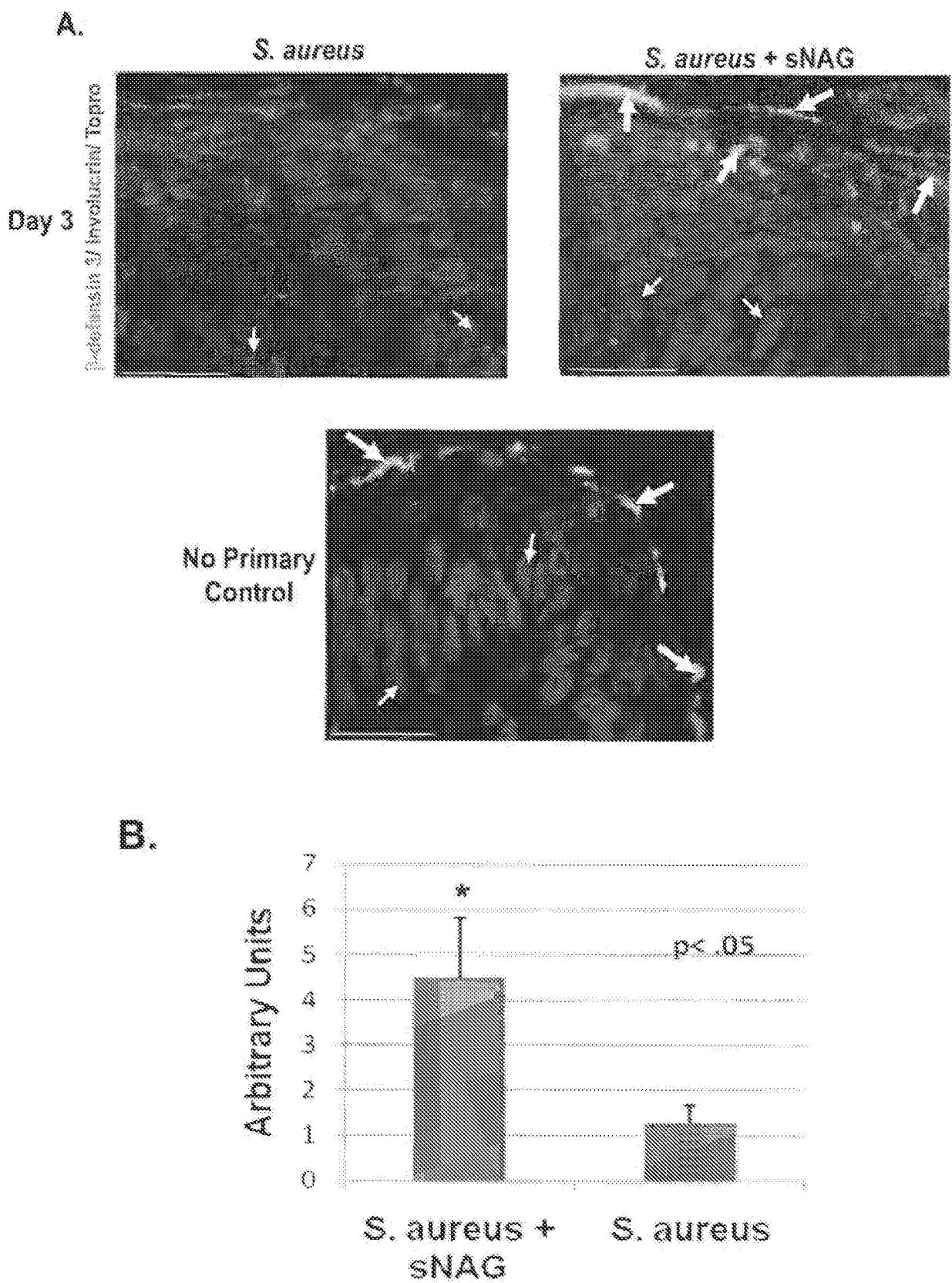


Fig. 12

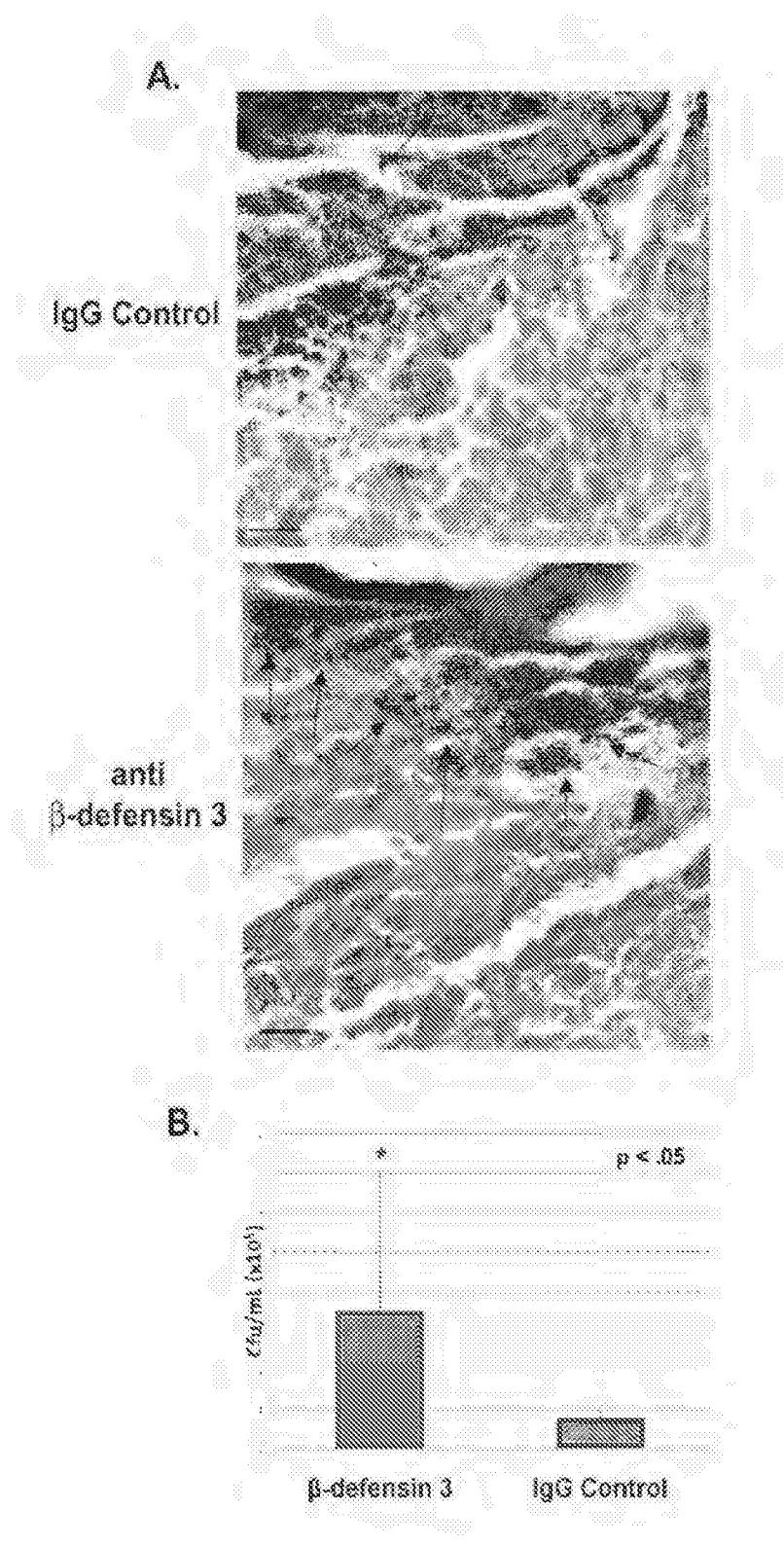


Fig. 13

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$\text{cfu}/\text{ml} \times 10^{11}$

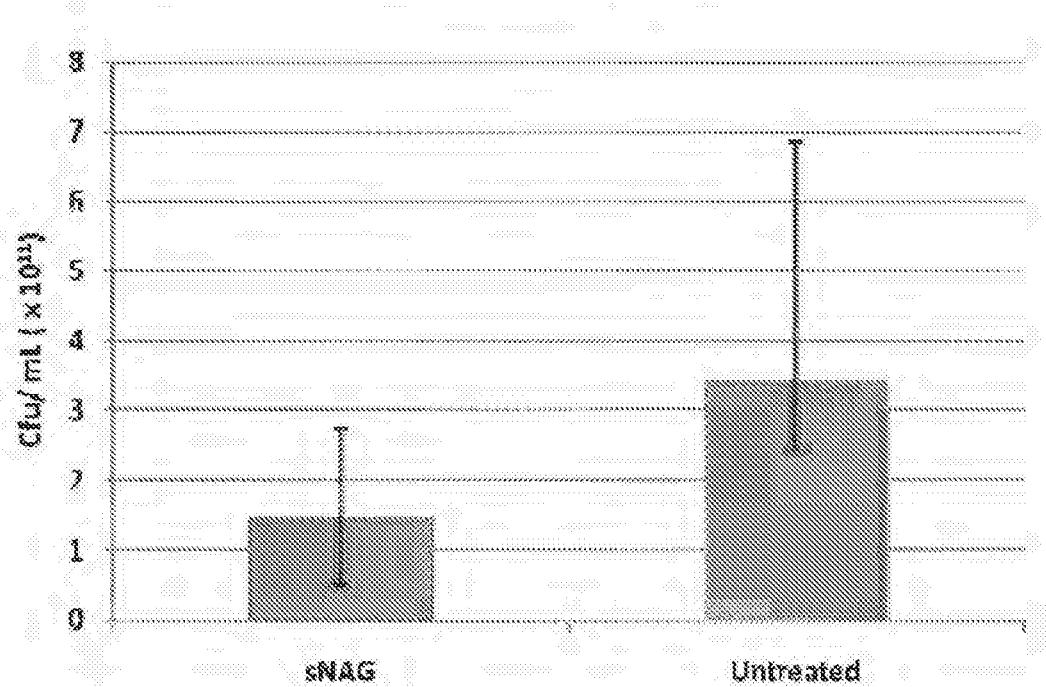


Fig. 14

Correlation between Molecular Weight of pGICNAC and Formulation for Irradiation

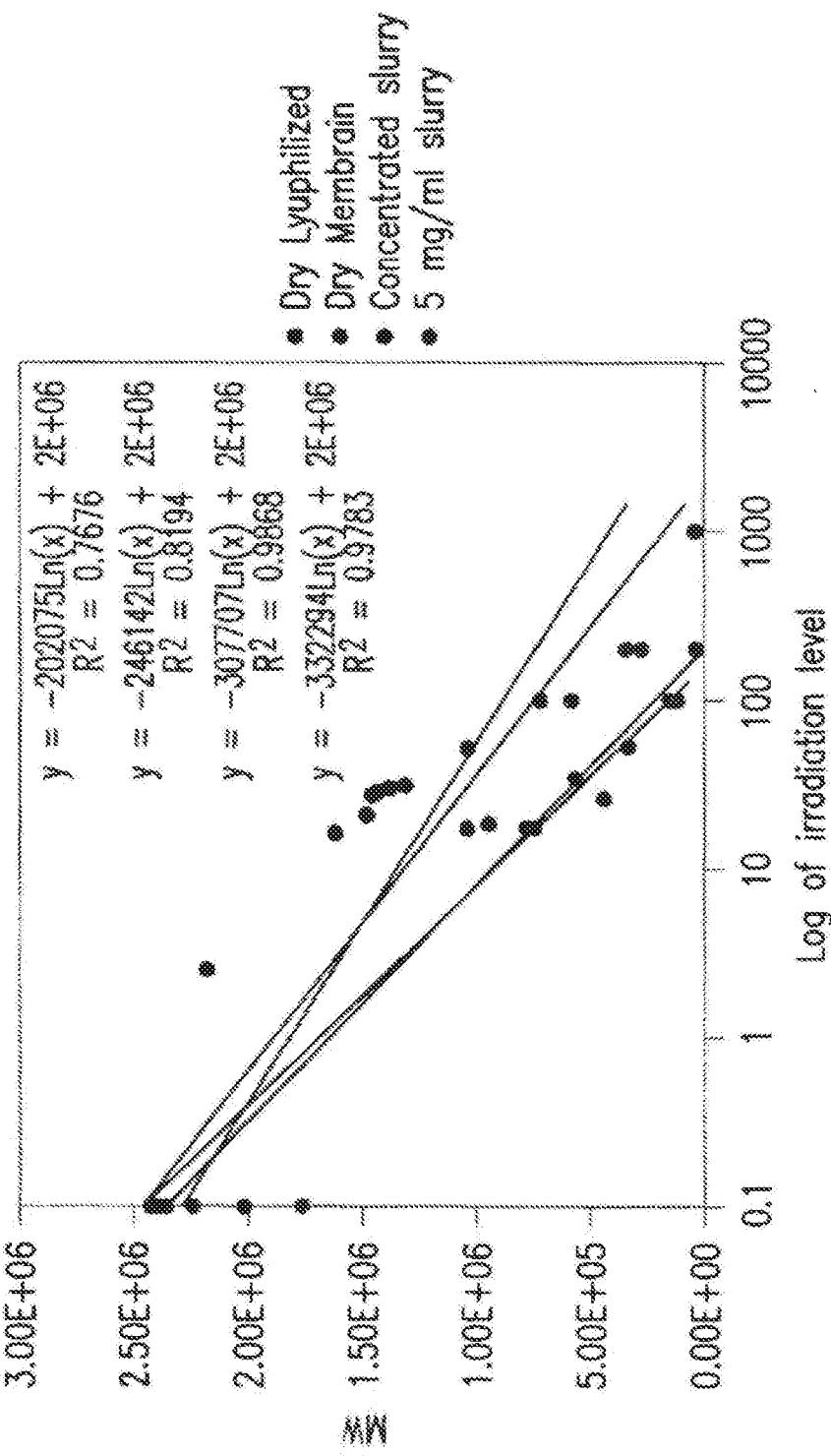


Fig. 15A

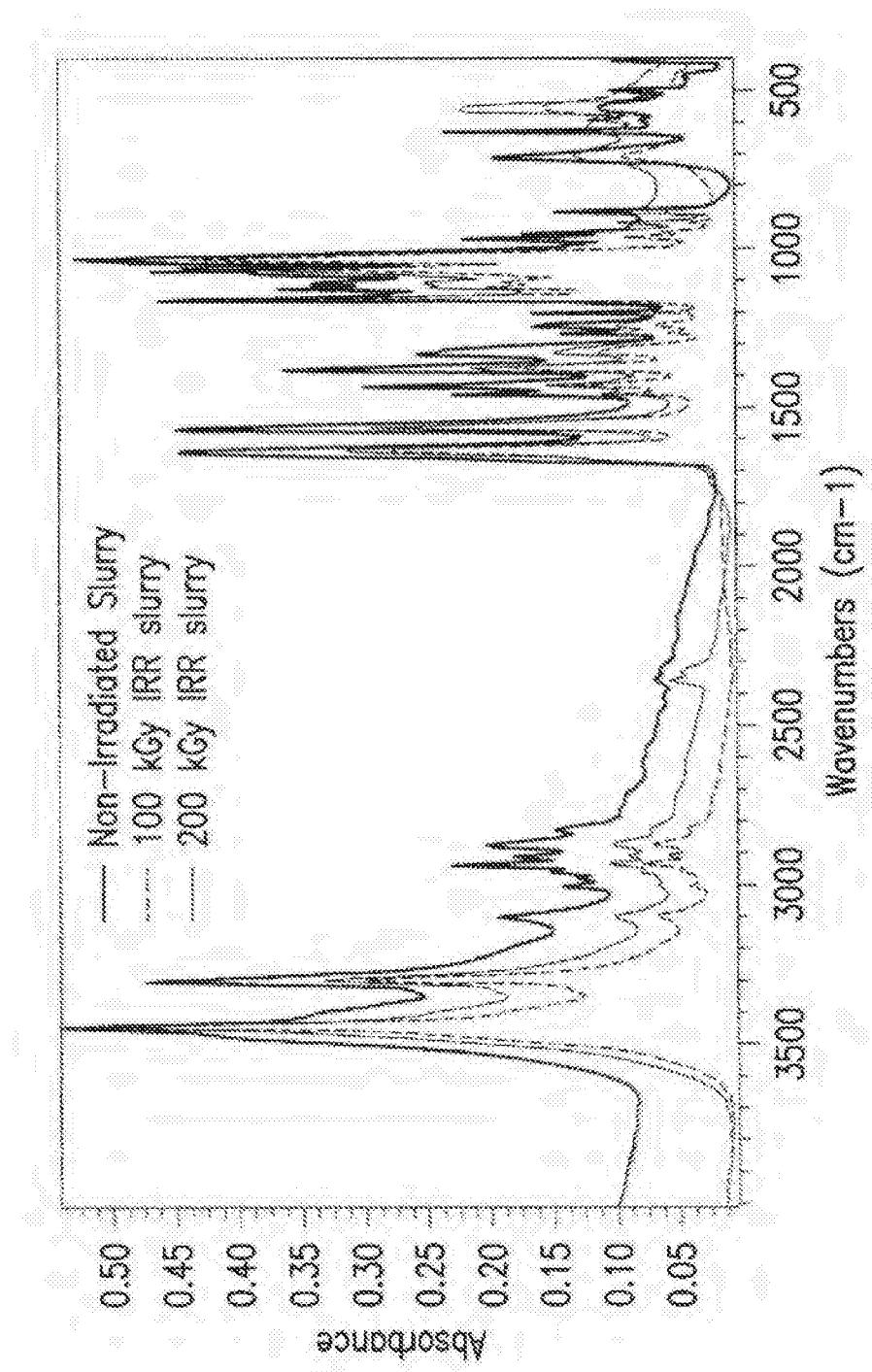


Fig. 15B

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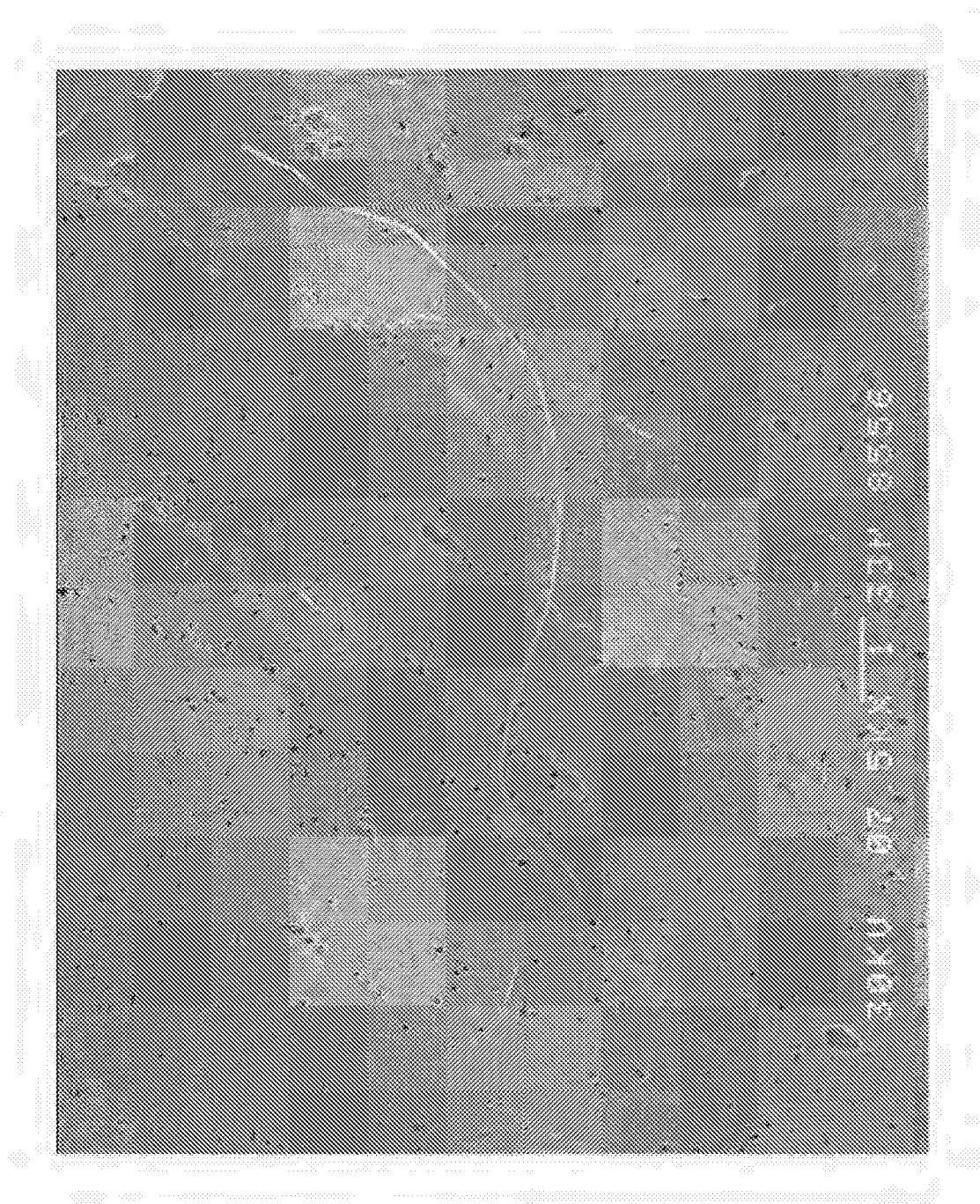


Fig. 15C

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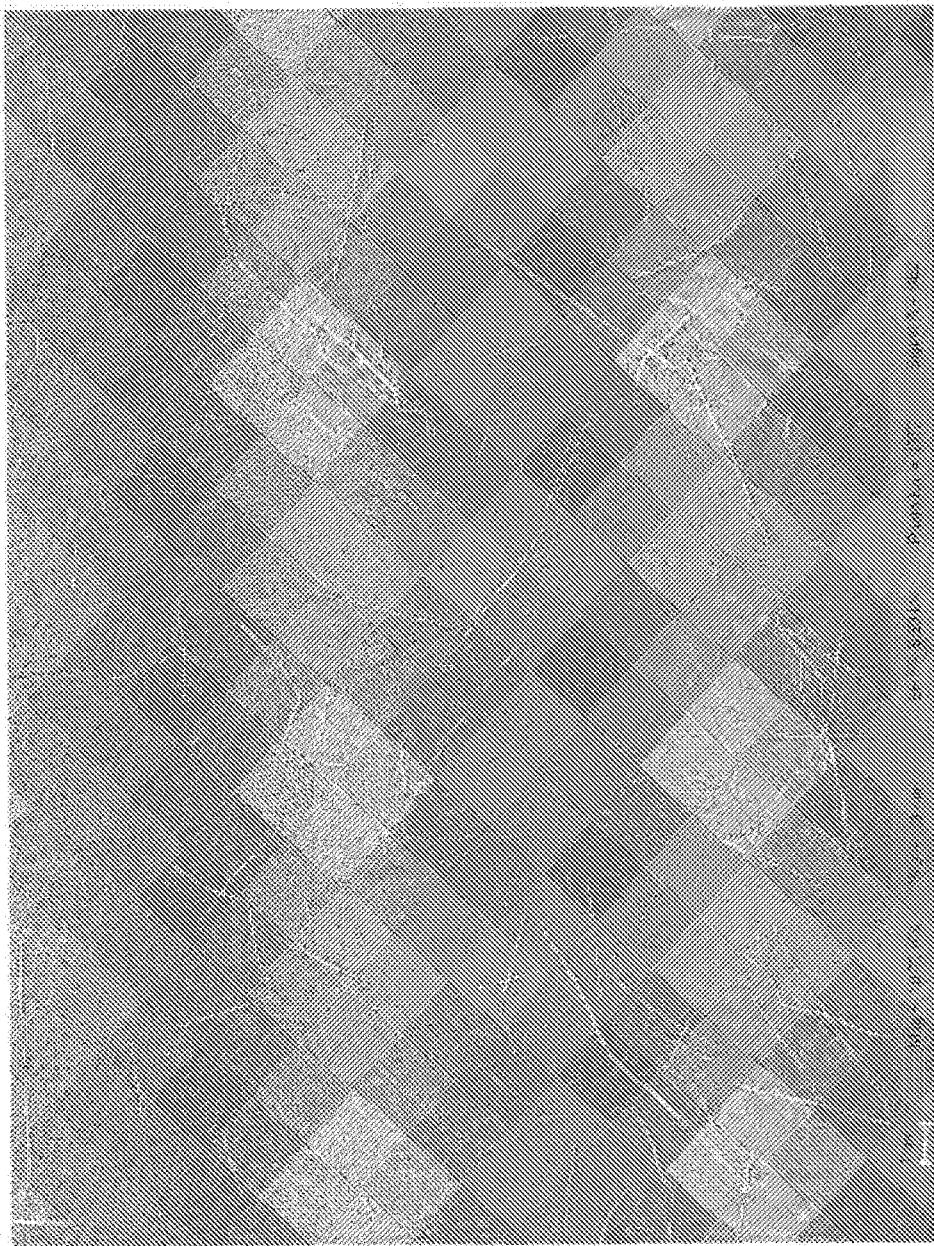


Fig. 15D

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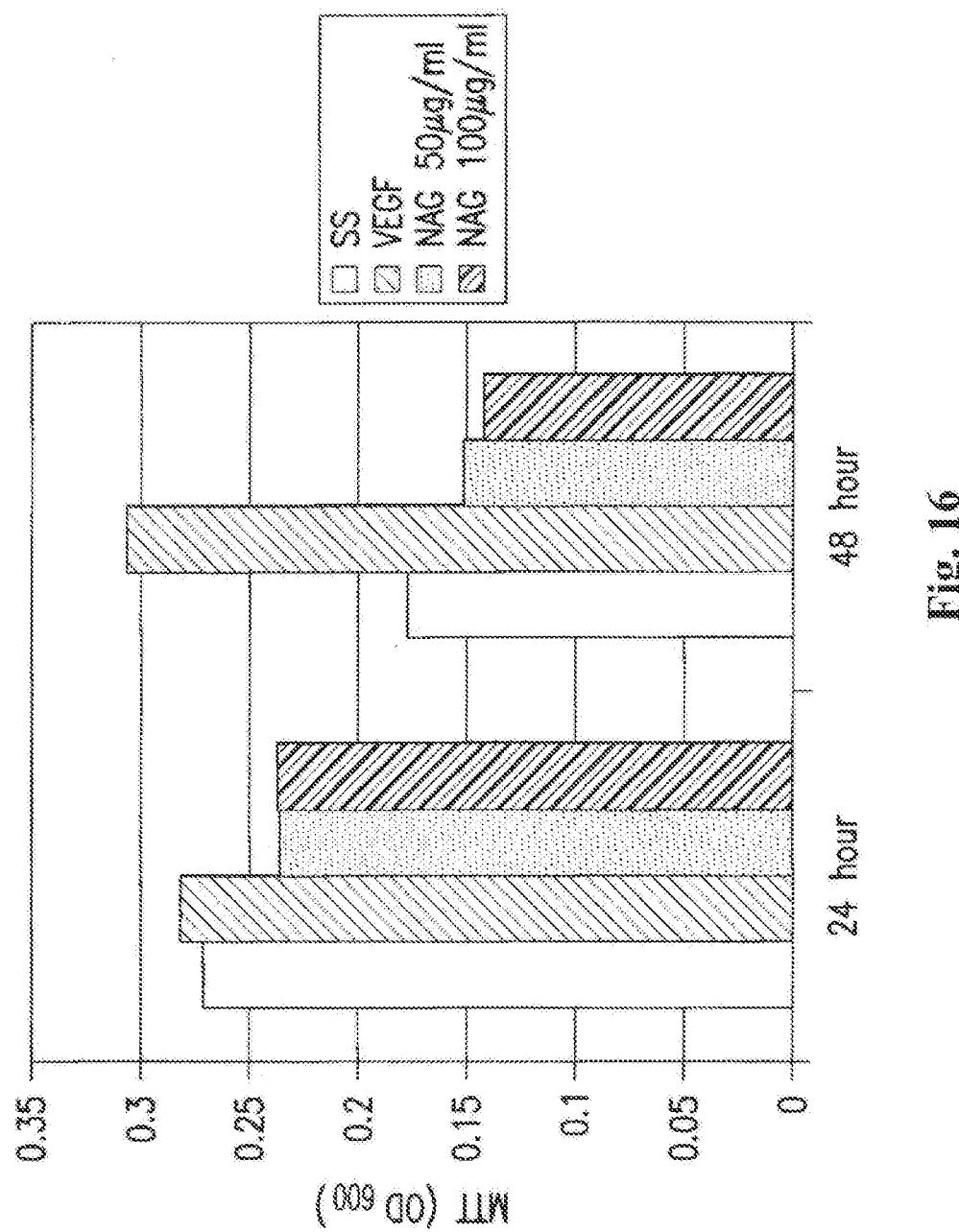


Fig. 16

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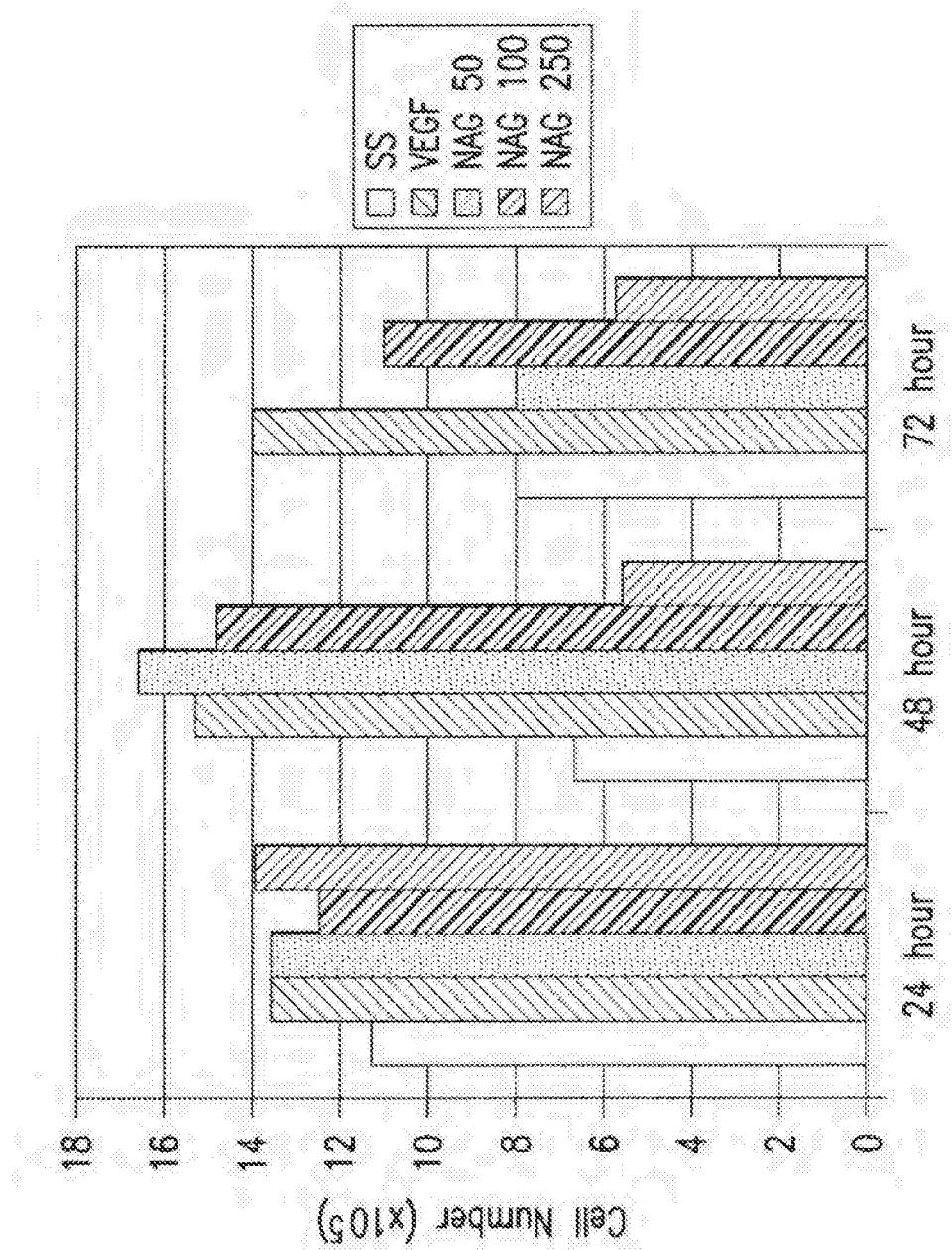


Fig. 17

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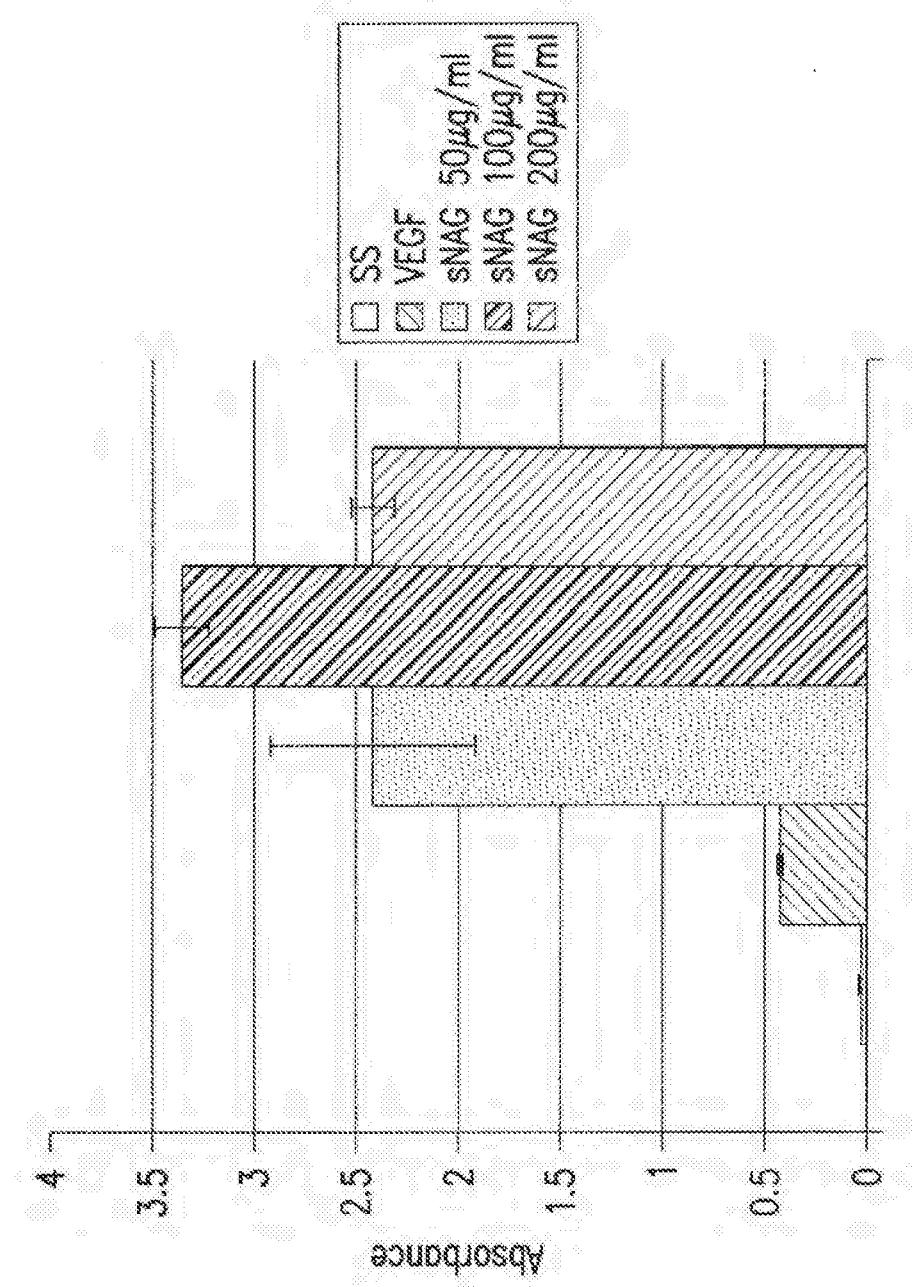


Fig. 18

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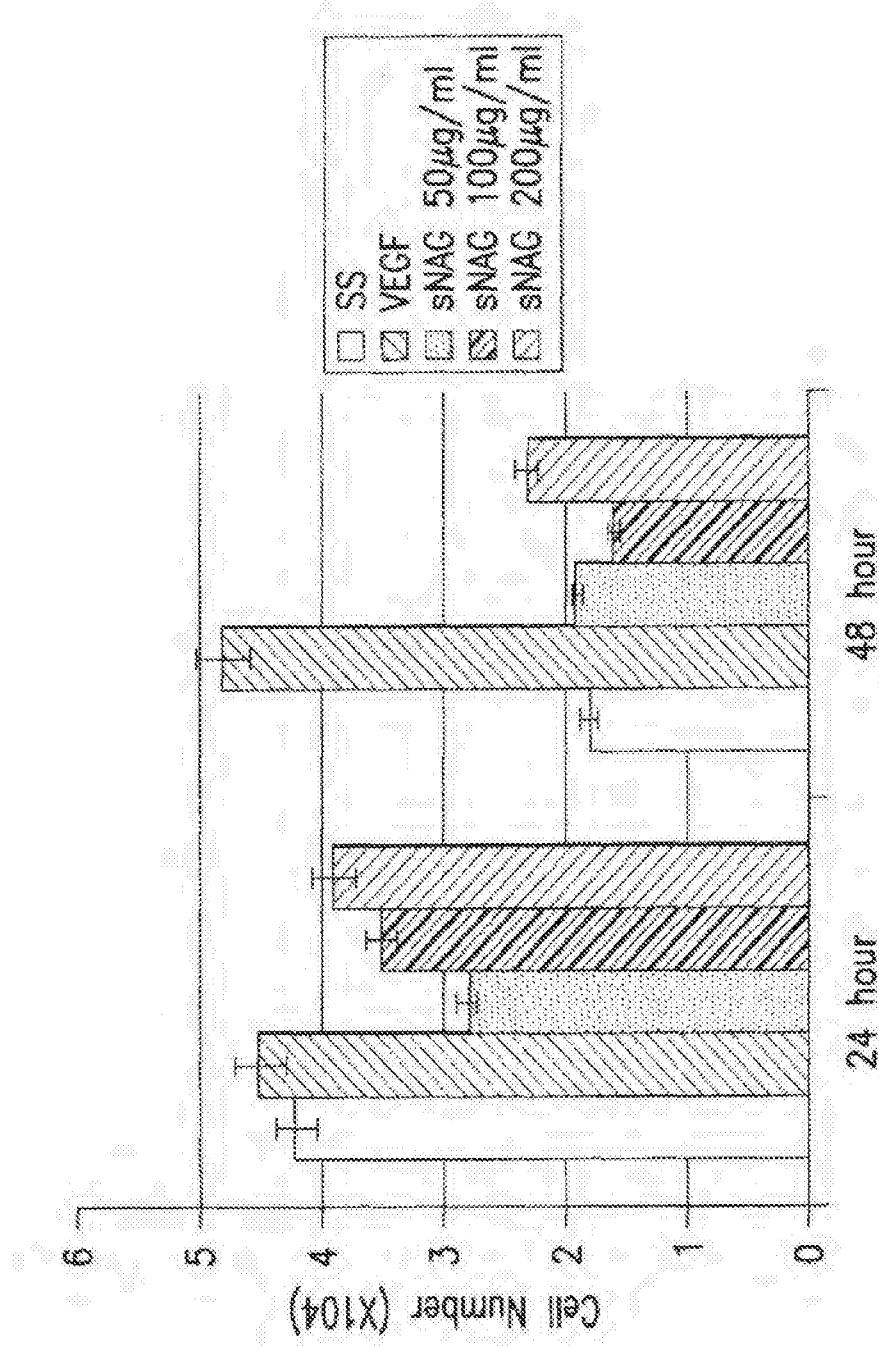


Fig. 19