# Protein Aggregation-Inhibition: A Therapeutic Route from Parkinson's Disease to Sickle Cell Anemia

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**Abstract**

Protein aggregation is implicated in multiple diseases, so-called proteinopathies, ranging from neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease (PD) to type 2 diabetes mellitus and sickle cell disease (SCD). The structure of the protein aggregates and the kinetics and mechanisms of aggregation have been the object of intense research over the years towards the development of therapeutic routes, including the design of aggregation inhibitors. Nonetheless, the design of drugs targeting aggregation inhibition remains a challenging endeavor because of multiple, disease-specific factors. Herein, we provide a perspective of this therapeutic route with emphasis on small molecules and peptide-based drugs in two diverse diseases, PD and SCD, aiming at establishing links among proposed aggregation inhibitors. The small and large length-scale regimes of the hydrophobic effect are discussed in light of the importance of hydrophobic interactions in proteinopathies. Some simulation results are reported on model peptides, illustrating the impact of hydrophobic and hydrophilic groups in water's hydrogen-bond network with an impact on drug binding. The seeming importance of aromatic rings and hydroxyl groups in protein-aggregation-inhibitor-drugs is emphasized along with the challenges associated with some inhibitors, limiting their development into effective therapeutic options, and questioning the potential of this therapeutic route.

## 1. Introduction

Protein aggregation is implicated in several human pathologies, ranging from sickle cell disease (SCD)[1–5], a red blood cell disorder, to various neurodegenerative diseases[6,7] (NDs), such as Alzheimer's (AD), Huntington's (HD), or Parkinson's disease (PD), commonly known as proteinopathies or protein conformational diseases. Although the root cause of most NDs, including PD, is not exactly known, compelling evidence[8–13] posits a relationship with protein misfolding and aggregation, resulting in the formation of abnormal protein aggregates, generally referred to as amyloids[6,7,14]. SCD, on the other hand, is probably the most well-known proteinopathy, coined the first molecular disease by Pauling and co-workers[1], being associated with the reversible aggregation of a mutated form of hemoglobin (i.e., sickle cell hemoglobin) most common in some parts of sub-Saharan Africa[15] and known to confer protection against malaria[16].

Several therapeutic routes have been concomitantly explored both in SCD and NDs in general. A common route includes the reduction of the monomeric precursor protein. This is because the kinetics of aggregation of both amyloid and HbS fibers depends on the concentration of the precursor as well as of the cell environment. Hydroxyurea, for instance, the most widely used drug to treat SCD, increases the levels of fetal hemoglobin (HbF), which does not polymerize[17–19]. The increase in HbF decreases the concentration of HbS, enhancing the delay time that precedes fiber growth, ultimately reducing the percentage of sickled erythrocytes.

Preventing or reducing protein aggregation in SCD[20–22] and NDs through a direct disruption of the aggregation mechanism is another possible therapeutic strategy since it could potentially prevent the diseases' progression without compromising the biological function of the protein. Ultimately, this route requires the rational design of drugs that can hinder or delay the aggregation process and, thus, a molecular knowledge of the aggregation mechanism of the proteins involved in the disease.

Herein, we provide an overview of the main features of two very different proteinopathies, PD (**Section 2**) and SCD (**Section 3**), along with some of the molecules, including small peptides, that have been found to exhibit some aggregation inhibitory activity and their proposed action mechanisms (**Section 4**).

Protein aggregation, much like protein folding, is deeply connected with hydrophobic interactions[23–25], which in turn are influenced by the surrounding environment, driving the conformational search towards a thermodynamic stable or native state. Hence, hydrophobic interactions are also discussed (**Section 5**) concerning protein aggregation and their influence on the design of aggregation inhibitors (**Section 6**).

The main goal of this review is two-fold: (i) expose putative similarities among aggregation inhibitors proposed for distinct proteinopathies, including their seeming "universal" or non-specific

action mechanism, and (ii) point out some of the main challenges concerning drug design associated with proteinopathies, with emphasis on the harnessing of the hydrophobic effect and the understanding of the action mechanism of some inhibitors and its relationship with specific chemical groups long reported to be pivotal to protein aggregation inhibition.

## 2. Parkinson's Disease

PD is the second most prevalent disease among NDs, next to AD, and the most common movement disorder. PD's etiology has been associated with the formation of cytotoxic oligomers[26,27], of which α-synuclein[28] (α-syn) is the primary component. These prefibrillar transient oligomers accumulate in intracellular inclusions called Lewy bodies and Lewy neurites[8,9], and are now[29–32] thought to be the main culprit, as opposed to the misfolded monomer and mature (insoluble) fibrils, in the loss of nigral dopaminergic neurons[10,11]. Despite the cell toxicity mechanism remains elusive, this is believed to be connected with perturbations in the neuronal membrane[30,33,34]. In addition, α-syn aggregates spread through a prion-like mechanism across brain cells, accelerating disease's progression[35–37].

### 2.1. α-synuclein Aggregation

α-syn is a 140 amino acid intrinsically disordered protein (IDP) mainly expressed in the central nervous system and whose function is not completely understood, although several putative functions have been put forward[26,38–42]. α-syn is comprised of three distinct domains (see **Fig. 1**), the N-terminal (N-term), a membrane-binding domain that tends to form α–helices, encompassing amino acids 1-60[43], the so-called non-amyloid-β component[44] (NAC), a highly hydrophobic and amyloidogenic domain comprising amino acids 61-95, and the C-terminal (C-term) domain, a more disordered region comprised of the amino acids 96-140.[26] Besides PD, α-syn aggregates are at the center of dementia with Lewy bodies[45] and multiple system atrophy[46,47], jointly known as synucleinopathies or Lewy body diseases[14,33,48].

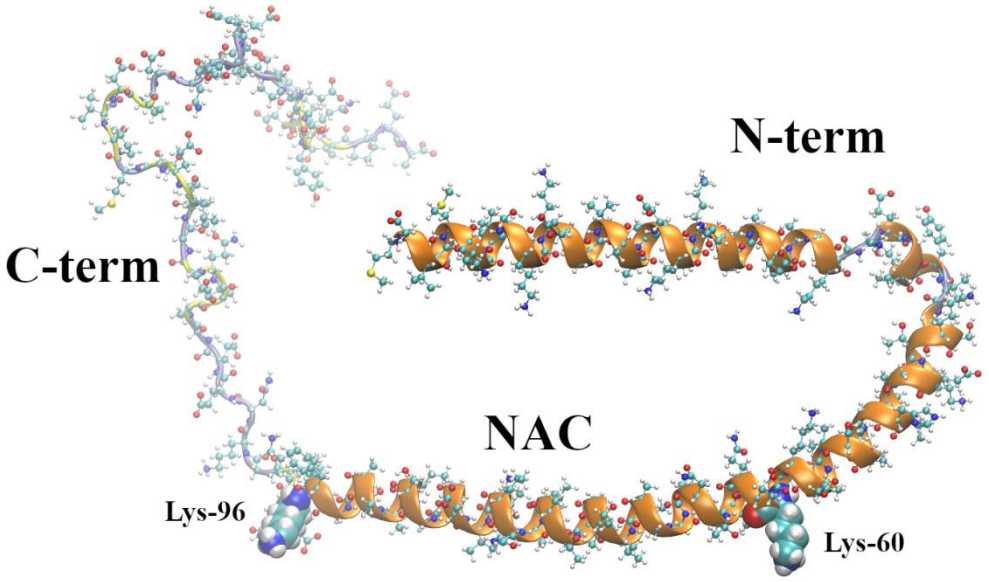


**Figure 1** - Membrane-bound α-syn structure (2kkw.pdb)[49]. The last amino acid of the N-term domain (Lys-60) and the first amino acid of the C-term domain (Lys-96), encompassing the NAC, are shown as vdW spheres. The chain colors correspond to the following secondary structures: α-helix (orange), turn (yellow), and coil (ice blue).

The structure of α-syn amyloid polymorph fibrils has been assessed by cryo-electron microscopy and NMR[50–53] (see **Fig. 2**) including membrane-bound α-syn[49,54] (**Fig. 1**). In addition, the structure of toxic oligomers[55,56] has been assessed through multiple experimental techniques, including circular dichroism, small-angle X-ray scattering, and atomic force microscopy-infrared spectroscopy[57–59].

The transient[60] and heterogeneous aggregational nature of α-syn poses a challenge to a comprehensive understanding of the aggregation mechanism(s) and kinetics[61], and their relationship with the onset of PD[26,27,62]. Nucleation–polymerization and nucleation–conversion–polymerization kinetic mechanisms have been proposed to describe the formation of soluble oligomeric species and the growth into insoluble mature fibrils[27]. The latter foresees a conversion stage where disordered oligomers with little or no stable β-sheet structure convert into more orderly and stable oligomers before they grow into fibrils[27]. Furthermore, the trigger behind the nucleation stage is thought to be associated with a conformational transformation (i.e., misfolding) of the natively unfolded protein into a more aggregation-prone partially folded intermediate[63,64]. The latter induces the formation of β-sheet-rich structures, a hallmark of the cytotoxic oligomers, nearly absent in the native α-syn. Further, the kinetics of aggregation is promoted by its binding with lipid membranes[65,66], in addition to the temperature, pH, or osmolytes.

In addition to the wild type α-syn, several pathogenic missense mutations[67–69] (e.g., A53T, A30P, E46K) have been implicated in early-onset familial PD, as opposed to the most common sporadic

form of PD, with some mutated forms accelerating the kinetics of oligomerization[70–74].

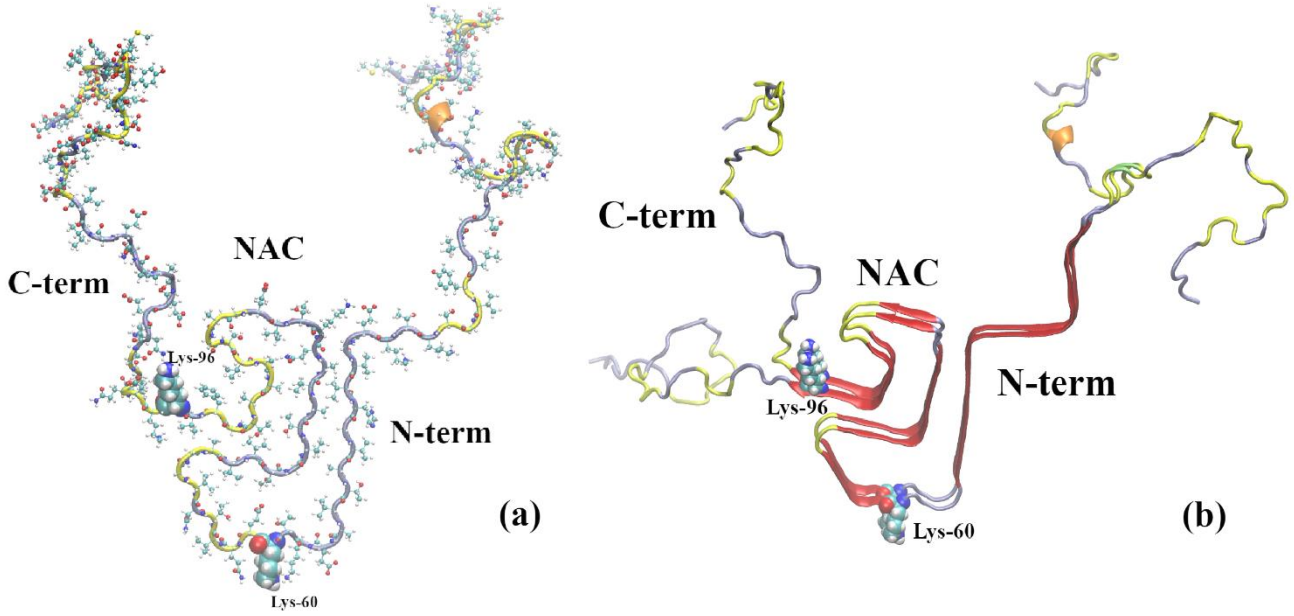


**Figure 2** – **(a)** α-syn monomer extracted from the α-syn experimental (NMR spectroscopy) protofibril reported by Tuttle *et al.*[50] (2n0a.pdb) **(b)** α-syn dimer extracted from the same experimental protofibril; cartoon representation showing a β-sheet-rich region in the NAC (amino acids 61-95) domain; some β-sheet is also visible in the N-term region. The last amino acid of the N-term domain (Lys-60) and the first amino acid of the C-term domain (Lys-96), encompassing the NAC, are shown as vdW spheres. The chain colors correspond to the following secondary structures: $3_{10}$ helix (orange), β-sheet (red), turn (yellow), and coil (ice blue).

Hydrophobic interactions (**Section 5.**), although insufficient to promote the formation of secondary and tertiary structures, are thought to be pivotal to IDP's aggregation. Thus, several domains within the NAC region have been shown to be key to the aggregation process[75–82]. That is to say that oligomerization decreases when specific changes in this region are promoted, opening the way for the development of drugs targeting these NAC sub-domains or stabilizing conformations of monomeric α-syn where intramolecular interactions shield the NAC region[78,83,84]. In addition, small domains in the NAC flanking regions have also been associated with α-syn's aggregation mechanism and function[85,86], including the domain comprised by amino acids 46-53 where the abovementioned missense mutations, implicated in familial PD, are found. The fact that these mutations are located in the N-term region suggests this segment could be equally important as the NAC domain to the aggregation process. **Figure 2b** shows a β-sheet-rich domain in this region of the N-term. This is also consistent with molecular dynamics results which indicate that the dimerization process already involves regions beyond the NAC segment[87].

Several other proteins, IDPs and globular proteins, are connected with the formation and

deposition of aggregates implicated in different NDs. Well-known examples include aggregates of mutated forms of the Huntingtin protein, forming intranuclear inclusions in HD[88], an autosomal dominant inherited disease, and the amyloid-β peptide and the tau protein, which form, respectively, extracellular amyloid plaques and intracellular neurofibrillary tangles in the brain, implicated in AD[6,7]. Although not discussed in this work, many of the drugs discussed in the **Section 4** (e.g., polyphenols) were found to inhibit aggregation of the proteins and peptides implicated in these diseases.

## 3. Sickle Cell Disease

At the other end of proteinopathies' spectrum is SCD, an autosomal recessive inherited disorder that affects hemoglobin, the protein responsible for the transport of $O_2$ and $CO_2$ in the red blood cells. Opposite to IDPs, hemoglobin (normal adult Hb; HbA) has well-defined secondary, tertiary, and quaternary structures[89]. HbA is an allosteric protein, existing in low-oxygen affinity and high-oxygen affinity quaternary conformational states, known as the T-state (tense) and the R-state (relaxed), respectively. HbA is composed of four polypeptide chains, 2 α subunits (α-globin) and 2 β subunits (β-globin); each α-globin is formed by 141 amino acids and a Heme group whereas β-globin is formed by 146 amino acids and a Heme group (see **Fig. 3a**).

### 3.1. HbS Aggregation

SCD is caused by a monogenic mutation in the β-globin gene that results in the substitution of a surface glutamic acid (charge -1) for valine (neutral and hydrophobic) at the 6th position of the β-globins of HbA[1–5]. This mutation, while not significantly changing the conformation of HbS[90], relative to HbA, reduces the solubility[5] of deoxygenated sickle cell hemoglobin (deoxy-HbS) from 7.0 $g \cdot cm^{-3}$ to 1.7 $g \cdot cm^{-3}$, inducing the (reversible) aggregation of deoxy-HbS into 14-stranded helical fibers[5,91–94,20,21,95–98]. These fibers are ~20 nm in diameter and distort the red blood cells into a stiff, non-deformable sickle-like shape, disrupting microcirculation and causing hemolysis[99]. Vaso-occlusion is responsible for pain crisis and organ failure, which ultimately can lead to death[100].

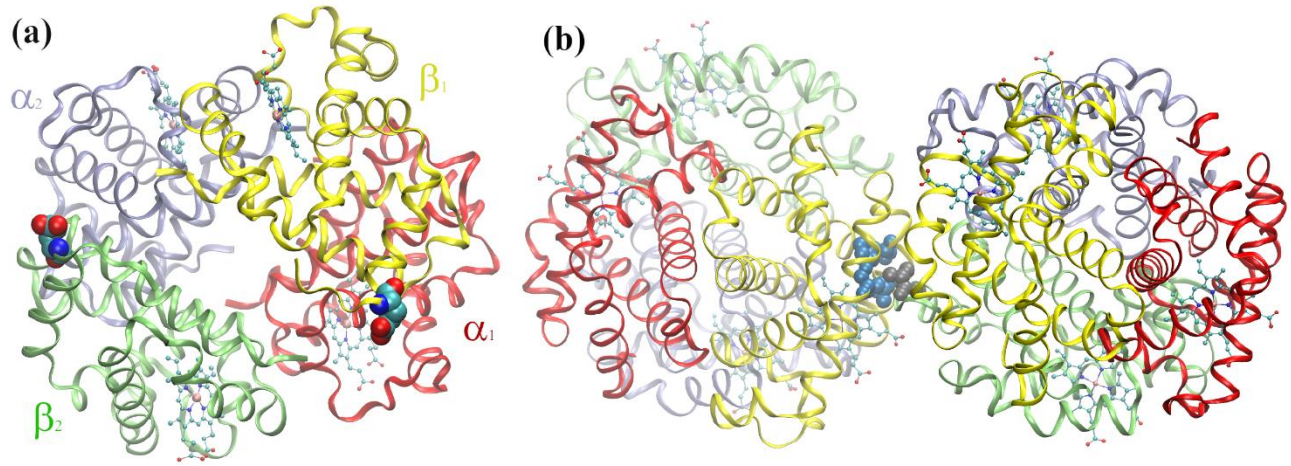


**Figure 3** – **(a)** Normal deoxygenated (T-state) hemoglobin, deoxy-HbA (2dn2.pdb)[101], showing the four heme groups and the Glu-β6 amino acid in $\beta_1$ and $\beta_2$, replaced by Val-β6 in HbS; **(b)** Sickle-cell deoxy-HbS dimer (2HBS.pdb)[92] showing the Val-β6 (grey spheres) in $2\beta_2$, lodged in a hydrophobic cavity in $1\beta_1$ formed by Ala-β70, Phe-β85, and Leu-β88 (blue spheres)[91–94,96].

HbS fibers involve a lateral contact where Val-β6 has its hydrophobic side chain lodged in a hydrophobic cavity of a neighbor HbS tetramer (see **Fig. 3b**). Further, the structure of the fibers has been shown to be similar to that of the deoxy-HbS crystal[102]. The kinetics of polymerization of HbS is characterized by a delay time and an exceedingly large dependence of the HbS intra-cellular concentration.[5,103–105] This delay time has been shown to be highly correlated with HbS supersaturation[106]. The kinetics is thought to proceed via a double nucleation mechanism[107,108] involving a stochastic homogeneous nucleation stage[109] in which the formation of HbS fibers occurs, followed by a heterogeneous nucleation stage involving the nucleation of additional polymers on the surface of the original fibers[110]. A kinetic model based on classic nucleation theory, which accounts for such a mechanism, aimed at probing the effectiveness of potential anti-sickling drug candidates was recently reported[111]. Another recent kinetic study found the HbS polymerization process to be a rather rapid and inefficient process, namely, fiber growth, re-opening the window for drugs directly targeting aggregation[112].

Whereas *in vitro* polymerization is commonly studied in a high concentration phosphate buffer, allowing reducing the solubility of HbS[113] and therefore the amount of HbS required to observe polymerization, a similar structure[114] and double nucleation mechanism is seemingly found. Thus, although some differences have been reported (see for instance ref.[115] and refs therein) polymerization is characterized by a lag time, similar to that observed at physiological conditions[105,116]. A similar solubility decrease is found in 2,3-bisphosphoglycerate (2,3-DPG) solutions[117–119] ; 2,3-DPG is an allosteric effector found in erythrocytes that enhances oxygen delivery, shifting the hemoglobin R-T equilibrium to the T conformational state, thus, enhancing

aggregation by increasing the concentration of deoxy-HbS. In addition 2,3-DPG stabilizes the fibers by decreasing the solubility of HbS[20].

Upon oxygenation the fibers disassemble without a delay period and most (5-10% erythrocytes sickle irreversibly[120]) erythrocytes recover their biconcave disk shape[5]. However, the rate of uptake of $O_2$ of deoxygenated sickled erythrocytes (82 ± 4.7 msec) is slower than that of normal erythrocytes (135 ± 17.6 msec), reflecting the time of depolymerization, among other possible factors[120,121]. These sickling-unsickling repeated cycles possibly damage the erythrocytes' membrane leading ultimately to extra- and intravascular hemolysis[5,122].

Concerning the molecular nature of the aggregation, this is believed to be primarily associated with hydrophobic interactions because of the nature of the abovementioned lateral contact[5,123,124]. A negative free energy around -3 kcal·mol$^{-1}$ along with a nearly zero enthalpy was found for the polymerization (i.e., gelation) process, in a 0.15 M potassium phosphate solution at 37 ºC.[125] The solubility was found to decrease with increasing temperature, with a minimum (0.16 g cm$^{-3}$) at 37 °C, increasing at higher temperatures. A monomer-polymer contact binding free energy of -7.5 kcal·mol$^{-1}$ was also reported by Cao and Ferrone[124] from nucleation theory and kinetic rate measurements. Assuming each Val-β6 and hydrophobic cavity in each β-globin contributes[126] ~1.5 kcal·mol$^{-1}$, Cao and Ferrone[124] estimated hydrophobic interactions to contribute 80% of the monomer-polymer binding free energy[124]. This estimative, however, assumes the host pocket as well as the other Val-β6 not involved in the lateral contact, also contribute ~1.5 kcal.mol$^{-1}$, an assumption that can be questioned because the pocket also exists in HbA and should be largely dewetted; MD simulations support this view[127].

Wang and Ferrone[123] studied through light scattering experiments, the aggregation of deoxy-HbS and several nonpolymerizable species, including deoxy-HbA, below the solubility for polymer formation. A positive binding free energy was found at 1 mM and 302 K for deoxy-HbA (4.0 kcal mol$^{-1}$) and deoxy-HbS (1.8 kcal mol$^{-1}$); the dimerization of deoxy-HbA involves, however, the (weaker) axial contact, as opposed to the lateral contact, which involves the β6 mutation site. Furthermore, HbS association was found to be entropically favored and enthalpically disfavored, consistent with the view that deoxy-HbS aggregation is impelled by hydrophobic interactions.

Nonetheless it has also been suggested that electrostatic interactions could be at least equally important[92,127–129]. Kuczera *et al.*[128] in fact long suggested that "for a fiber-like dimer structure, it is not the stabilizing hydrophobic interaction of Val in HbS that is the dominant factor, but the loss of the destabilizing interaction of Glu in HbA." Galamba and Pipolo[129] provided evidence, based on molecular dynamics (MD) simulations, that aggregation could be triggered by the formation of

hydrogen-bonded ion pairs (i.e., salt bridges) between several surface residues (Lys, Asp, Glu) as well as heme groups. A subsequent study[127] provided evidence that the absence of Glu-β6 was more important than the presence of Val-β6, consistent with Kuckzera *et al.*[128]. In particular, it was shown that the Glu-β6→Val-β6 mutation favors aggregation through the elimination of strong electrostatic repulsions involving Glu-β6 and several residues, noteworthy, Asp-β73 and Glu-β90, as well as heme, whereas a mild attractive potential energy was found to be connected with Val-β6[127]. Asp-β73 was found to be especially relevant, being associated with strong electrostatic repulsions with Glu-β6 in the deoxy-HbA dimer, while forming a major attractive residue pair with Val-β6 in deoxy-HbS (see **Fig. 4**), supporting the view that damping of electrostatic repulsions involving Glu-β6 in deoxy-HbA could explain the polymerization of deoxy-HbA at high potassium phosphate concentrations[5].

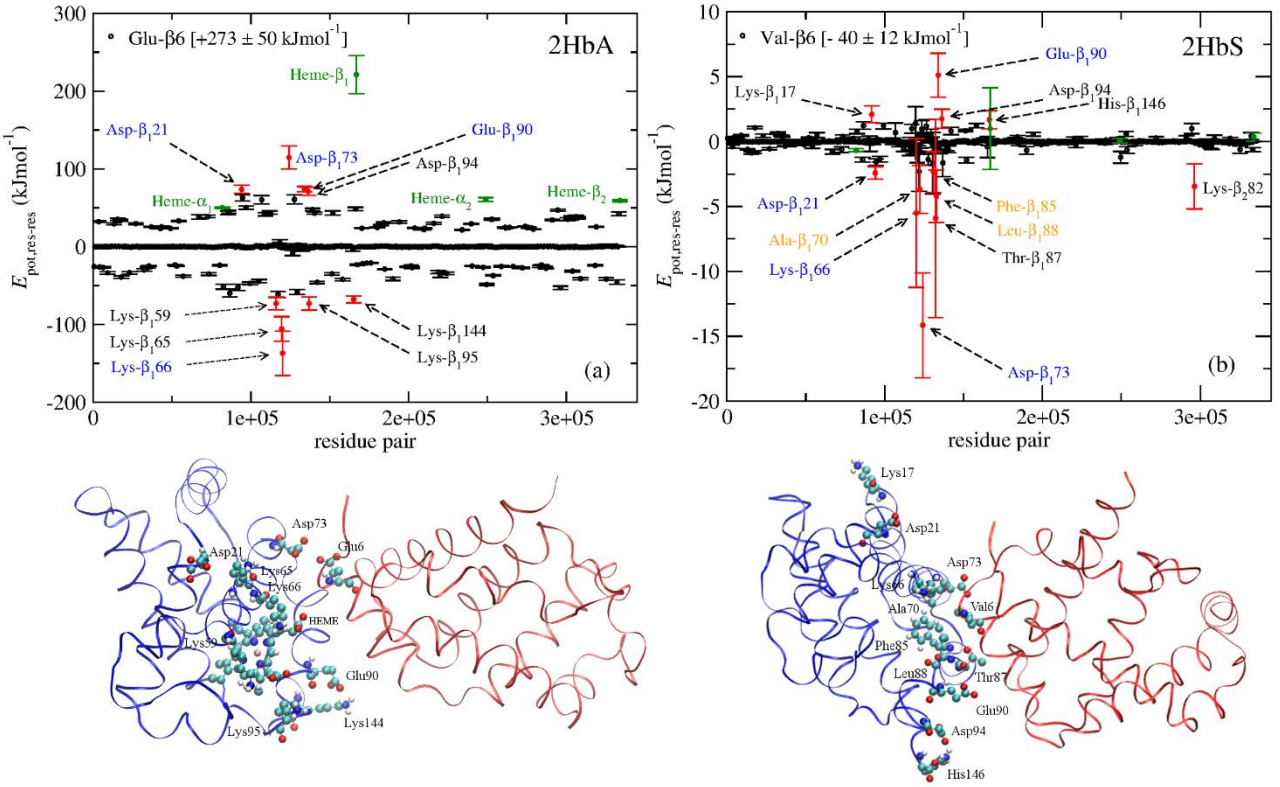


**Figure 4 – (a)** Glu-β6 (HbA) and (b) Val-β6 (HbS) interactions with every residue from adjacent HbA and HbS tetramers, respectively. The residues forming the hydrophobic pocket in HbS-1, Ala-β70, Phe-β85, and Leu-β88, are shown in orange in **(b)**. The most important residues in the $1\beta_1$ (blue) and Glu-β6 and Val-β6 in the $2\beta_2$ (red) polypeptides from a MD snapshot are shown below. The potential energy, and not the electrostatic and vdW energy, are plotted in (a) and (b), respectively. Reprinted from ref. [127].

Interestingly, Asp-β73 is a point mutation in HbC-Harlem (Glu-β6→Val-β6; Asp-β73→Asn-β73) which differs from HbS in this single amino acid (i.e., Asp-β73→Asn-β73)[130]. This mutation was shown to have a pronounced effect in the aggregation of HbC-Harlem, relative to HbS, resulting in

the formation of crystals as opposed to polymers, seemingly through a similar mechanism, although with a much slower kinetics than HbS gellation[131,132]. Adachi *et al.*[133] related some of these differences with the formation of a hydrogen bond between Asp-β73 and Thr-β4, further influencing the hydrophobic interaction between Val-β6 and the hydrophobic pocket in deoxy-HbS.

A binding free energy of -14 kcal·mol$^{-1}$ and -4 kcal·mol$^{-1}$ were found, respectively, for the HbS and HbA lateral contact dimers in the abovementioned MD studies[127,129]. These studies further suggested that possible aggregation inhibitors could target several salt bridges found in the HbS dimer, instead, or in addition to the hydrophobic contact.

MD simulations of sickle and normal hemoglobin and hemoglobin fibril models were also recently reported[134–136]. Maity and Pal[135] argued that the presence of hydrophobic residues without a bulky side chain at β6 in hemoglobin explained the stability of the fibrils, consistent with the experiments by Adachi *et al.*[137], which showed that some substituents in the β6 position, such as phenylalanine and tryptophan, polymerized less readily compared to deoxy-HbS and that when oversaturated polymerization occurred without the delay time observed for HbS. Adachi *et al*. argued that the difficulty of insertion of the bulky side chains of phenylalanine and tryptophan into the hydrophobic acceptor pocket on an adjacent tetramer could inhibit nuclei formation prior to polymerization. Interestingly, however, phenylalanine, tryptophan and derivatives and analogues[138–141] are themselves aggregation inhibitors, although it is not known whether these interact with the amino acids that form the hydrophobic pocket that lodges Val-β6.

Olagunju *et al.*[136] found through MD simulations that both electrostatic interactions and hydrophobic interactions involving the mutation site are important in the HbS aggregation. The authors suggested that a potential aggregation inhibitor could, in addition to target HbS–HbS interactions involving Val-*β*6, aim to interrupt an electrostatic contact involving Lys-*β*17 and the Glu-*β*90 of a neighbor tetramer. This same contact was identified as being among the lowest energy contacts in the deoxy-HbS and deoxy-HbA dimers (see SI of ref. [127,129]).

While not an exhaustive road map through the drugs developed for NDs and SCD, in what follows we aim at providing a broad perspective on some of the early and most recent molecules shown to have aggregation inhibitory activity, and their putative action mechanism, in the context of SCD and NDs, especially PD. In addition, we aim to establish some contact between aggregation inhibitors found for such different diseases, involving archetypes of an intrinsically disordered protein and an allosteric globular protein.

## 4. Protein Aggregation Inhibitors

### 4.1. Parkinson's Disease

A potential disease-modifying drug for PD, that is, one beyond the symptomatic treatment, should aim at reducing the α-syn expression, aggregation, membrane affinity and cytotoxicity, and/or propagation[41]. In addition, reversing α-syn aggregation could be pivotal to treating several proteinopathies because of delayed diagnosis. Despite several molecules being found to induce at least one of these responses *in vitro* and/or *in vivo*, these either have some major drawback, ruling out their therapeutic potential, or have not passed clinical trials. Hence, the most effective drug still used today against PD is L-3,4-dihydroxyphenylalanine (aka levopoda or L-dopa)[142], a dopamine precursor developed in the late 1960s. L-dopa increases the dopaminergic flux in the striatum, without, therefore, curing the disease nor influencing non-dopamine-associated symptoms[143–145].

Thus, several alternative therapeutic routes continue to be actively explored[143,146]. These include, for instance, drugs that target non-dopaminergic neurotransmitters[147,148], associated with symptoms not alleviated by L-dopa and other related drugs, neuroprotective therapies[149], aggregation inhibitors, gene therapies, or cell-based treatments[150]. The latter, as discussed in the next section (**Section 4.2**), were already successfully applied in the cure of SCD, although several challenges persist.

Concerning protein aggregation inhibitors, covered herein, although there are still no approved drugs that preclude or even delay the formation of α-syn oligomers[143], major advances have been achieved in recent years both in unraveling the structure of the multiple putative cytotoxic oligomers and in the design of new drugs targeting aggregation-inhibition. Molecules that showed aggregation-inhibition potential in PD and other proteinopathies encompass peptides[75,82,151–154], peptidomimetics and macrocycle peptides[155,156], antibodies[157], heat-shock proteins[158,159], and small organic molecules, including natural products[160–165]. Here, we discuss various small molecules and peptide-based aggregation inhibitors developed through *in silico*, *in vitro*, and *in vivo* model studies.

#### 4.1.1. Small Molecule Drugs

Several small organic molecules were found to exhibit anti-amyloid activity, although their action mechanism is not always completely understood. Some of the most explored small molecule anti-amyloid agents, including natural products, are catecholamines[166–173], phthalocyanines[174–177], and polyphenols[163,178–192], and among the latter, flavonoids[58,181,182,193]. Many other small molecule drugs[183,194–202], were, however, reported to inhibit the aggregation of α-syn. In addition, many such anti-amyloid drugs are not protein specific, exhibiting anti-amyloid activity for different proteins involved in several proteinopathies[161,163,174,183,188,203]. Some of these small molecules stabilize the

monomeric form, inducing the formation of disordered non-toxic oligomers, whereas others prevent aggregation into toxic and non-toxic oligomeric species either by interacting with specific domains or by displacing the protein away from the lipid membrane. In addition, several molecules were shown to disrupt preformed fibrils[182,193,202–204]. The mechanism of several drugs (e.g., polyphenols) generally considered neuroprotective agents does not necessarily involve, however, protein aggregation inhibition alone, being also or exclusively associated with other PD pathogenic events such as increased oxidative stress and defective mitochondrial function[205].

Among the most studied catecholamines is dopamine (**Fig. 5**), in addition to several oxidation derivatives and analogs[171,172,206], which have been shown to inhibit aggregation through different mechanims[166–170]. A non-covalent aggregation-inhibition mechanism of dopamine, in particular, was associated with non-specific interactions with the $_{125}$YEMPS$_{129}$ sequence region in the C-terminal tail, and with long range electrostatic interactions involving E83 in the NAC domain[169,171].

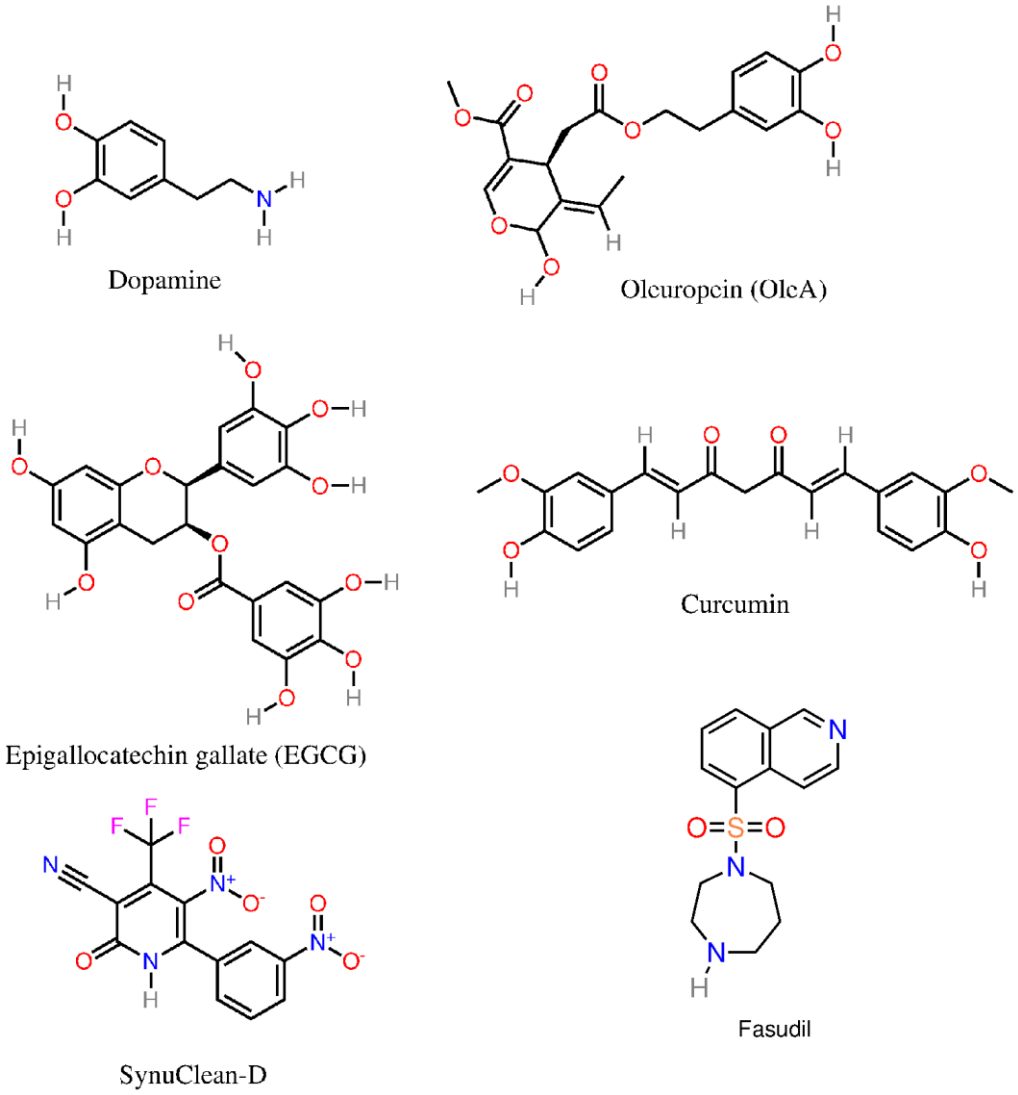


**Figure 5** – Molecular structure of several α-syn aggregation-inhibitors: dopamine, OleA, EGCG, Curcumin, SynuClean-D, and Fasudil.

Oleuropein and derivatives[179], including oleuropein aglycone (OleA)[178–180] (**Fig. 5**), a phenolic compound found in olive oil, were shown to stabilize less aggregation-prone conformations of monomeric α-syn, favoring the growth of stable nontoxic aggregates[179,180]. A cytotoxicity reduction was also connected with a reduction of the propensity of oligomers to bind to cell membrane components by interacting with the membrane-binding N-terminal domain of α-syn[178,180]. In addition, it was argued[180] that OleA stabilized the NAC and C-terminal regions of α-syn, preventing long-range and hydrophobic interactions between these sub-domains, which could favor aggregation.

Interestingly, however, long-range (tertiary) interactions involving the NAC and C-terminal regions were found in other studies to prevent, not promote, aggregation[78,83,84]. Furthermore, truncation of the C-term region has been shown to induce an acceleration of fibril formation *in vitro*[207,26]. This illustrates the apparent wide spectrum of monomeric α-syn conformations which might be involved in aggregation pathways of toxic and non-toxic oligomers.

Epigallocatechin gallate (EGCG) (**Fig. 5**), a polyphenol within the group of flavonoids, and an aggregation inhibitor not specific to α-syn, was also suggested to induce the formation of less toxic disordered oligomers[203] and remodel mature α-syn fibrils into less toxic aggregates.[188] Another study, however, reported that EGCG binds to the oligomers without changing either the secondary structure or its size distribution. In this study, the EGCG-induced toxicity reduction was linked with a decrease in the oligomers' membrane affinity[208].

The molecular mechanism underlying the aggregation-inhibition of α-syn by several flavonoids (e.g., baicalein) was shown to be associated with the restriction of conformational changes as well as with stabilization of α-syn's monomeric and oligomeric species[58,181,182,193]. Furthermore, flavonoids with three vicinal hydroxyl groups exhibited enhanced inhibitory effects on α-syn fibrillation[181]; this was related with the flavonoids anti-oxidant activity, although limitations of this correlation were pointed out[181]. In addition, the oxidized species (e.g., baicalein quinone) rather than the polyphenol (e.g., baicalein) were found to be the main fibrillation inhibitors; aggregation inhibition of baicalein is significantly reduced under anaerobic conditions[181,193]. A complex mechanism encompassing the auto-oxidation of baicalein and other flavonoids and the subsequent covalent bonding to α-syn, through the formation of a Schiff base between the quinone of baicalein and a Lys of α-syn were, in fact, found to be the key factors for the inhibition of α-syn fibrillation[181,193]. Caruana *et al.*[184] also suggested that the main factors underpinning α-syn self-assembly inhibition and destabilization are the existence of aromatic elements that bind to α-syn monomers/oligomers, and neighbor hydroxyl groups on a single phenyl ring.

A drawback of some potential drugs found in small molecule libraries, in particular several aggregation inhibition polyphenols (e.g., curcumin; see **Fig. 5**), quinones, and catechols, is the fact that these might be pan-assay interference compounds[209,210] (PAINS), that is, molecules that give false positives in high-throughput screening assays for several possible reasons. Since the action mechanism of these molecules is not always completely understood, neither as PAINS nor as aggregation inhibitors, and because of their non-specificity, it is difficult to predict whether molecules such as curcumin can both be PAINS and effective aggregation inhibitors[186,189].

In addition, polyphenols at dietary concentrations have been connected with the prevention and attenuation of PD through alternative mechanisms, including oxidative stress (i.e., a reduction of reactive oxygen species) and neuroinflammation reduction[211,212].

Another class of compounds explored, concerning anti-amyloid activity, are phthalocyanines, which suppress aggregation through the interaction of the aromatic rings with aromatic amino acids via π–π interactions (π−stacking)[174]. This mechanism led to the suggestion that aromatic interactions could be key players in the aggregation mechanism of α-syn[176,177], in spite of a relatively reduced number of aromatic amino acids. α-syn has only four tyrosine (Tyr39, Tyr125, Tyr133, Tyr136), two phenylalanine (Phe4, Phe94), and no tryptophan amino acids, of which only Phe94 is in the NAC segment. The anti-amyloid activity of these compounds also depends on the type of metal ion coordinated to the tetrapyrrolic system[174].

A series of pyridinyl-triazole derivatives were also recently reported to inhibit α-syn aggregation from *in vitro* screening and docking studies[197]. Fasudil (5-(1,4-diazepan-1-ylsulfonyl)isoquinoline) (**Fig. 5**), a small isoquinoline derivative, was shown to inhibit α-syn aggregation through direct binding to tyrosine residues Y133 and Y136 in the C-terminal region of α-Syn[196]. SynuClean-D (2-hydroxy-5-nitro-6-(3-nitrophenyl)-4-(trifluoromethyl)nicotinonitrile), (**Fig. 5**) an aromatic compound, was shown to reduce the in vitro aggregation of wild-type α-syn and the A30P and H50Q variants in a sub stoichiometric molar ratio[204]. In addition, this compound was found to disrupt mature amyloid fibrils and prevent fibril propagation.

Squalamine[194] (**Fig. 6**), a natural product isolated from the dogfish shark was shown to inhibit the aggregation of α-syn *in vitro* and *in vivo* by blocking the nucleation of α-syn. The mechanism of action of squalamine is not linked with specific protein-drug interactions but instead with a competition with α-syn for binding the membrane. The latter stimulates nucleation[65] and, thus, the displacement of α-syn from the membrane hamper the first steps of the aggregation process. A later study by Dobson and co-workers[195] showed that the related compound, trodusquemine (**Fig. 6**), interferes not only with the nucleation of α-syn but also with fibril-dependent secondary pathways. In

addition, trodusquemine was shown to suppresses the toxicity of α-syn oligomers in neuronal cells[195]. These molecules are already relatively long, potentially binding to larger protein domains relative to most small molecules previously discussed. In this sense these are more similar to peptides, discussed in the next section.

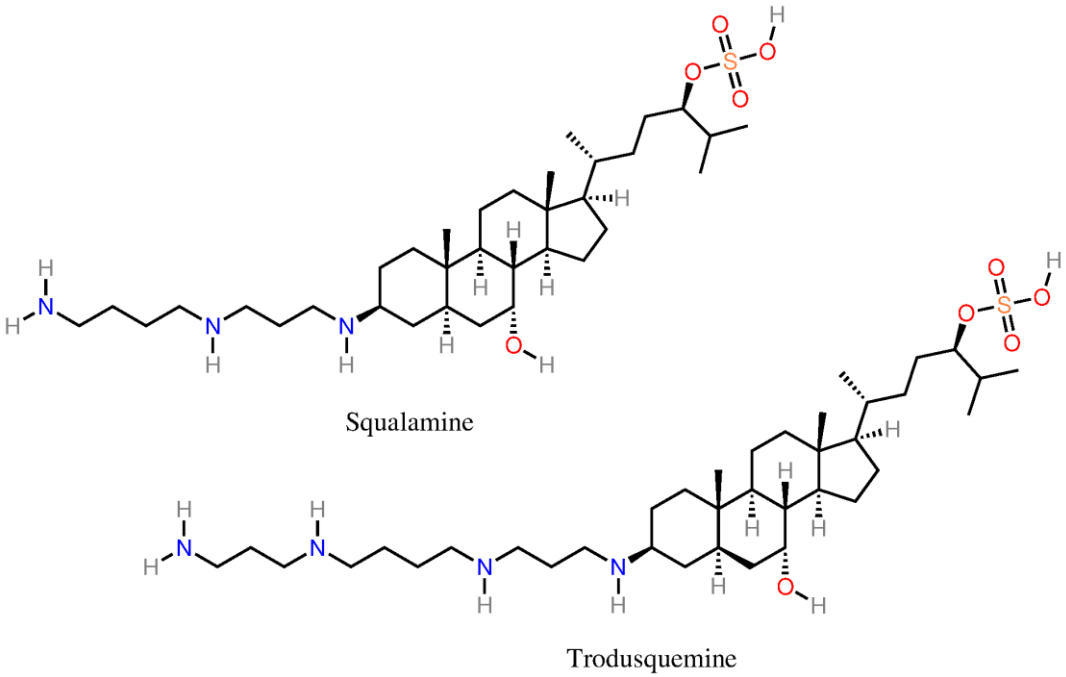


**Figure 6** – Molecular structure of the α-syn aggregation inhibitors squalamine and trodusquemine.

While small molecules remain appealing because they have a good metabolic stability and can, in principle, more easily cross the blood-brain barrier, in addition to other reasons, including economic, they suffer in general from poor selectivity, specificity, and potency, regarding protein aggregation inhibition. Thus, alternative aggregation-inhibitors have been explored. Among these, we focus on some small peptide-based drugs[82,153,156,213,214].

### 4.1.2. Peptide Drugs

Peptide-based drugs re-emerged as a promising alternative to small molecules concerning proteinopathies because of their specificity and potency[151,215,216]. The enhanced potency of small peptides is directly related with their larger interaction surfaces, allowing, in principle, to interfere with extended protein domains linked to the aggregation process[216]. Linear peptide drugs, however, suffer from other drawbacks such as bioavailability and proteolytic instability[151]. This has stimulated the development of macrocycle peptides and peptidomimetics to overcome some of these limitations.

Anti-amyloid designed peptides are often β-sheet breakers or blockers, disrupting or inhibiting the formation of cross-β structures, a structural hallmark of toxic oligomers[162,217–219,154]. Furthermore, in

addition to aggregation inhibitors, since peptides are often modified segments of the amyloid proteins, they can provide insight into pivotal aggregation-prone domains[215]. Peptide modification strategies include, for instance, peptide termini modifications, insertion of prolines, which are potent β-sheet breakers, backbone modifications, or peptide cyclization.[219] In addition, polar amino acids can be inserted to enhance the solubility.[82]

El-Agnaf[82] showed that modified peptides containing amino acid sequences $_{68}$GAVVT$_{72}$ from the NAC inhibit aggregation into oligomers and mature amyloid fibrils. The peptides modification included the insertion of RG and GR amino acids in the N- and C-terminals. Aggregation inhibition was observed at (α-syn:peptide) 2:1, 1:1, and 1:2 molar ratios and the shortest peptide that inhibited α-syn aggregation had the central sequence $_{69}$AVVT$_{72}$. These peptides were also observed to inhibit NAC aggregation suggesting that aggregation-inhibition is due to the binding of the peptides to their homologous sequence in α-syn.

Madine *et al.*[75] showed that a peptide from NAC ($_{77}$VTGVTAVAQKTV$_{82}$), N-methylated in the C-terminus, disrupted the aggregation of α-syn. This peptide is a sub-domain of the 72-84 region of α-syn, absent in β-syn, which in spite of sharing 78% of similarity with α-syn, does not aggregate[220].

Kim *et al.*[154] also proposed a small peptide ($_{72}$PGVTAV$_{77}$) able to block fibrillation and to dissolve preassembled fibrils. This is a modified ($T_{72} \rightarrow P_{72}$) sub-domain of NAC ($_{72}$TGVTAV$_{77}$), part of the NACore ($_{68}$GAVVTGVTAVA$_{78}$) an 11 amino acid peptide later shown[80] to play an important role in the aggregation and cytotoxicity of α-syn.

A 10 amino acid peptide (KDGIVNGVKA) was proposed by Cheruvara *et al.*[152] from intracellular screening of a peptide library based on α-syn residues 45–54, involved in several familial PD mutations (E46K, H50Q, and A53T). The peptide was shown to inhibit the aggregation of α-syn and the associated toxicity. Interestingly, Torpey *et al.*[153] found, through NMR, that although this peptide precludes oligomerization of the wild-type and several mutations associated with familial PD, it does not bind to the monomer neither to low-n ($n < 4$) oligomers. Thus, the aggregation-inhibition mechanism of this peptide is not completely understood.

A cyclic peptidomimetic (NPT100-18A) with a seemingly similar mechanism to squalamine was proposed by Wrasidlo *et al.*.[156] This peptidomimetic has the ability to displace α-syn from the membrane by interacting with domains in the C-terminus of α-syn.

Rezaeian *et al.*[221] proposed two peptides, KISVRV and GQTYVLPG, which suppressed the aggregation of α-synuclein *in vitro*. These peptides were chosen based on their binding affinity to the amino acids 70 to 75 (VVTGVT) and 46 to 53 (EGVVHGVA) of α-syn. The first region is among the several regions of NAC found to be pivotal in the aggregation process. The second corresponds

to the region where several missense mutated forms of α-syn (E46K, H50Q, G51D, A53T), implicated in familial PD, were found, and shown to induce an acceleration of the aggregation process, as previously discussed.

**Table 1** – Examples of α-syn aggregation inhibition peptides. Hydrophobic amino acids with aliphatic side chains are displayed in blue; hydrophobic amino acids with aromatic side chains are displayed in red. The second column identifies either the region of α-syn used in the peptide design and/or the believed target region/species.

| Aggregation Inhibition Peptide | α-syn parent domain/target | ref. |
|---|---|---|
| $_{68}$GAVVT$_{72}$ | NAC | 82 |
| $_{77}$VTGVTAVAQKTV$_{82}$ | NAC | 75 |
| $_{72}$PGVTAV$_{77}$ | NAC | 154 |
| $_{45}$KDGIVNGVKA$_{54}$ | N-term | 152 |
| KISVRV | NAC | 221 |
| GQTYVLPG | N-term | 221 |
| SUMO1(15–55)[(i)] | N-term | 214 |
| PSMα3[(ii)] | oligomers/fibrils | 222 |
| K84s[(iii)] | oligomers | 223 |
| K102s[(iv)] | oligomers | 223 |

[(i)] $_{15}$DKKEGEYIKLKVIGQDSSEIHFKVKMTTHLKKLKESYCQRQ$_{55}$
[(ii)] MEFVAKLFKFFKDLLGKFLGNN
[(iii)] FLVWGCLRGSAIGECVVHGGPPSRH
[(iv)] FLKRWARSTRWGTASCGGS

More recently, a small ubiquitin-related modifier 1 (SUMO1) derived peptide SUMO1(15–55) (see **Table 1**), which targets two SUMO-interacting motifs within the N-terminal region flanking the NAC was shown to inhibit α-syn aggregation[214]. Another recent study[222] proposed the use of α-helical peptides with a low affinity towards the monomeric form, avoiding perturbing the natural function of α-syn, while interrupting aggregation by binding to toxic oligomers and fibers. The PSMα3 peptide was found to have a high affinity towards a large number of binding sites in the oligomers, inhibiting aggregation.[222]

Popova *et al.*[223] also found two synthetic peptides, through a high-throughput screening study, (**Table 1**), 25 and 19 residues-long, that suppress α-syn aggregation. While the action mechanism was not disclosed the peptides were shown to significantly suppress the first steps of oligomerization. The peptides were also shown to be specific to α-syn. Furthermore, the larger and more potent peptide, was shown to reduce α-syn aggregation in human cells.

We now discuss the nature of some of the drugs, including amino acids and peptides, found to inhibit the aggregation of HbS, aiming at establishing some contact points between potential common drugs and aggregation inhibition mechanisms.

### 4.2. Sickle Cell Disease

Opposite to NDs, SCD can be cured, either through hematopoietic stem cell transplantation[224] or gene therapy[225], although several limitations and challenges, including economic, persist, preventing these treatments' widespread[20,21,226–228]. Thus, great interest remains in the development of drugs that can be used in the treatment of SCD.

In addition to hydroxyurea, long used in the treatment of SCD, other drugs became recently available. These include L-glutamine[229–231], whose action mechanism, although thought to reduce the oxidative stress in the erythrocytes, remains largely unknown, and voxelotor[232], an allosteric modulator (i.e., an oxygen affinity modifying drug) aimed at stabilizing the nonpolymerizing R quaternary structure of HbS. L-glutamine was approved in 2017 by the Food and Drug Administration (FDA) for adult and pediatric patients 5 years and older. Voxelotor was approved by the FDA and the European Medicines Agency (EMA) and recently extended to the treatment of children of ages 4-11 years old. Nonetheless, in spite of representing important alternatives and/or potential co-adjuvants to hydroxyurea, these drugs have limitations[21]. Allosteric modulators are among the most studied anti-sickling agents, and several other potential drugs were recently reported[233–239]. Other anti-sickling drugs that neither bind to HbS nor change the HbS oxygen affinity were also recently reported[240].

Various SCD therapeutic strategies have evolved over the years (see refs [20,21,227] for recent reviews). These include[20,21]: (i) increase of HbF or the increase of the erythrocyte volume to decrease the intra-cellular HbS concentration, (ii) decrease the concentration of the allosteric effector, 2,3-DPG, increasing the solubility and decreasing the fibers' stability, (iii) shift the allosteric equilibrium towards the R-state, and (iv) block protein-protein contacts by binding to HbS. In addition, several drugs aiming to reduce adhesion of erythrocytes to the vascular endothelium, decreasing transit times, have been investigated[20,227,241]. Although most of these approaches started being explored in the 1970s (see Table 1 of ref. [139]), only recently there have been some important advances in the development of effective drugs falling within the purview of at least one of the above action mechanisms, as briefly discussed above.

Our main focus herein is on non-covalent stereospecific aggregation inhibitors that block protein-protein contacts. Again, our approach is not an exhaustive one, but rather to provide some examples of small molecules, including amino acids and peptides, that may share common features across the drugs studied in other proteinopathies.

#### 4.2.1. Small Molecule Drugs

A foremost obstacle to the design of an effective anti-sickling molecule addressing the

polymerization process, concerns the high concentration of HbS in the erythrocytes[120] (~5mM) and therefore the putative high concentration required of any effective aggregation inhibition drug[20]. Nonetheless, the fact that a small change in the solubility of HbS can have a major impact in the lag period that characterizes the polymerization process, motivated the continuous exploration of this therapeutic strategy[20]. Furthermore, a recent study provided evidence that HbS fiber growth is a rather inefficient process (~4% efficiency), as previously mentioned in the Introduction, where monomer addition and loss are nearly equal. This led to the suggestion that HbS fiber growth is a viable therapeutic target even at drug concentrations below the total hemoglobin concentration. Nonetheless, this concentration will depend further on drug selectivity, among other factors, since many drugs will bind to additional domains of HbS, not affecting or even enhancing polymerization.

Many small molecules[18,139,242–249] including some amino acids[140,139,250,138,251], were long found to decrease the deoxy-HbS aggregation *in vitro* by increasing the solubility of HbS. While these molecules, many reported in the 1970s and 1980s, failed their purpose as effective drugs for the treatment of SCD it is of interest to contrast some of these molecules with those proposed more recently to address SCD and other proteinopathies such as PD.

Examples of small molecules long shown to increase the HbS solubility include for instance[18,139]: alkyl ureas[252–254], Hofmeister (lyotropic) salts[255], aromatic compounds with a phenyl group and a pendant side chain terminating in a hydrogen bond donor/acceptor (e.g., $NH_3^+$, $COO^-$, OH)[256], benzyl and phenoxy acids[257], and clofibric acid and gemfibrozil[243] (see **Fig. 7**.).

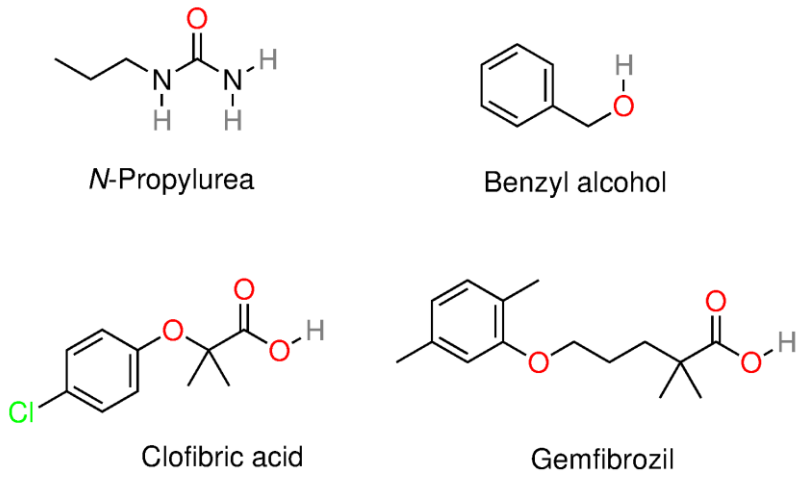


**Figure 7** – Molecular structure of several α-syn deoxy-HbS aggregation inhibitors: n-propyl urea, benzyl alcohol, clofibric acid, and gemfibrozil.

Ross and Subramanian[256] provided a comprehensive analysis of several small molecules and concluded that deoxy-HbS aggregation inhibition (i.e., solubility increase) was promoted by the

combination of a hydrophobic and a (single) hydrophilic group. In this regard, aromatic rings were more potent than aliphatic chains, and the hydrophilic group should be located on an aliphatic side chain attached to the aromatic ring to provide proper flexibility and/or distance to interact with deoxy-HbS. The latter was based upon the fact that molecules such as phenol, aniline, and salicylic acid do not inhibit aggregation. According to Ross and Subramanian a similar logic should apply to alkyl ureas. They proposed an aggregation inhibition mechanism where the deoxy-HbS lateral contact was blocked through the interaction of the aromatic ring with the hydrophobic pocket (i.e., Phe-β85 and Leu-β88) whereas the hydrophilic group formed a hydrogen bond with the Asp-β73.

This mechanism is at odds with the later study by Adachi *et al.*[137], which showed that phenylalanine and tryptophan in the Val-β6 position largely precluded aggregation, presumably due to stereo hindrance, as previously discussed. With regard to the requirement of an aromatic ring and a hydrophilic moiety at a given distance, this is common to many small molecules found to inhibit aggregation, although their specific action mechanism is not always understood.

Among amino acids, phenylalanine and tryptophan (see **Fig. 8**) were the only amino acids found to exhibit aggregation inhibition activity *in vitro* (i.e., gelation inhibition)[139,258]. Again, following Ross and Subramanian[256] this was consistent with their findings regarding aromatic small molecules. A study in 1975, nonetheless, showed that 3.8 mM homoserine, asparagine, and glutamine but no other amino acid, reversed the erythrocytes sickling[259]. This suggested at the time that these amino acids should inhibit sickling through a completely different (unknown) path than that observed in *in vitro* antigelling experiments.

More recently an experimental and simulation/docking study investigated alizarin[246], a hydroxyl anthraquinone (see **Fig. 8**) found in the *plant Rubia* cordifolia (aka Indina Madder). HbS polymerization was shown to decrease with the concentration of alizarin. The proposed mechanism involved the perturbation of the Val-β6 binding pocket through hydrogen bonding and hydrophobic interactions of HbS with alizarin. Many other natural products found in plants and long used in traditional medicine have also been shown to have some anti-sickling activity[260–264]. For instance, piperine, capsaicin, and cubebin (see **Fig. 8**) have been pointed out[263] as possible aggregation inhibitors present in Niprisan[244] (drug Nix-0699), a product of the extracts of four different plants.

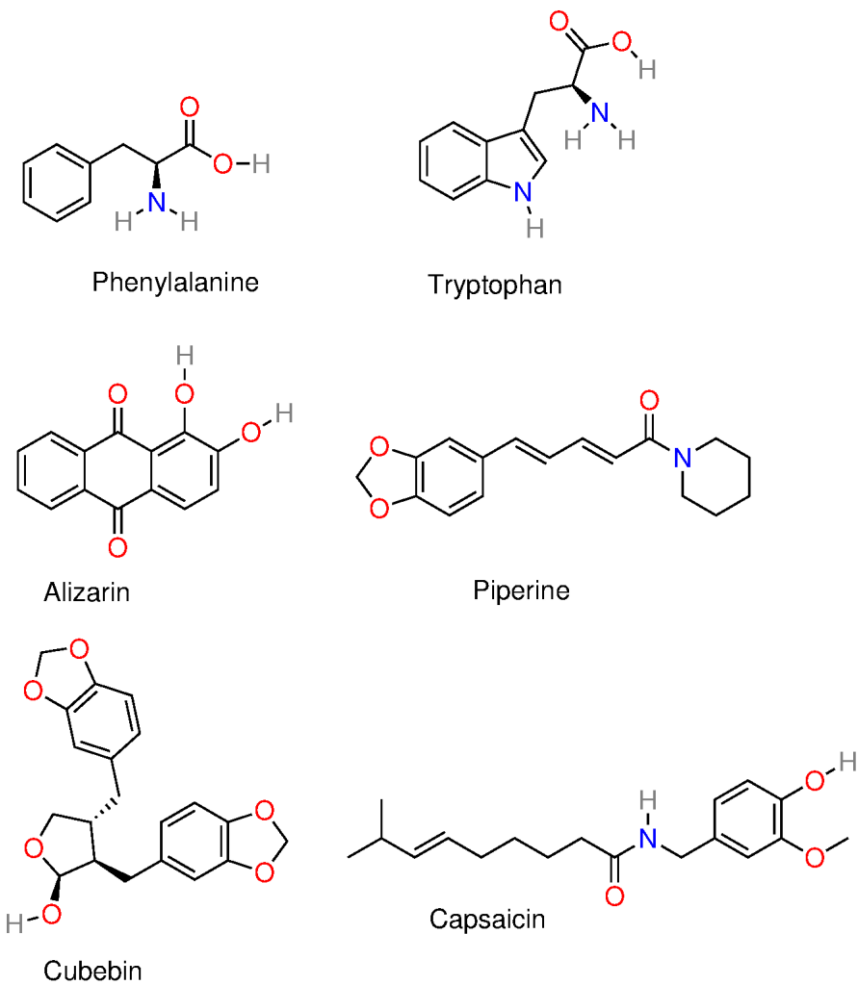


**Figure 8** – Molecular structure of several α-syn deoxy-HbS aggregation inhibitors: phenylalanine, tripthophan, alizarin, piperine, capsaicin, and cubebin molecular structures. Phenylalanine and tripthophan exhibit some aggregation inhibition activity and were used as building blocks in aggregation inhibition peptides[139,258]. Alizarin (hydroxyl anthraquinone) is a bioactive compound from the plant *Rubia Cordifolia*. Piperine, capsaicin, and cubebin have been pointed out[263] as possible aggregation inhibitors present in Niprisan[244] (drug Nix-0699) a product of the extracts of four different plants.

Several potential drugs were also recently investigated through docking studies[265,266]. A recent *in silico* drug repurposing study[266] identified a series of compounds (praziquantel, losartan, ketoprofen, glipizide, rosuvastatin, atorvastatin, ergotamine, and risperidone) interfering in the Val-β6 lateral contact with the hydrophobic cavity in the neighbor HbS tetramer. This perturbation involved mostly the interaction of the drugs' aromatic rings with the pocket formed by Ala-β70, Phe-β85, and Leu-β88.

Similar to other proteinopathies the deoxy-HbS aggregation inhibitory effect of small peptides in SCD, now discussed, has long been probed.

### 4.2.2. Peptide Drugs

Several peptides were also long shown to exhibit anti-sickling activity[139,251,258,267–270]. The latter were the object of a recent comprehensive review[271]. Herein, we highlight the main features that characterize the inhibition mechanism of peptides as opposed to small molecules, as well as putative resemblances with some of the peptide-drugs explored within the context of α-syn aggregation, discussed in **Section 4.1.2.**.

Gorecky *et al*.[269] found, based on a study of over 30 peptides, that the hydrophobicity of the side chains was the most important feature with respect to the HbS aggregation inhibition (i.e., antigelling activity). Votano and Rich[270] later reported that "compounds containing bicyclic or multi-aromatic residues have a higher activity than those that carry a single aromatic or aliphatic side chain". In addition, an increase in the apolar content of the aromatic residue and ring polarizability further enhanced the antigelling activity of such compounds. The peptides in these studies commonly comprised one to two Phe or Trp and a Gly, and were succinylated to enhance the solubility[270]; additional amino acids comprising the peptides included Arg, Nle, and Lys[269]. Whereas the binding sites in HbS were not completely disclosed it was argued that peptide-HbS contacts should involve several amino acids near the hydrophobic cavity where Val-β6 is enclosed in the fibers. The fact that peptides containing several aromatic residues are more potent that those with a single Phe or Trp suggest that the aggregation inhibition mechanism should involve a larger domain than that comprised by the principal amino acids that form the Val-β6 binding pocket.

A pivotal advantage of peptide-based drugs concerning SCD is the possibility to enhance the protein-drug contact area, decreasing, in principle, the drug:HbS ratio. The low stereoselectivity of small molecules towards protein surfaces can translate into the interaction with several protein domains, which can disfavor, promote, or exert no significant effect on aggregation. In principle, larger peptides should depict a greater aggregation inhibitory activity, relative to single amino acid or di- and tri-peptides because of the increased contact area. However, some critical length should exist above which aggregation inhibition "saturates". The latter, however, will depend on the contact area which in turn depends on the chemical nature of the peptide. Several works[138,268,272] addressed this problem concluding that the aggregation inhibitory activity increased with the peptide size, although large concentrations were still required to observe effective antigellation effects (see also ref. [271] for a broader discussion of this aspect).

A possible explanation for the large concentrations required for observing antigellation activity by small molecules and peptides involving aromatic rings (Phe and Trp) is the fact that these may not interact with the hydrophobic pocket where Val-β6 is inserted. The latter can be speculated based on

the results of Adachi *et al.*[137], previously discussed. Thus, such a pocket could only be blocked at high drug concentrations when interactions with neighbor sites imped the entrance of Val-β6 from a neighbor tetramer. An alternative explanation is that some of these peptides and or small molecules are neither selective enough nor the aggregation inhibition mechanism is associated with the Val-β6 binding pocket. The abovementioned studies, point, nevertheless, to a common direction, that is, aromatic amino acids are key to the blockage of protein-protein contacts behind nucleation (increasing the lag time), and heterogeneous nucleation, preventing the formation of fibers. In the following sections, we attempt to rationalize some of the above information regarding the role of hydrophobic interfaces, aromatic rings, and hydroxyl groups, all seemingly playing a part in protein aggregation inhibition in SCD, PD, among other proteinopathies.

## 5. Hydrophobic Effect and Protein Aggregation

The design of a drug to oppose or delay protein aggregation could, in principle, aim at increasing the solubility of the protein, in principle, by weakening the hydrophobic effect. The hydrophobic effect comprehends two related phenomena, hydrophobic hydration, associated with the low solubility of apolar molecules and groups in water, and hydrophobic interactions, the spontaneous association of apolar molecules in water. The most remarkable feature of hydrophobic hydration is, perhaps, its system size dependence, with the hydration of small ($R_S$ < ~1 nm) and large radius ($R_S$ > ~1 nm) spherical solutes being dominated, respectively, by entropy and enthalpy[273–279].

### 5.1. Hydrophobic Hydration

#### 5.1.1. Small Length Hydration

Hydration of small apolar molecules is governed by the formation of a suitable cavity to lodge the solute. For hard spheres this probability is related to the hydration free energy (i.e., excess chemical potential) by $\Delta G_{\text{hyd}} = -k_B T \ln p_0(R)$, where $R$ is the solvent accessible radius, given by $R = R_S + R_W$, and $R_S$ and $R_W$ are the radius of the solute and water, modeled as hard spheres, whereas $p_0(R)$ is the probability that a sphere of radius $R$ randomly inserted in water is devoid of water molecules[277,280,281].

Whereas solute-water interactions are favorable, the hydration enthalpy is smaller in magnitude than the hydration entropy and the process is, therefore, entropic. Although displaying differences relative to bulk water, the water structure (and dynamics) and, therefore, water-water interactions, produce a small net effect on the hydration enthalpy and entropy. For instance, a tetrahedral enhancement[282–285] of a sub-population (e.g., ~70 % for methane) of water molecules in the hydration layer of small apolar solutes has been found, along with an enhancement of the hydrogen

bond strength between those water molecules and the water molecules that comprise the vertices of the imperfect "tetrahedrons" [286–288]. This water population illustrated in **Fig. 9(a)** is denoted here W-4W. This is opposed by water molecules (e.g., ~30 % for methane) that are not tetrahedral (i.e., water molecules closer to the solute than to the four nearest water neighbors (W-3W; see **Fig. 9(a)**), which cannot, therefore, form up to four hydrogen bonds), where a weakening of the average water-water interactions with the third and fourth water neighbors is observed, making a positive contribution to the hydration enthalpy. These populations (i.e., tetrahedral and non-tetrahedral; see **Fig. 9(a)** and the discussion in the next section) decrease and increase, respectively, with the solute size[287]. The excluded volume induced weakening of water-water interactions (i.e., hydrogen bond breaking), nonetheless, outweighs the tetrahedral enhancement, except for small solutes at low temperatures[288], making a small positive contribution to the enthalpy. Furthermore, notwithstanding these contributions to the hydration entropy and enthalpy, the water reorganization around the cavity is characterized by a nearly exact entropy-enthalpy compensation, thus, not contributing to the hydration free energy[289–293].

For long linear hydrophobic solutes such as n-alkanes, a behavior similar to small solutes is observed as these can be accommodated in water almost as an ensemble of small apolar molecules and the hydration free energy is a linear function of the carbon number ($n_C$) up to $n_C$ ~20 [274,288,294]. Folding in long alkanes ($n_C$ > ~ 20) was recently shown to result in the violation of entropy and enthalpy convergence[288]. This was demonstrated to result from the release of water molecules in the hydration layer of methylene groups upon folding, increasing the hydration entropy and reducing solute-water interactions, thus, lowering the enthalpy[288]. Entropy convergence violation is observed for low curvature ($R_S$ > ~1 nm) hard spheres[277,295] and for many globular proteins upon unfolding[296], because of the heterogeneous nature of protein-water interactions[297] and probably folding/unfolding differences[288].

### 5.1.2. Large Length Hydration

For low curvature solutes ($R_S$ > ~1 nm), hydration is governed instead by the formation of a solute-water interface, as opposed to density fluctuations and spontaneous cavity formation, resulting in a substantial loss of water-water HBs and, therefore, an enthalpy-dominated hydration free energy and a positive hydration entropy[274,278]. Hydrophobic hydration of such large surfaces is characterized by an increase of the local water fluctuations and isothermal compressibility, relative to bulk water[274,278]. Furthermore, a more favorable binding of hydrophobic molecules has been observed because of facilitated cavity formation next to the surface, favoring the binding of hydrophobic drug-groups[278].

The above picture, nonetheless, applies to hydrophobic molecules or interfaces, as opposed to (amphiphilic) proteins and other biomolecules where chemical heterogeneities result in heterogeneous solvation, with water molecules next to hydrophilic groups sharing the hydration layer of hydrophobic side chains of neighbor amino acids, leading to quenching of density fluctuations[274,278]. In addition, topological (microscopic) irregularities influence protein hydration and density fluctuations. Hydrophobic hydration has, for this reason, been more difficult to characterize in biomolecules than in hard spheres, hydrocarbons, or flat planar self-assembled monolayers[278,298].

To illustrate some of the above points **Fig. 9** displays some molecular dynamics results on the size of the water populations next to hydrophobic and hydrophilic amino acids in 11-mer peptides along with the tetrahedrality[299] and energetics of water-water interactions. The peptides and water were described by the AMBER99sb[300] and TIP4P-Ew[301] force fields, respectively. Further details about these simulations can be found elsewhere[302]. **Figure 9(b)** shows a hydration increase by 15 % of an Ile amino acid when sided by Ser amino acids in an 11-mer peptide compared to an Ile amino acid in an 11-mer Ile peptide. This increase is related both with the hydrophilic nature of Ser and with a reduction of the excluded volume. The latter is confirmed by an even larger hydration increase in a C-terminal segment of NAC ($_{85}$AGSIAAATGFV$_{95}$), denoted herein NACterm peptide[78,302], where Ile is sided by a Ser and an Ala; although no significant folding is found for 11-mer peptides[302], hydration is also influenced by peptide structural fluctuations.

As previously discussed, excluded volume, either next to a hydrophobic or hydrophilic amino acid results in a tetrahedrality increase of a water population that retains four water neighbors (W-4W **Fig. 9(a)**). This can be seen in **Fig. 9(c)** for Ile in an 11-mer Ile and NACterm peptides, and Ser (Ser-6), in an 11-mer Ser peptide. **Figure 9(d-g)** also confirms the water HB enhancement next to Ile within the W-4W population, compared to bulk water, more pronounced for the 3rd and 4th potential HB partners. The W-3W population shows an enhancement of only the first HB, whereas solute exclusion volume hinders the formation of more than two HBs, in average, within this population. The fact that such a population dominates in low curvature solutes (**Fig. 9b**), along with the weak nature of dispersion interactions involved in hydrophobic group-water interactions, is responsible for a mild dewetting not found around molecules and groups that form HBs with water. However, hydrophobic segments in proteins are generally limited in length and may not favor hydrophobic drug binding over protein-protein aggregation at therapeutically relevant concentrations. The latter is among the main obstacles to the rational drug design targeting protein-protein hydrophobic contacts.

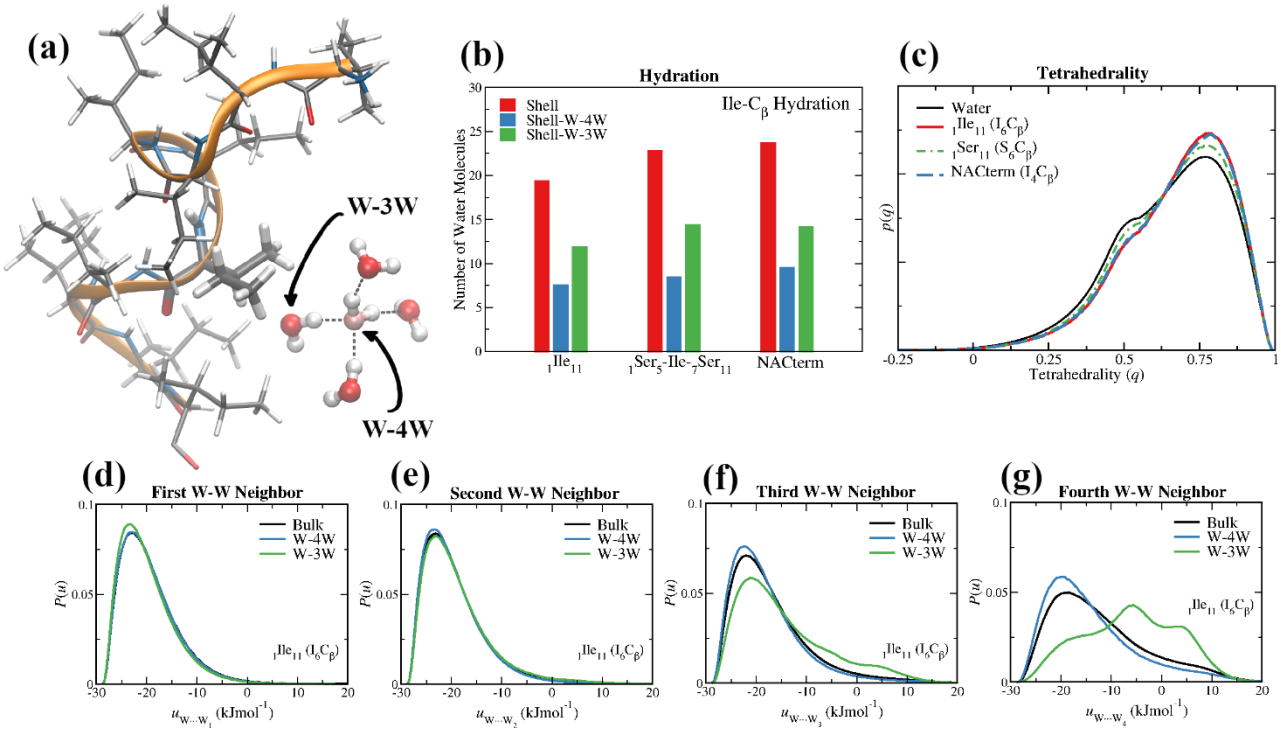


**Figure 9 – (a)** A water pentamer next to Ile-6 in an 11-mer isoleucine peptide ($_1$ILE$_{11}$) displaying a water in the $C_\beta$ coordination sphere with at least 4 nearest water neighbors (W-4W ) and a water with 3 or less nearest water neighbors (W-3W) because of the proximity of the solute; **(b)** Number of water molecules in the first hydration layer ($r_{min} \leq 6.45$ Å) of the $C_\beta$ of, Ile-6 in an 11-mer isoleucine peptide ($_1$ILE$_{11}$), Ile-6 in an 11-mer serine peptide with a single (middle) isoleucine ($_1$SER$_5$-ILE-$_7$SER$_{11}$), and Ile-4 in NAC-term ($_{85}$AGSIAAATGFV$_{95}$), an 11-mer peptide comprised of the last 11 amino acids of NAC; **(c)** tetrahedrality of the W-4W water populations in the first hydration shell of the $C_\beta$ of Ile and Ser, respectively, in isoleucine ($_1$ILE$_{11}$) and serine ($_1$SER$_{11}$) 11-mer peptides, compared with the tetrahedrality of bulk water; **(d-g)** Water pair interaction energy distributions, $P(W\cdots W_n)$ for $n$ = 1 to 4, for bulk water and W-4W and W-3W water populations in the first hydration shell of the $C_\beta$ of Ile in isoleucine ($_1$ILE$_{11}$).

### 5.2. Hydrophobic Interactions

Hydrophobic interactions, the other related manifestation of the hydrophobic effect, are indirect, solvent-induced interactions associated with the dewetting of apolar groups and the formation of a liquid-vapor-like interface that culminates in the so-called hydrophobic collapse for sufficiently large hydrophobic surfaces[274,279,303]. This microscopic dewetting, while not opposed by solute-water interactions in ideal hard (i.e., repulsive) flat surfaces is outweighed by van der Waals interactions in real hydrophobic systems bringing water closer to the hydrophobic surface[278,298], resulting in a small (i.e., less than the size of a water molecule[304]), although important, width depletion region[274,305]. A similar phenomenon is not observed for small solutes, unless an extended surface cluster can form[274]; even relatively large (e.g., neopentane) and long (e.g., dodecane) alkanes show small tendency to aggregate[306].

Hydrophobic interactions are the main driving "force" responsible for protein folding[23,24] and

intrinsically disordered proteins such as wild-type α-syn are thought not to fold because of their high net charge and low hydrophobicity. Hydrophobic interactions are also thought to be the main driving force in protein aggregation, as previously discussed for HbS and α-syn.

In light of the above discussion, small and large length-scale hydrophobic effects should be important to protein hydration, (mis)folding, and aggregation. In addition, water-mediated electrostatic interactions should also play an important part. For instance, rationalization of the hydrophobic interaction of Val-β6 with the host pocket in the neighbor HbS within the small length hydrophobic picture, would suggest a low tendency for aggregation since the hydrophobic pocket, which also exists in HbA, should be largely dehydrated in the monomer, as previously discussed. Thus, the main hydrophobic driving force should be associated with the dewetting of the side chain (isopropyl) of Val-β6. However, following the above discussion, other hydrophobic and electrostatic interactions resulting both from the departure of Glu-β6 and the insertion of Val-β6 should be important in triggering the aggregation process and the formation of a nucleus. Thus, although undoubtedly linked with the interaction of Val-β6 with the acceptor hydrophobic pocket (see **Fig. 10(a,b)**) and neighbor hydrophilic and hydrophobic amino acids, a comprehensive understanding of the initiation of the homogeneous nucleation at deoxy-HbS saturation conditions, remains elusive.

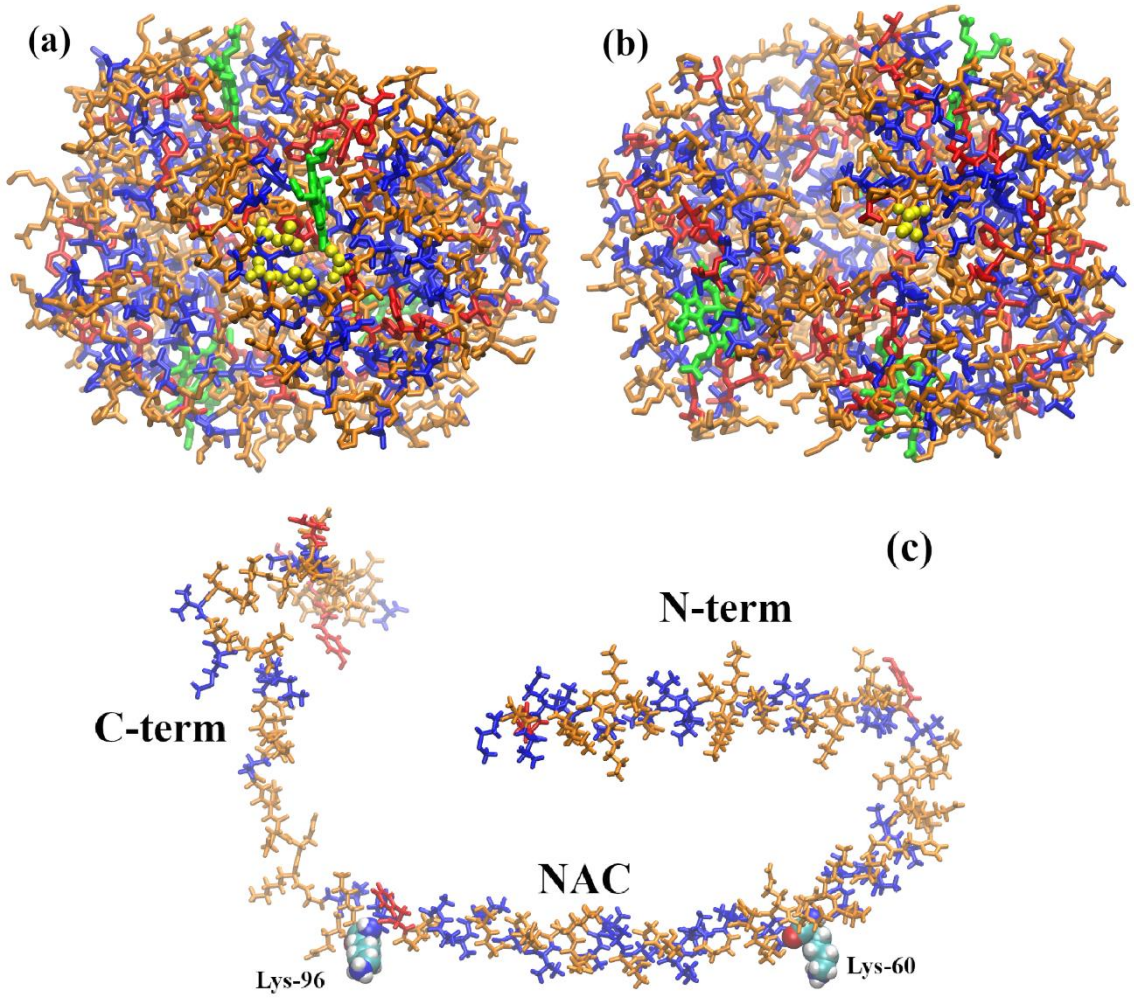


**Figure 10** – **(a)** deoxy-HbS monomer showing the hydrophobic pocket (yellow); **(b)** deoxy-HbS monomer showing Val-β6 (yellow); **(c)** α-syn. (blue) hydrophobic amino acids with aliphatic side chains (Ala, Ile, Leu, Met, and Val); (red) hydrophobic amino acids with aromatic side chains (Phe, Trp, Tyr); (orange) remaining amino acids; (green) Hem in deoxy-Hbs.

With respect to α-syn aggregation, again, in spite of copious differences relative to HbS, both hydrophobic length scales should be pivotal. For instance, NAC (35 amino acids) has 17 hydrophobic amino acids, mostly Val and Ala, but no more than four contiguous hydrophobic amino acids ($_{88}$IAAA$_{91}$), with the main hydrophobic domains being $_{88}$IAAA$_{91}$, $_{69}$AVA$_{71}$, $_{76}$AVA$_{78}$, and $_{94}$FV$_{95}$ **(Fig. 10c)**. The aggregation of the 11-mer domain[78] NACterm ($_{85}$AGSIAAATGFV$_{95}$) of α-syn, which contains this longer hydrophobic domain (i.e., $_{88}$IAAA$_{91}$) was recently studied through molecular simulations[302]. A binding free energy of ~ -10 kJ mol$^{-1}$ was found[302]. Urea, a protein denaturant, that also induces protein disaggregation at high concentrations (~6-8 M), was shown to strongly inhibit aggregation through the occupation of the dewetted interpeptide region precluding hydrophobic collapse. Whether some of the small molecules discussed in **Section 4**, including some polyphenols, inhibit aggregation by directly interacting with the NAC or other domains of α-syn and/or reduce

density fluctuations next to hydrophobic groups remains unknown. Furthermore, the relationship between polyphenols and other small molecules that stabilize specific oligomers and the hydrophobic effect, seemingly responsible for the oligomerization process, is also not clear. Nevertheless, some chemical groups such as aromatic rings and hydroxyl groups seem to play a key role in the aggregation and aggregation inhibition of distinct proteins. This is now briefly discussed, including some of the limitations and challenges associated with these ubiquitous groups and compound families, in drug design.

## 6. Hydrophobic Effect and Drug design

A small molecule or peptide aimed at inhibiting aggregation *in vivo* through some protein-protein contact impediment must (i) cross the cell membrane in intracellular aggregation-related diseases and the blood-brain barrier in NDs, (ii) selectively bind to some key protein aggregation site(s), and (iii) inhibit aggregation or induce the formation of non-toxic oligomers, and/or disrupt pathological oligomers or fibrils without compromising the protein's function(s). Furthermore, the drug should be soluble, have high selectivity, specificity and potency, and low toxicity, along with good bioavailability and metabolic stability. We pause to note that in AD and PD, the diseases further induce changes in the blood-brain barrier, altering drug permeability, that, in spite of possible menaces, can also lead to novel drug therapies[307]. In SCD, erythrocyte membrane alterations might also change drug permeability, in addition to known cation permeability perturbation, which results in erythrocyte dehydration[308], favoring aggregation through the increase of deoxy-HbS concentration.

With regard to the selective binding to specific protein sites, knowledge of the aggregation mechanism is pivotal to define suitable targets to block aggregation and/or promote oligomer and fiber disassembly. For proteinopathies the most common targets are hydrophobic regions such as the hydrophobic host pocket in deoxy-HbS and the NAC or the N-terminal region in α-syn because these are involved in the main contacts found in the respective fibers. However, structural changes affecting these regions, and thus, aggregation, can, in principle, also be driven by protein-drug interactions involving protein segments far-off from the main protein aggregation binding contacts.

A link between many protein aggregation inhibitors, including amino acids and peptides, is the existence of aromatic rings, which allow the formation of non-covalent stacked structures with aromatic amino acids and the interaction with aliphatic hydrophobic side chains. In addition, hydroxyl groups can interact with polar side chains and have been found to play a role in the aggregation inhibitory potency of small aromatic molecules[181,184,256]. In this sense and considering

the large collection of polyphenols that showed aggregation inhibitory activity *in vitro* for different proteins, although with reported diverse mechanisms, it could seem that finding a drug for therapeutic purposes would not be difficult. Nonetheless, although polyphenols occupy a relevant position among protein aggregation inhibitors, in addition to their long reported role as protective agents in NDs through various mechanisms[309,310], their general protective role remains elusive[311]. In particular, concerning their reputation as aggregation inhibitors, a more fundamental knowledge about the action mechanism of polyphenols and aromatic rings and hydroxyl groups, in general, seems important, namely, (i) their impact in protein solvation, including water density fluctuations and structural transformations, (ii) whether direct (i.e., protein-polyphenol) and/or indirect (i.e., polyphenol induced alterations of water-water and protein-water interactions) aggregation inhibition mechanisms are involved, (iii) their selectivity towards hydrophobic and hydrophilic domains, or (iv) their influence on the structure of the monomeric proteins. This could allow unraveling the fundamental intermolecular interactions through which some small molecules induce the formation of stable (not-toxic) oligomers, precluding fibrillation and even disrupt mature fibrils[58,188,193,208], whereas others may inhibit nucleation by stabilizing the monomer, although not disrupting preformed fibrils.

We stress that, the fact that some polyphenols were found to inhibit aggregation of distinct proteins[310], behaving almost as universal-aggregation-inhibitors, could mean these are not effective therapeutic drugs because of low selectivity, requiring concentrations too high to hamper key contacts. For instance, there is a common view that a drug, either a small molecule or a peptide-based drug[151], that can shield specific domains of the NAC[78,83] is of potential therapeutic interest in PD. The concentration necessary to block some pivotal sub-domain of the NAC, however, may not be therapeutically relevant because of a selectivity deficit. Indeed, looking at the hydrophobic-hydrophilic amino acid sequence in α-syn (**Fig. 10c**) it is difficult to argue on the putative preferential binding of a polyphenol towards a specific domain, let alone its influence on the structure, solubility, and aggregation propensity of the monomer.

A similar rationale applies to peptide-based drugs. While the importance of aromatic rings was long reported in the context of deoxy-HbS aggregation, concentrations too high for therapeutic purposes were required to observe significant solubility increases[271]. This suggests that, despite peptides carrying aromatic rings can compete more effectively than small molecules with proteins for specific extended amphiphilic surfaces involved in fiber contacts, they still lack enough selectivity. In addition, some peptides may also aggregate, a characteristic possibly shared with some (low solubility) polyphenols.

Thus, although the rational design of aggregation inhibitors continues to hold the promise of developing effective therapeutics for several proteinopathies, the fact that many small molecules in drug libraries are soluble polyphenols or other amphiphilic molecules with aromatic rings (and OH groups), some of which have been considered PAINS, can taint drug screening assays[312], unless the aggregation inhibition mechanisms of some of these molecules, at a molecular-level, is unraveled. In this sense, it seems particularly promising to investigate the molecular origin of the seeming "universal" aggregation inhibitory activity of some small molecule drugs, either to improve drug design strategies based on these molecules or to rule them out as promising leads possibly for reasons that put them into the category of PAINS.

**Conclusions**

The failure of many potential drugs for NDs, especially Alzheimer's disease, propelled the discussion[313] on whether the amyloid hypothesis is, in fact, correct, and protein aggregation is the primary cause of NDs. However, unlike NDs, the molecular origin of SCD is well-known and there has also been limited progress in finding effective aggregation inhibitors. This naturally raises questions concerning the efficacy of this approach in SCD, NDs, and other proteinopathies. However, given the large number of diseases that involve some form of protein aggregate and the economic burden associated with future stem cell and gene therapies, the development of small molecule or peptide-based drugs remains a worth-exploring therapeutic route.

Herein, we provided an overview of the drugs developed to treat two completely different proteinopathies, namely, PD and SCD, aiming to establish some links associated with the importance of hydrophobic interactions in protein aggregation. The small and large length-scale regimes of the hydrophobic effect were discussed, along with the apparent relevance of aromatic rings and hydroxyl groups to aggregation inhibition. Although the ubiquity of polyphenols as potential small molecule drugs concerning a multitude of diseases, ranging from cardiovascular diseases to cancer and neurodegenerative diseases, stimulates their study, their mechanisms as protein aggregation inhibitors remain poorly understood. As a consequence, the design of improved drugs that could dodge intrinsic drawbacks of polyphenols that showed aggregation inhibitory activity *in vitro* even at non-toxic concentrations, including low bioavailability and selectivity, has proven difficult.

Significant advances might emerge in a near future, nevertheless, linked with improved machine learning-assisted drug design methodologies[314] and a more fundamental understanding of the action mechanism of potential drugs, polyphenols and others, concerning their influence on the impediment of intertwined hydrophobic and electrostatic interactions and protein solvation. In addition, the design of cyclic peptides represents a promising route towards the development of alternative drugs

with enhanced selectivity while avoiding some of the limitations of linear peptides.

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