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Review

Interactions of intrinsically disordered proteins with the unconventional chaperone human serum albumin: From mechanisms of amyloid inhibition to therapeutic opportunities

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ABSTRACT

Human Serum Albumin (HSA), the most abundant protein in plasma, serves a diverse repertoire of biological functions including regulation of oncotic pressure and redox potential, transport of serum solutes, but also chaperoning of misfolded proteins. Here we review how HSA interacts with a wide spectrum of client proteins including intrinsically disordered proteins (IDPs) such as Aβ, the islet amyloid peptide (IAPP), alpha synuclein and stressed globular proteins such as insulin. The comparative analysis of the HSA chaperone – client interactions reveals that the amyloid-inhibitory function of HSA arises from at least four emerging mechanisms. Two mechanisms (the monomer stabilizer model and the monomer competitor model) involve the direct binding of HSA to either IDP monomers or oligomers, while other mechanisms (metal chelation and membrane protection) rely on the indirect modulation by HSA of other factors that drive IDP aggregation. While HSA is not the only extracellular chaperone, given its abundance, HSA is likely to account for a significant fraction of the chaperoning effects in plasma, thus opening new therapeutic opportunities in the context of the peripheral sink hypothesis.

1. Introduction

Pathogenic protein misfolding, self-association, and amyloid formation are linked to the onset and progression of several neurodegenerative disorders such as Parkinson's disease (PD) and Alzheimer's disease (AD) [1,2]. These conditions often involve the aggregation of intrinsically disordered proteins (IDPs) into toxic oligomeric species, including the amyloid β peptide (Aβ) and alpha synuclein (αSyn), associated with AD and PD, respectively [3–8]. Amyloid formation is not only limited to IDPs, but other folded peptides such as the hormone insulin can also form amyloid fibrils [9]. Although the mechanisms of amyloid aggregation and cytotoxicity are not clearly understood, it has been proposed that protein misfolding in general and the exposure of hydrophobic residues specifically can lead to cross-β sheet formation, the hallmark of amyloid fibrils [10]. In addition, several physiological and *in vitro* conditions such as pH, oxidation, ligand binding, and ionic strength can also contribute to IDP self-association [4,11–17].

A viable therapeutic approach against amyloidogenic disorders relies on the identification of amyloid inhibitors that disrupt the self-association of IDPs and their related cellular toxicity [2,18–20]. To this date, several classes of amyloid inhibitors have been characterized from peptides and small molecules to antibodies, nanoparticles, and chaperones, including plasma proteins such as albumin and transferrin [14,21–31]. Human serum albumin (HSA) has received great interest for its potential neuroprotective effect in the treatment of AD [26,32]. HSA has been shown to inhibit amyloid aggregation by directly binding to aggregation-prone IDPs and their toxic oligomers [33]. In addition, HSA displays antioxidant properties by chelating toxic redox metal ions that enhance amyloid aggregation [34,35].

Here we review the mechanisms underlying the inhibition of amyloid self-association and cytotoxicity by HSA based on work by others [36–43] and our own [25,33,44–46]. We start by focusing on Aβ as a model IDP whose interactions with HSA have been widely characterized. In addition, we discuss how other HSA ligands such as fatty acids can affect the amyloid inhibitory potency and the binding to Aβ species [46–51]. Examples of HSA as a therapeutic approach for the treatment of AD are provided including the Alzheimer Management By Albumin Replacement (AMBAR) trial [32,52,53]. Besides Aβ, we also reviewed

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other amyloidogenic proteins whose aggregation is inhibited by HSA, these include αSyn [54–56], insulin [57], and the islet amyloid polypeptide [58].

2. Human serum albumin

HSA, the most abundant protein in blood plasma and cerebrospinal fluid (CSF), is a monomeric multi-domain globular protein of 66 kDa that is synthesized in the liver [59]. HSA serves several physiological functions including the binding and transport of exogenous and endogenous solutes such as hormones, fatty acids, metals, and drugs (Fig. 1a). In addition, HSA maintains colloid osmotic pressure and regulates the extracellular redox potential by binding and carrying radical scavengers, and chelating toxic transition metal ions (e.g. Cu^{2+}, Ni^{2+}, Co^{2+}) [60]. With a relatively long lifetime of approximately 30 days, HSA may undergo several posttranslational modifications that may affect its ligand-binding and anti-oxidant properties. Such modifications include glycation, oxidation, and truncation [61].

Emerging evidence suggests that besides the previously mentioned roles, HSA may also serve as an extracellular chaperone [62,63]. The chaperone function of albumin becomes particularly evident under "stressing" conditions caused by oxidative stress, shear stress from blood flow, heat, or UV light, under which proteins are more prone to unfold and aggregate [64]. The albumin chaperone preferentially binds stressed over unstressed client proteins [47] and shows a potent inhibitory effect on the self-association of several IDPs, such as the prototypical Aβ peptide and extracellular αSyn (Fig. 1 b and c) [25,45,46,58,63,65,66]. Indeed, one of the earliest accounts of the interactions between HSA and IDPs dates back to 1996 [67], when Biere and colleagues reported that the majority of the Aβ peptide present in biological fluids (≈89%) is bound to albumin, a small amount is free, and up to 5% is bound with specific lipoproteins [67]. Further studies have confirmed such interactions and how the binding of HSA inhibits the aggregation and toxicity of Aβ [25,33,39,40,44–46].

Besides the chaperoning effect of HSA through direct binding to aggregation-prone IDPs such as Aβ, HSA can also prevent amyloid formation through its ligand-binding and anti-oxidant properties [68]. For example, HSA chelates toxic metals ions such as Cu^{2+}, a divalent cation whose role in Alzheimer's and Parkinson's has been widely described [60]. The following sections will focus on the prototypical Aβ peptide and how the chaperoning and chelating properties of HSA prevent its aggregation and related cell toxicity. In addition, we also discuss how modulators, such as fatty acids, affect HSA-Aβ interactions and therefore the amyloid inhibitory potency of the HSA chaperone.

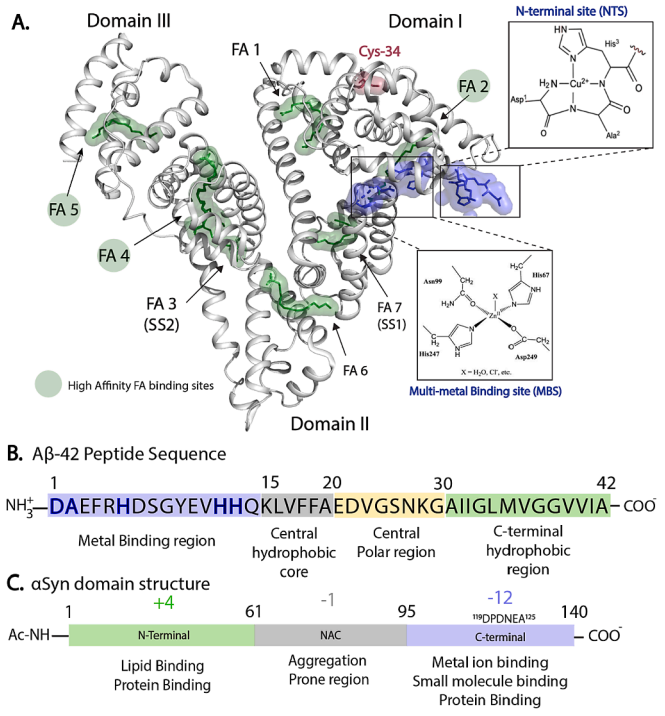

Fig. 1. ***Human serum albumin and model IDPs.*** **(a)** Crystal Structure of fatty acid (oleic acid) bound HSA (PDB: 1GNI). Fatty acids (FAs) bound to HSA are colored in green. FAs bind HSA at seven different binding sites, the labels of the high affinity binding sites (FA 2, FA 4, FA 5) are highlighted with a green circle. The Sudlow sites 1 and 2 are represented in FA 7 and FA3, respectively. Cu(II) binds to HSA at the N-terminal binding site (NTS) but can also bind at the multi-metal binding site (MBS); however, under physiological conditions the MBS is occupied by Zn(II). Both metal binding sites are highlighted in blue. **(b)** Amino acid sequence of Aβ-42 peptide and its distinctive regions. Residues that directly coordinate Cu(II) ions are bolded. **(c)** Alpha synuclein (αSyn) is characterized by three regions. NAC stands for non-amyloid-β component, which is highly hydrophobic and aggregation prone. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Mechanisms of inhibition of Aβ peptide self-association by HSA

3.1. HSA chelates toxic metal ions that enhance Aβ self-association and toxicity

Post-mortem analyses of amyloid plaques from AD patients have revealed an excessive accumulation of copper, iron, and zinc, suggesting a link between AD and redox metal dysregulation [69]. In particular, copper was found in amyloid plaques approximately at six-fold higher levels than normal brains [69,70]. When Cu ions bind Aβ amyloidogenic species in a reducing environment, they catalyze the reduction of dioxygen into reactive oxygen species (ROS), leading to oxidative stress damage and neuronal death [71,72]. Dysregulation of copper homeostasis in plasma is also thought to play a role in AD. In addition, the amount of serum Cu (II) not bound to ceruloplasmin ("free" copper) is higher in AD patients [73]. Since Aβ is found primarily in the extracellular space, it is suggested that an increase of Cu(II) concentration in this medium enhances Aβ aggregation [38].

HSA has been proposed to prevent copper toxicity by sequestering free serum Cu from Aβ [36]. Cu(II) ions bind to HSA at two sites, *i.e.* the N-terminal site (NTS, Kd ~ pM) and the multi-metal binding site (MBS, Kd ~10 nM; Fig. 1a). However, under physiological Cu(II) plasma concentrations, only the NTS is occupied by Cu(II) while the MTB is instead occupied by Zn(II) ions, which display a higher affinity for this site [35]. A study in 2010 by Perrone et al. found that the transfer of copper from the Cu-Aβ complex to HSA inhibits the aggregation and cell toxicity of Aβ, as well as radical production [36]. Using X-band electron paramagnetic resonance (EPR) the authors were able to monitor the transfer of Cu(II) from Aβ to HSA. Removal of Cu(II) ions occurred effectively and stoichiometrically from Aβ when HSA was added. In addition, turbidity assays and transmission electron microscopy (TEM) showed that HSA inhibits the Cu-induced aggregation of Aβ40 and is also capable to reverse it. Finally, the authors measured the hydroxyl radical production and cell toxicity of Cu-Aβ in the presence and absence of HSA. ROS production in neuroblastoma cells is significantly reduced and cells are rescued from Cu-Aβ toxicity when HSA is added [36]. Similar results were obtained when the authors repeated the previously mentioned experiments with the tetrapeptide DAHK, which corresponds to the N-terminal Cu-binding site (NTS) of HSA, suggesting that the chelating function of HSA arises primarily from the NTS.

Another study in 2015 by Lu and colleagues reached similar conclusions about the inhibitory effect of HSA on Cu-induced Aβ-40 aggregation and toxicity [37]. Using a competitive binding assay, the authors found that Cu(I) can also be chelated away from Aβ. Furthermore, the thioflavin T (ThT) fluorescence assay revealed that HSA

suppresses the Cu(I)/Cu(II)-induced *in vitro* formation of Aβ amyloids. In addition, Lu et al. confirmed that in the presence of the reducing reagent ascorbic acid, HSA decreases Cu-Aβ ROS generation and rescues cell viability, as assessed by the colorimetric MTT assay [37].

More recent evidence from Choi et al. using a combination of small-angle X-ray scattering (SAXS), ion mobility mass spectrometry (IM-MS), TEM, and cell viability assays suggests that HSA is able to sequester Cu (II) and Zn(II) ions while maintaining its interaction with Aβ [74]. This observation demonstrates that HSA is able to act simultaneously as a chelator and a chaperone while inhibiting the aggregation of Aβ [74]. Similar to previous results they found that HSA inhibits the aggregation (Fig. 2 a–c, f), cytotoxicity (Fig. 2 d and g), and also the cellular transport of metal-bound Aβ species, but is not able to disassemble preformed metal-Aβ aggregates (Fig. 2e) [74]. These findings point to albumin behaving similarly regardless of the presence or absence of metal ions, binding directly to Aβ species and affecting its cellular transport [74].

It is notable that HSA is not the only extracellular protein eliciting chaperoning and chelating effects on Aβ aggregation. For example, the iron-transporting transferrin prevents Aβ self-association and toxicity by sequestering Fe(III) ions and reducing oxidative damage [75]. Transferrin direct binding to Aβ also plays a role in its inhibitory potency [29]. In 2013 Raditsis et al. using solution nuclear magnetic resonance (NMR) techniques, demonstrated that transferrin is able to inhibit Aβ self-association by binding preferentially to Aβ oligomers, thus preventing the recruitment of monomers and the formation of larger amyloid assemblies [29]. The chaperoning mechanism of Aβ aggregation based on direct binding has also been described for HSA and is described in the following section.

3.2. *The chaperoning effect of HSA through direct binding to Aβ*

3.2.1. *HSA-Aβ monomer interactions - The "monomer stabilizer" model*

After HSA was discovered to be the major carrier of Aβ peptides in blood plasma [67,76,77], several subsequent studies focused on finding the affinity and stoichiometry of the HSA-Aβ complex. It was hypothesized that the direct binding between HSA and Aβ monomers was one of the mechanisms by which HSA could inhibit the self-association of Aβ [39]. In this model HSA selectively binds and stabilizes Aβ monomers, preventing their aggregation into oligomeric and fibrillar forms. In 2007 Rózga et al. monitored the titration of HSA with the Aβ40 peptide using circular dichroism (CD) spectroscopy and obtained a dissociation constant (K_d) of 5 ± 1 μM [39]. While CD may not offer sufficient selectivity to fully separate monomer *vs.* oligomer contributions to affinity, the authors also pointed out that upon Aβ40 binding to HSA the IDP adopts a partially α-helical conformation. In addition, immunoassays studies by Kuo et al. showed that the Aβ monomer:HSA complex was consistent with an apparent 1:1 stoichiometry [77]. These early studies supported the "monomer stabilizer" mechanism of HSA inhibition of Aβ fibrillization. While this model offers a viable explanation of the chaperoning effect elicited by HSA, other studies revealed that more complex mechanisms are also at play. For example, it was found that HSA binds preferentially Aβ oligomers instead of monomers [25,33,44,78], but at physiological HSA plasma concentrations (~sub-mM) the weak Aβ monomer/HSA interaction is still relevant.

Algamal et al. used solution NMR experiments to map the interactions of Aβ monomers with HSA at residue resolution [45]. ^{1}H-^{15}N Heteronuclear Single Quantum Coherence (HSQC) is a useful NMR experiment to probe protein-ligand interactions [79,80]. The HSQC experiment allows to monitor the chemical shift changes and NMR intensity losses upon protein binding, and provides a residue resolution-map of ligand binding regions [79]. Taking advantage of this approach the authors generated a binding isotherm for the HSA/Aβ monomer interaction by monitoring the loss of intensity in the Aβ NMR

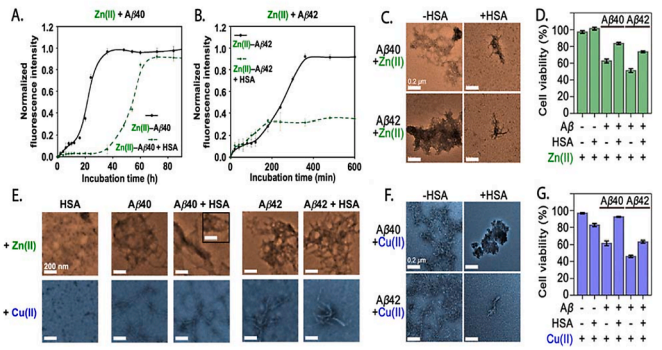

Fig. 2. ***Effects of HSA on aggregation, cytotoxicity, and disassembly of metal − Aβ species.*** (**a**) ThT fluorescence assay of 20 μM Aβ40:Zn(II) in the presence (green) or absence (black) of 20 μM HSA. (**b**) Same as (a), but for Aβ42. (**c and f**) TEM images of (c) Zn(II)- and (f) Cu(II)-induced Aβ40/Aβ42 fibrils with and without HSA. Conditions: [HSA:Aβ:Metal] = 20 μM; 37 °C; 24 h; constant agitation. Scale bar = 0.2 μm. (**d and g**) MTT cell viability assay of SH-SY5Y cells treated with samples generated upon 8 h preincubation of (d) Zn(II) or (g) Cu(II) with Aβ and HSA. Conditions: [HSA:Aβ:Metal] =10 μM and 24 h incubation. Error bars represent the standard error from four independent experiments ($P < 0.05$) (**e**) Effect of HSA on further aggregation or disassembly of preformed metal Aβ aggregates revealed by TEM. Aggregates were preformed by incubating 20 μM Aβ with 20 μM metal ions (Zn^{2+} or Cu^{2+}) for 24 h at 37° C. After the first incubation, 20 μM of HSA was added and the samples were incubated a second time for 24 h at 37 °C. pH was adjusted to 7.4 for metal-free and Zn(II) experiments and 6.6 for Cu(II) experiments. Scale bar = 200 nm. Panels (a)-(g) are adapted from [67]. Reprinted (adapted) with permission from The American Chemical Society. *J.Am.Chem.Soc.* **2017**, 139, 43, 15,437–15,445. Copyright (2017) American Chemical Society.

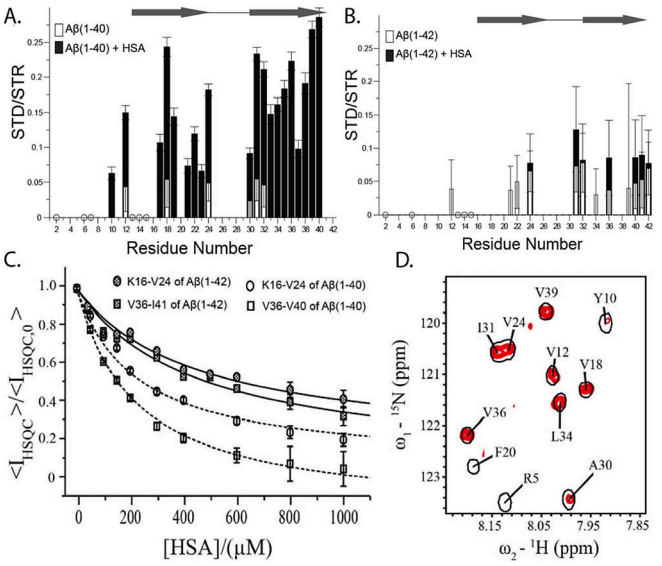

Fig. 3. ***Probing the interactions of HSA with monomeric Aβ40 and Aβ42 through NMR.*** (**a**) Residue-specific ratio between the STD and the saturation transfer reference (STR) intensities of 40 μM Aβ40 in the presence (black) or absence (white) of 200 μM HSA. (**b**) As in (a) but instead for 40 μM Aβ42 in the presence (black) or absence (white) of 200 μM HSA. (**c**) Average ^{1}H-^{15}N HSQC intensity losses of Aβ40 (open symbols and dashed fitted lines) and Aβ42 (dotted symbols and solid fitted lines) for the C-terminal region (square symbols) and the central hydrophobic core (circular symbols) as a function of increasing HSA concentration. The data were fitted using a Scatchard-like model (dashed lines). (**d**) Representative expansion of the STD-HSQC (red) spectrum used in (a) superimposed to the STR-HSQC (black) of Aβ40 in the presence of HSA. [38] Adapted with permission from *J.Biol.Chem.* jbc. M117.792853. DOI: https://doi.org/10.1074/jbc.M117.792853. Copyright 2017. The American Society of Biochemistry and Molecular Biology. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

signal upon increasing concentrations of HSA (Fig. 3c) [45]. Both the central hydrophobic core and the C-termini of Aβ displayed reduced intensity upon addition of HSA. However, the Aβ C-terminus displayed a greater loss of intensity suggesting its role as the main binding site for HSA.

Besides the HSQC experiment, Algamal et al. used a combination of STD NMR experiments with ^{1}H-^{15}N HSQC as a more direct approach to probe the binding of HSA to Aβ monomers [45]. This time the binding of HSA to Aβ monomers was monitored as the enhancement in the STD NMR signal of Aβ upon HSA addition (Fig. 3 a and b). The STD experiments confirmed the previously observed binding of HSA at the C-terminus and the STD/HSQC-based binding isotherm provided an effective K_d in the sub-mM range. The study by Algamal et al. also found that the HSA-Aβ binding affinity is isoform specific with HSA displaying a higher affinity for Aβ40 than Aβ42 monomers. However, for both Aβ isoforms the effective K_d values are still comparable to physiological HSA plasma concentrations, suggesting that in plasma the "monomer stabilizing" model is still relevant, along with other mechanisms, to account for the inhibition of amyloid aggregation and toxicity by HSA. The other mechanism by which HSA inhibits Aβ self-association includes direct binding of HSA to oligomeric Aβ species, which is described in the next section.

3.2.2. *HSA-Aβ oligomers interactions - The "monomer-competitor" model*

Besides the direct binding of HSA with monomeric Aβ, the HSA chaperone also binds Aβ oligomers [33,40,45,46,81]. STD-based NMR and intrinsic albumin fluorescence quenching showed that HSA preferably binds cross-β-sheet rich Aβ oligomers as opposed to Aβ monomers [33]. Milojevic et al. found K_d values in the μM to sub-μM range for the HSA/Aβ oligomer complexes [33]. Based on these results the authors proposed the "monomer-competitor" model as an effective mechanism to explain the inhibition of Aβ aggregation by HSA.

In the "monomer-competitor" mechanism the chaperone binds selectively to the oligomers, preventing further addition of Aβ monomers and growth into larger Aβ aggregates [81]. Further studies in 2011 assessed the binding and stoichiometry of oligomeric Aβ and HSA [44]. A complex experimental strategy that included the comparative analysis of STD and off-resonance NMR experiments on domain-deletion mutants of HSA, combined with dynamic light scattering (DLS) and ultrafiltration, showed that Aβ oligomers bind HSA at multiple independent and equivalent binding sites located across the three albumin domains with similar K_d values in the 1–100 nM range. A similar K_d value in the μM-nM range was reported in an orthogonal study using surface plasmon resonance (SPR) for Aβ42 oligomers [82].

The sub-μM K_d values highlight the relevance of HSA/Aβ oligomers interaction not only in plasma, but also in the CSF, where the HSA concentration (low μM) is orders of magnitude lower than in plasma (~ sub-mM). The role that HSA plays in the CSF was confirmed by Stanyon et al. using ThT fluorescence based fibrillization assays [40]. The effect of HSA at physiologically relevant CSF concentrations was investigated in the context of Aβ aggregation, showing that at micromolar concentrations HSA is still able to reduce both Aβ40 and Aβ42 fibril formation by increasing the lag time and reducing the amount of aggregated species. The authors also pointed out that the amount of fibrils formed in the presence of HSA correlates to the fraction of Aβ not bound to albumin [40].

Algamal et al. further mapped the interactions between Aβ oligomers and HSA at subdomain and peptide resolution [46]. The authors found that each subdomain of domain 3 of HSA, subdomain 3a and 3b, is able to inhibit the self-association of Aβ. Therefore, they mapped the binding of Aβ oligomers to HSA subdomains using a bioinformatic approach. They focused on subdomain 3b because in isolation it retains the ability to inhibit Aβ-self association and includes only a single well-defined FA binding site [46]. In order to find which sites within HSA subdomain 3b bind to Aβ oligomers it was hypothesized that such sites should resemble the recognition sites of Aβ monomers for oligomers [46]. Based on this hypothesis the authors used the Waltz algorithm to select HSA regions that are prone to form amyloid aggregates (Fig. 4 a and b). In addition, only the HSA sites that align with the Aβ self-recognition sites were considered (Fig. 4c). For example, several residues in the 494–515 segment of HSA were found to align with the central hydrophobic core of Aβ and also displayed amyloid propensity as revealed by the Waltz algorithm (Fig. 4 b and c). Algamal and colleagues additionally found that the HSA (494–515) peptide in isolation retains the ability to inhibit Aβ self-association through specific binding to the Aβ oligomers (Fig. 4 d and e) [46].

Further solution NMR studies by Algamal and colleagues in 2017 demonstrated that HSA perturbs how monomeric Aβ recognizes oligomeric Aβ species [45], consistent with the "monomer competitor" mechanism. In conclusion, both "the monomer stabilizer" and the "monomer competitor" models can explain the chaperoning effect of HSA and the inhibition of Aβ aggregation in plasma. However, the "monomer competitor" mechanism which focuses on the HSA/Aβ oligomers interaction is also relevant in the CSF. Recent studies have used molecular dynamics (MD) simulations to provide a higher-resolution picture of Aβ aggregation in the presence of HSA using both models, and is discussed in the next section.

3.2.3. *HSA inhibition of Aβ aggregation: general mechanism revealed by MD simulations*

The interactions of the Aβ42 monomer with HSA was analyzed also through MD simulations [43]. According to MD modeling, Aβ42 can bind HSA at different sites but displays a tighter affinity towards domain III. In addition, the presence of HSA shifts monomeric Aβ conformational

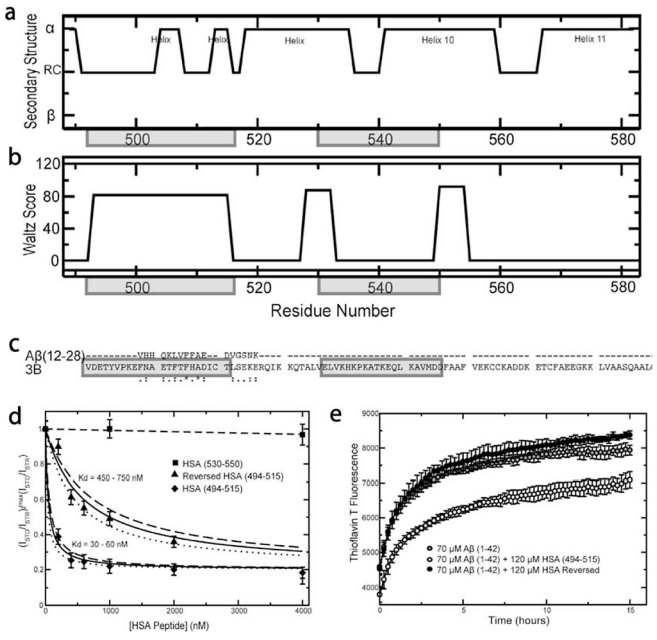

Fig. 4. ***Determining the binding sites of Aβ oligomers in subdomain 3B of HSA.*** (**a**) Secondary structure of subdomain 3B of HSA (PDB: 1E7G). (**b**) Amyloid prediction by Waltz scores. (**c**) Sequence alignment of Aβ (12–28) and subdomain 3B. (Dashed line) no consensus residues; (star) fully conserved residues; (semicolons) strong conserved residues; (dots) weak conserved residues. The shaded area represents HSA domain-3 region peptides 494–515 and 530–550 used in d and e. (**d**) STD-based profiles of Aβ (12–28) inhibition upon addition of increasing HSA domain 3 peptides: HSA 494–515 (solid diamonds), and the negative controls HSA 494–515 reversed (solid triangles), and HSA 530–550 (solid squares). (**e**) ThT fluorescence monitoring the inhibition of Aβ42 aggregation in the absence (gray circles) and presence of HSA 494–515 (white circles) or HSA 494–515 reversed (black circles). Panels (a) to (e) were adapted from [39].*Biophys.J.* **2013**, 105, 7, 1700–1709. Copyright © 2013 Biophysical Society. Published by Elsevier Inc.

states.

away from patterns prone to β-sheet association. One example is the frequent formation of aggregation-prone β-hairpins in the regions spanning Q15-V24 and N27-V36 of Aβ42, which is disrupted in the presence of HSA. The authors also stress that when HSA is added, the Aβ intrapeptide interactions needed for β-sheet formation are disrupted [43]. MD simulations further revealed that the binding of HSA with monomeric Aβ is characterized by salt bridges and hydrophobic interactions with the Aβ hydrophobic core, in agreement with previous NMR results [46].

Another MD simulation study by the same research group focused on the role that HSA plays at early stages of Aβ aggregation [42]. This time the authors analyzed not only the interaction of HSA with monomeric Aβ, but also with Aβ protofibrils. Interestingly, HSA is able to bind and sequester multiple Aβ monomers thus preventing the formation of fibrillar oligomers in water. This result explains how HSA lengthens the lag phase of fibrillization by reducing the concentration of free monomers in solution. The authors also highlight that once protofibrils are formed, HSA disrupts the β-strand spanning the central hydrophobic core, therefore blocking the addition of monomers to the protofibril end [42]. This result supports the "monomer competitor" model discussed earlier. Besides the "monomer competitor" and "monomer stabilizer" models, to recapitulate the complexity of Aβ-HSA system in plasma it is essential to consider also how the Aβ-HSA interactions are modulated by other plasma solutes carried by HSA.

4. Modulators of Aβ-HSA interactions – Effect of fatty acids, drugs and hormones

Under physiological conditions HSA binds on average up to two equivalents of unesterified fatty acids and is capable to accommodate up to six moles in selected pathological conditions [83]. Crystal structures of fatty acid-bound HSA display seven different binding sites dispersed through the different HSA domains (Fig. 1a). Two out of the three high affinity binding sites are located in domain III, while the other can be found in domain I [54,84]. Besides fatty acids (FA), HSA also serves as carrier of other hydrophobic ligands including cholesterol, drugs and hormones [83]. The following section will discuss how the binding of HSA ligands such as long chain fatty acids, cholesterol, warfarin, and serotonin affects the interaction of HSA with Aβ and amyloid formation.

4.1. Fatty acids affect the interactions of Aβ with HSA

The realization that Aβ oligomers bind to several binding sites across the three different domains of HSA with comparable affinities led Algamal et al. to study how FAs affect such interaction [46]. The authors worked on domain 3 of HSA because it contains two high affinity binding sites for FA and it remains structured and soluble when isolated from the other two domains [46]. Furthermore, myristic acid (MA) was selected as a model FA because its binding to domain 3 of HSA has been previously well described. The effect of domain 3 on the aggregation inhibition of Aβ in the presence or absence of MA was probed through one-dimensional NMR. The loss of monomeric Aβ42 NMR signal over time can be used to probe the formation of NMR-invisible high molecular weight aggregates (self-association; Fig. 5). The results from the NMR experiments revealed that the amyloid inhibitory potency of domain 3 is significantly reduced upon addition of excess MA [46]. This suggests that FAs may compete with Aβ42 for the binding to HSA or FAs may promote Aβ aggregation on their own. To discard the latter possibility, Algamal et al. monitored the aggregation of Aβ in the presence or absence of MA and did not find significant differences. Further NMR-based competition experiments and comparative mutagenesis analyses revealed that MA and Aβ compete for similar binding sites in domain 3, as both bind to subdomains 3A and 3B; however, at least some residues critical for FA binding are not involved in the HSA-Aβ oligomers interaction.

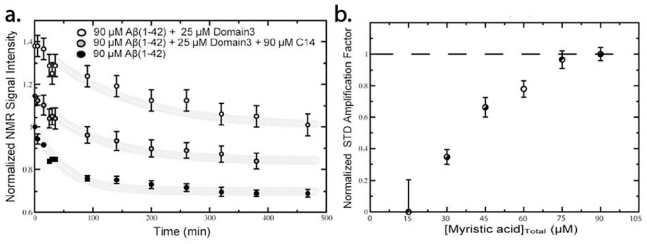

Fig. 5. ***Myristic acid (MA) affects the inhibition of Aβ42 aggregation by domain 3 of HSA.*** **(a)** 1D ^{1}H-NMR intensities of 90 μM Aβ42 recorded over time in the absence (black circles) and in the presence of domain 3 with (gray circles) and without (white circles) MA. (**b**) Domain 3 is fully saturated by MA at 90 μM. The STR and the STD spectra of apo Domain 3 were subtracted from the respective spectra at each MA titration point. Panels (a) and (b) were adapted from [39].*Biophys.J.* **2013**, 105, 7, 1700–1709. Copyright © 2013 Biophysical Society. Published by Elsevier Inc.

The fibrillization of Aβ was also studied in the presence of palmitic acid loaded HSA [49]. Using ThT fluorescence and TEM, Bode et al. revealed that HSA loaded with seven molar equivalents of palmitic acid is not able to inhibit the aggregation of Aβ40. When palmitic acid was present the ThT plateau fluorescence of Aβ increased and the lag phase shortened which suggests enhanced production of amyloid fibrils and aggregation, respectively. A similar effect was observed when other hydrophobic HSA ligands were added, such as cholesterol and the anticoagulant drug warfarin [49].

The effect of FA addition to HSA on its interaction with monomeric Aβ has been investigated also through MD simulations [48]. First, the simulations confirmed previous results by Algamal et al. [45], indicating that the C-termini of Aβ is the primary site for interactions with apo HSA (fatty acid free). However, addition of seven equivalents of palmitic acid changes the HSA residues involved in direct Aβ contacts. In addition, the authors point out that FA addition to HSA results in an evenly distributed affinity in each HSA domain for Aβ, while in apo HSA, domain 3 displays the highest Aβ binding propensity [48]. As a general mechanism Guo and Zhou propose that the flexibility displayed by apo HSA allows promiscuous interactions with ligands such Aβ and such flexibility is quenched upon FA binding.

Lastly, the development of a quantitative method based on surface plasmon resonance (SPR) allowed Litus and colleagues to study the binding between monomeric Aβ40/42 and HSA loaded with the major plasma unsaturated FAs [50]. The authors used a series of HSA ligands including oleic (OA), arachidonic (ArA), linoleic (LA) and docosahexaenoic (DHA) acids. Contrary to other reports [48], the authors found that the HSA ligands analyzed do not change considerably the affinity of Aβ40 monomers for HSA, while the addition of ArA and LA promote HSA-Aβ42 interactions. However, the authors point out that specific HSA-Aβ affinities do depend on the details of the Aβ preparation protocols and Aβ immobilization.

4.2. Serotonin-bound HSA interactions with the Aβ peptide

Immobilization of Aβ40/Aβ42 on the surface of a SPR sensor chip allowed Litus et al. to study its interaction with HSA bound to another set of ligands: serotonin and its structurally homologous precursor tryptophan [51]. The study revealed that serotonin increases the affinity of monomeric Aβ for HSA as indicated by a reduction of the K_d value by a factor of 7 and 17 for Aβ40 and Aβ42 respectively. On the other hand, the addition of tryptophan had little effect on the Aβ/HSA interactions. Additional dynamic light scattering and molecular docking experiments explained the previous results in terms of serotonin-bound HSA showing variations in quaternary structure relative to tryptophan-bound HSA [51].

5. HSA as a potential treatment for AD

5.1. The peripheral sink hypothesis and AMBAR

It has been suggested that Aβ travels from the brain to blood following a decreasing concentration gradient [85]. Such finding is supported by studies in which a decreased concentration of Aβ in the brain was observed after administering a monoclonal anti-Aβ antibody to transgenic mice models for AD ensuring that antibodies did not cross the blood brain barrier (BBB) [86,87]. Similarly, it has been proposed that HSA contributes to the preservation of a healthy balance of free-Aβ between the blood and the brain by binding Aβ monomers in plasma [85]. In AD patients the plasma levels of HSA-bound Aβ are reduced suggesting that the fine-tuned balance of free Aβ between the brain and the blood is disrupted [85]. In addition, AD progression is characterized by posttranslational modifications (*e.g.* oxidation, nitration, glycation) of several proteins including HSA [88,89]. Such modifications can affect how HSA interacts and inhibits Aβ aggregation contributing to AD pathophysiology [88]. For instance, HSA was found to be mostly oxidized in the CSF of AD patients compared to healthy controls [90] and oxidated-glycated and cysteinylated-glycated isoforms of HSA were found to be increased in the plasma and CSF of AD patients [91].

Based on the hypothesized dynamic equilibrium between brain and plasma Aβ, the replacement of posttranslationally modified and Aβ-bound HSA from plasma should in turn shift the balance between CSF and plasma Aβ, directing Aβ from the CSF to plasma and reducing the Aβ concentration in the brain. This is the principle of the "peripheral sink" hypothesis, which proposes that HSA can act as a "sink" to remove Aβ from the brain of AD patients [92]. The peripheral sink hypothesis is the rationale underlying the Alzheimer Management By Albumin Replacement (AMBAR) trial (NCT01561053), a program developed by Grifols [32]. This program aims at replacing AD patients' HSA with therapeutic-grade HSA characterized by strong Aβ binding and antioxidant capacities. After the therapeutic plasma replacement, Aβ levels should decrease in plasma shifting the Aβ equilibrium from the CSF to plasma and subsequently decreasing pathological Aβ concentrations in the brain [32,92].

The AMBAR study has shown promising findings in the treatment of AD after completing a phase IIb trial in the United States and phase III in Europe [32]. The trials were conducted in patients with mild-to moderate AD progression. Patients were treated weekly with therapeutic plasma exchange containing Albutein® (therapeutic grade HSA) over a period of six weeks. The frequency of the sessions was later reduced to monthly sessions over 12 months with low-volume plasma exchange [93]. In general, the results of the AMBAR study indicate that patients treated with plasma exchange exhibit a reduced deterioration in the cognitive scale compared with the placebo group. In addition, this effect is more prominent in patients with moderate AD progression than mild [32,93,94]. Although the AMBAR results are encouraging and the "peripheral sink" hypothesis is one of the mechanisms that may explain them, the exact mechanism responsible for slowing down AD progression is not known. Other viable mechanisms are related to decreased tau pathology and/or the antioxidant capacity of HSA. In addition, mechanisms that are independent of HSA and Aβ might be also be at play in the plasmapheretic treatment, such as the removal of cytokines and neurotoxic auto-antibodies [92].

5.2. Other therapeutic approaches based on HSA-Aβ interactions

Based on the promise of the previously discussed therapeutic plasma exchange method, Ishima et al. developed HSA mutants with enhanced Aβ binding capacities [52]. The authors first identified the highest binding affinity site on HSA for Aβ monomers, the 187–385 fragment (domain 2), and with the use of the phage display method, several mutants with enhanced binding capacities for Aβ were found. *In vitro* equilibrium dialysis of the HSA mutants with Aβ revealed that compared to the WT, the mutants were able to sequester Aβ more effectively [52].

Another approach by Wang et al. also illustrates the potential therapeutic effects of selectively modified albumin with enhanced amyloid inhibitory potency [53]. This time HSA was basified through the conversion of carbonyl groups to amino groups at different degrees using ethylenediamine. The experimental approach was inspired by the basic protein lysozyme and its strong inhibition of Aβ aggregation and toxicity [62,95]. ThT fluorescence and atomic force microscopy (AFM) imaging revealed that higher degrees of HSA basification were positively correlated with higher inhibitory potency of Aβ self-association. In addition, cell viability assays also displayed a similar pattern, where the highest degree of albumin basification led to an increase of cell viability [53]. Through fluorescence quenching analyses the authors determined that upon HSA basification, the Aβ42 peptide decreased its binding affinity with the hydrophobic pockets of HSA. At the same time, the binding constant between Aβ42 and basified HSA as probed by quartz crystal microbalance experiments increased significantly. These results led Wang and colleagues to suggest that binding between Aβ monomers and modified HSA is based on electrostatic interactions [53]. Taken together, these examples illustrate the potential of HSA and its derivatives as therapeutics against Aβ. Furthermore, HSA is an effective chaperone also for other client amyloidogenic proteins, such as α-synuclein (αSyn) as well as insulin [57] and transthyretin [47].

6. Effect of HSA on the aggregation of other amyloidogenic proteins

6.1. HSA interacts with monomeric and oligomeric αSyn

The hallmark of Parkinson's disease are Lewy bodies, abnormal protein inclusions comprised primarily of alpha-synuclein (αSyn), an intrinsically disordered protein [96,97]. Similarly to Aβ, αSyn can self-associate into toxic oligomers and fibrils which in turn lead to neuronal damage and death [98,99]. While αSyn is considered an intracellular protein, αSyn has also been detected in cerebrospinal fluid and blood plasma. Unconventional exocytosis of intracellular αSyn into the extracellular space and subsequent trafficking into recipient cells, via a prion-like mechanism, contributes to cell-to-cell transmission of synucleinopathies [100,101]. Once in the extracellular environment, αSyn interacts with a wide array of ligands and proteins that affect its structure, its subsequent entry to cells, and aggregation propensity [102,103]. Such ligands could be amyloid enhancers, such as redox metal ions [104,105], or amyloid inhibitors, such as the extracellular chaperone HSA [54,55]. Unlike Aβ, the interactions of HSA and αSyn have not been extensively studied until recently [54,55].

In 2019, Bellomo et al. characterized the interactions between HSA and αSyn and the effect of the chaperone on αSyn self-association [55]. ThT fluorescence experiments under both low and physiological salt concentrations revealed that albumin, at plasma concentrations (~sub-mM), is able to slow significantly αSyn aggregation (Fig. 6a). To dissect the mechanism underlying the inhibition of αSyn aggregation by HSA, the authors studied the αSyn-HSA interactions using 2D ^{1}H-^{15}N HSQC NMR experiments [55]. By monitoring the changes in intensity of 15-N labelled αSyn NMR signal upon titration with different HSA concentrations, the authors found that HSA interacts with αSyn at the N and C-termini. The decrease in intensity of αSyn residues at the C-terminus is ionic strength-dependent, which led the authors to hypothesize the electrostatic nature of such interaction. Calculations using the Adaptive Poisson-Boltzmann Solver revealed a positively charged pocket in HSA as the candidate partner for αSyn C-termini. The authors concluded that binding of HSA at the N-terminal region of αSyn is hydrophobically driven and at physiological ionic strength represents the primary relevant binding site [55].

Ahmed et al. further investigated the interactions between αSyn and HSA beyond αSyn monomers [54]. The authors focused on αSyn oligomers, the key players of amyloid-related toxicity and pathology [100].

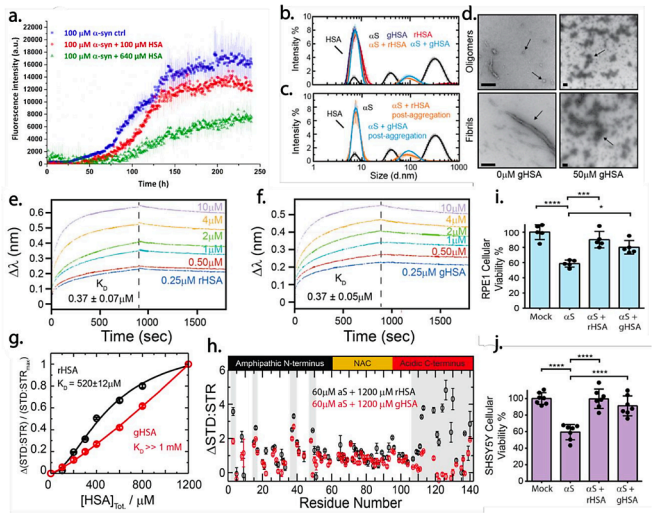

Fig. 6. ***HSA interactions with monomeric and oligomeric αSyn, effects on aggregation and cell toxicity.*** (**a**) ThT fluorescence of 50 μM αSyn at different HSA concentrations. (**b**) DLS intensity measurements of oligomeric αSyn prepared in the presence and absence of 50 μM fatty acid free HSA (rHSA) and fatty acid bound HSA (gHSA). The experiments started from monomeric αSyn. (**c**) Same as (a) except rHSA and gHSA were added to preformed αSyn oligomers. (**d**) TEM images of preformed αSyn species without (left) and with (right) 50 μM gHSA from (c). Scale bar =100 nm. (**e**) Biolayer interferometry (BLI) analysis of rHSA binding to αSyn oligomers. (**f**) Same as (e) but for gHSA. (**g**) Normalized 1D methyl STD-HSQC-based binding isotherms for the interactions of αSyn monomers with rHSA (black) and gHSA (red). Experimental points were fitted to a Hill-like (solid line) model to obtain the Kd values. (**h**) Residue-specific 2D STD-HSQC intensities of αSyn monomers with rHSA (black) and gHSA (red). (**i**) Presto-Blue cell viability assays of retinal pigment epithelial (RPE1) cells after treatment with αSyn oligomers in the absence or presence of rHSA and gHSA. (**j**) Same as (i) but measured for SH-SY5Y neuroblastoma cells. Panel (a) was adapted from [48]. Reprinted (adapted) with permission from The American Chemical Society. *J.Phys.Chem.B.* **2019**, 123, 20, 4380–4386. Copyright (2019) American Chemical Society. Panels (b-j) were adapted from [47]. Reprinted (adapted) with permission from The American Chemical Society. *J.Am.Chem.Soc.* **2020**, 142, 21, 9686–9699. Copyright (2020) American Chemical Society. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

In addition, Ahmed et al. characterized the interactions of fatty acid bound HSA (gHSA), extracted from plasma, and fatty acid-free (rHSA) with both αSyn monomers and oligomers. They also evaluated how such binding affects the contacts of αSyn with membranes, since previously it was shown that membrane disruption is one of the key mechanisms by which αSyn oligomers confer cellular toxicity [98]. A combination of solution and solid state NMR, DLS, size exclusion chromatography with multiangle light scattering (SEC-MALS), biolayer interferometry, cell viability assays among other methods revealed that HSA inhibits the aggregation and amyloid toxicity of αSyn through three different mechanisms [54].

The first mechanism involves the binding of HSA to αSyn oligomers at exposed hydrophobic sites with a K_d in the sub-μM range (Fig. 6 e and f), inhibiting the growth of the oligomers into larger assemblies. The second mechanism posits that HSA remodels αSyn toxic oligomers into aggregates with intermediate molecular weight and reduced toxicity (Fig. 6 b-d). The third mechanism proposes that HSA disrupts the interactions of αSyn with membranes, therefore exercising a protective role. Another notable finding of this study is that the binding of HSA to αSyn oligomers is not affected by the load of FAs in HSA. However, the binding of HSA to monomeric αSyn at the C-termini is supressed in the presence of FAs, while the binding at the N-termini is not affected (Fig. 6 g and h) [54]. Additionally, cell toxicity assays revealed that both fatty acid bound HSA, and fatty acid free HSA are able to rescue cells from αSyn-induced toxicity (Fig. 6 I and j). This study is an example of how a chaperone can suppress harmful interactions of toxic aggregates with membranes, in addition to binding and stabilizing a client protein.

The integrated experimental approach proposed by Ahmed et al. represents a useful tool to map at different degrees of resolution protein-protein interactions related to amyloidogenic inhibition systems. In fact, αSyn serves as an excellent model system to develop methods for characterizing IDP interactions in general. For example, using αSyn it was possible to test an approach to establish hierarchical self-assembly maps at atomic resolution [56]. This method is called "Self-Assembly monitored by Temperature-Induced Relaxation Enhancement" or SATIRE in brief and is based on the simple idea that the temperature dependence of ^{15}N-R_2 relaxation rates is a sensitive indicator of early self-association events [56]. In the case of residues not directly involved in self-association, the ^{15}N-R_2 relaxation rates monotonically decrease as the temperature increases due to enhanced flexibility of monomeric IDPs in the picosecond (ps)- nanosecond (ns) timescale at higher temperatures (Fig. 7 a and b). On the contrary, residues involved in self-association exhibit a non-monotonic temperature dependence due to self-association induced relaxation enhancements occurring at higher temperatures (Fig. 7 a and b), which typically amplify the entropic contribution to the free energy of hydrophobic collapse.

6.2. HSA inhibits the aggregation of insulin

Insulin is a peptide hormone that regulates carbohydrate and fat metabolism. It consists of 51 amino acids and adopts a mostly α-helical structure [106]. Insulin insufficiency and resistance plays a key role in diabetes, a disease in which the regulation of blood glucose levels and its cellular uptake are compromised [107]. The treatment for diabetes often involves insulin therapy, which consists of exogenous insulin injected directly into the blood stream or administered through a pump [106,107]. Unfortunately, during the process of manufacturing and administering insulin, its globular structure can be perturbed leading to the formation of amyloid aggregates in the vicinity of the site of application, a condition termed injection amyloidosis [108–111]. Similar to other amyloidogenic proteins, insulin amyloid formation involves a shift of hydrophobic buried residues to the surface of the peptide. This, in turn, leads to the formation of oligomers and characteristic insoluble cross β-sheeted fibrillar deposits [108,109].

The role of albumin in the inhibition of other amyloidogenic proteins, as discussed in the previous sections, led researchers to test if the abundant chaperone also binds insulin and inhibits its self-association

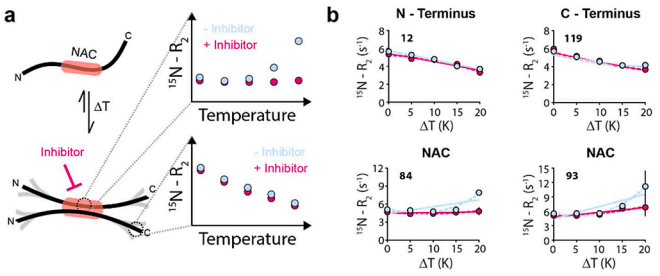

Fig. 7. ***Mapping IDP Self-Association through SATIRE NMR.*** (**a**) In monomeric IDPs, ^{15}N-R_2 relaxation rates decrease upon heating due to enhanced ps-ns dynamics. Such decrease is in first approximation linear. However, when IDP self-associate upon heating the temperature dependence of ^{15}N-R_2 is no longermonotonic, with increases in ^{15}N-R_2 observed at higher temperatures for residues involved in self-association. Upon addition of a self-association inhibitor, this effect is attenuated (magenta *vs.* blue plots). **(b)** Application of SATIRE NMR to α-Syn showing that the NAC region is more directly involved in early self-association than the terminal regions and that HSA interferes with the self-association of the NAC region [49]. Reprinted (adapted) with permission from The American Chemical Society. *J.Am.Chem.Soc.* **2021**, 143, 12, 4668–4679. Copyright (2021) American Chemical Society.

[57]. Finn et al. utilized turbidity measurements to show that the closely related bovine serum albumin (BSA) at physiological concentrations acts as a chaperone for insulin stressed through reducing agents [47]. Further insight on the albumin-insulin interactions was provided more recently by Wasko et al., who studied how HSA binds with native insulin and specific fragments of the peptide that are responsible for its amyloid formation ("hot-spots") [57]. To this end, Wasko et al. attached the fluorophore 4-(1-pyrenyl) butyric acid to the N-termini of insulin and two hot-spot peptides, A13-A19 and B12-B17. Upon HSA addition, the fluorophore displayed increased fluorescence lifetimes in the excited state, suggesting binding of the chaperone to the peptides [57]. Using microscale thermophoresis (MST) assays, HSA was found to bind the aggregation hot-spot fragments A13-A19 and B12-B17 with K_d values of 14.4 μM and 246 nM, respectively. Furthermore, using CD the authors revealed that the binding of HSA to insulin and its hots-spots stabilizes their α-helical conformation. Lastly aggregation analyses based on ThT fluorescence and congo red showed that the interaction of HSA with insulin and the hot-spot peptides inhibits their self-association into amyloid fibrils [57]. This study shows promising results for the therapeutic use of HSA in injection amyloidosis, as a natural and effective inhibitor of insulin aggregation. Overall, these results point to the potential of HSA as a drug delivery system that targets the hot-spots of insulin and inhibits its self-association [57].

6.3. HSA inhibits the amyloid formation of other client proteins

HSA acts as an effective chaperone for several client proteins besides Aβ, αSyn, and insulin. For example, HSA acts as an effective inhibitor of wild type and mutant transthyretin (TTR), stressed alcohol dehydrogenase [47] as well as the human islet amyloid polypeptide (IAPP). For the sake of brevity, here we focus on the latter, given its close functional relationship to insulin discussed in the previous section. Co-secreted with insulin, IAPP is also a peptide hormone of 37 residues produced in pancreatic β cells [112]. The aggregation of IAPP has been linked to the loss of islet β-cells and the onset of type 2 diabetes [113–115]. IAPP plaques form extracellularly around pancreatic islet β-cells [114]. Since IAPP can be released into the blood stream its exposure and interaction to several ligands including lipids and proteins has been hypothesized to affect its aggregation [58,116,117]. Therefore, the most abundant extracellular chaperone was studied as one of the possible inhibitors of IAPP aggregation in plasma [58].

Through the use of ThT fluorescence, TEM, and hemolysis assays Kakinen et al. showed that HSA is able to inhibit the fibrillization of IAPP and reduce the damage caused by IAPP aggregates to biological membranes [58]. Briefly, ThT fluorescence results revealed that the lag phase of IAPP aggregation is extended upon addition of increasing HSA concentrations. The authors proposed that the inhibitory potency of HSA is related to its electrostatic interactions with positively charged IAPP residues and/or hydrophobic contacts with the amyloidogenic region of IAPP. Such binding is thought to decrease the availability of free IAPP necessary for the nucleation and elongation processes [58]. This is related to the "monomer-competitor" model previously proposed for the inhibition of Aβ by Milojevic and colleagues [33]. Furthermore, TEM images revealed that HSA changes the morphology of IAPP fibrils, while an excess of HSA leads to its co-aggregation with IAPP fibrils. Lastly, the authors found that HSA protects red blood cells (RBCs) from hemolysis induced by toxic oligomeric species of IAPP, hinting to the chaperone's protective role possibly through direct binding to RBCs or its antioxidant capacity [58]. Overall, based on these examples, HSA emerges as a versatile extracellular chaperone capable of inhibiting the self-association of a wide spectrum of client proteins, from IDPs such as Aβ, αSyn and IAPP, to globular structures, such as insulin and TTR through a variety of concurrent mechanisms (Fig. 8).

Although HSA functions as an effective inhibitor of amyloid aggregation and its related toxicity, its inhibitory potency may be affected *in vivo* by several factors including molecular crowding as well as simultaneous interactions with several IDPs leading to cross-seeding [118]. In this respect, it is notable that BSA can form amyloid-like assemblies at physiological temperatures induced by the cross-seeding with HEWL amyloid aggregates, albeit only at low pH (pH ~ 3) [119]. In addition, the promiscuous profile of interactions of HSA with several metal ions can also influence how the chaperone chelates specific metal ions that enhance the aggregation of IDPs.

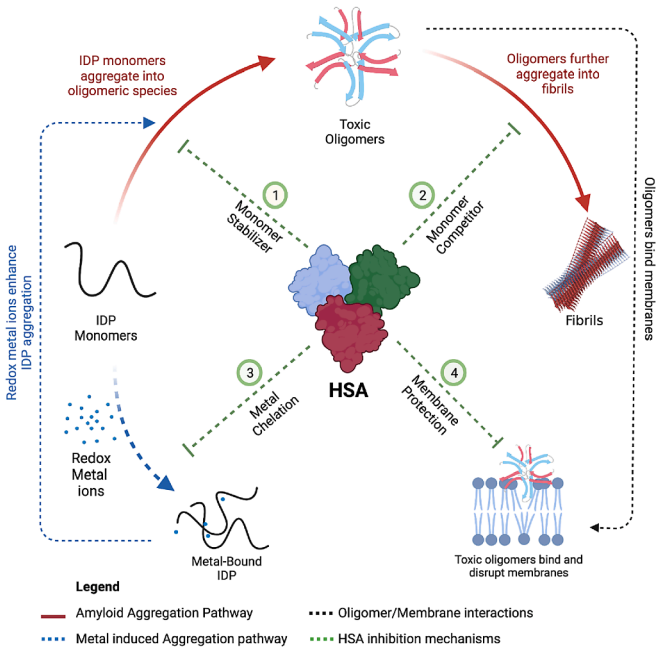

Fig. 8. ***Mechanisms of inhibition of amyloid aggregation and cytotoxicity by HSA***. (1) The monomer stabilizer-model: HSA binds IDP monomers at physiologically relevant plasma concentrations and inhibits their aggregation into oligomers and fibrils. (2) The monomer-competitor model: HSA binds IDP oligomers and prevents the further addition of monomers, inhibiting the oligomer's further aggregation into fibrils. (3) Metal chelation mechanism: HSA sequesters redox metal ions from IDP-metal complexes that enhance IDP aggregation. (4) Membrane protection: HSA prevents the binding of toxic oligomers to membranes, inhibiting oligomer-mediated membrane damage and cell toxicity. This figure was generated with BioRender. https://biorender.com/

7. Conclusions

Besides functioning as a solute carrier and oncotic pressure regulator, HSA acts as an ATP-independent chaperone for a wide spectrum of client proteins, from IDPs to folded proteins. IDPs include Aβ, IAPP, the Atrial Natriuretic Peptide (ANP) and αSyn. Folded proteins include insulin and TTR, especially when subject to stress induced by mutations or changes in environmental conditions (*e.g.* redox potential, temperature, shear stress from blood flow). HSA inhibits the aggregation of client proteins through a combination of direct binding (Fig. 8, schemes 1–2) and indirect mechanisms (*e.g.* metal chelation and membrane protection; Fig. 8, schemes 3–4). Direct binding mechanisms include both monomer-competitor and monomer-stabilizer mechanisms (Fig. 8, schemes 1–2), although higher affinity is typically observed for oligomers than monomers of client proteins, suggesting a predominance of the former mechanism especially in CSF where [HSA] is low (~μM). However, in plasma where [HSA] is high (~sub-mM), both mechanisms are likely at play. In addition, the binding of IDPs to HSA is modulated by HSA ligands (*e.g.* FFAs), which in turn are modulated by metal binding, suggesting that the direct and indirect mechanisms are not fully decoupled.

The complex profile of interactions displayed by HSA makes it difficult to conclusively establish the dominant mechanism *in vivo*, and

to what extent some HSA ligands such as fatty acids and metal ions affect its chaperoning capability. As previously discussed, only the monomer-competitor mechanism and the chelating effect of HSA are relevant at CSF concentrations. However, in plasma where HSA is more abundant the other discussed mechanisms can be simultaneously at play. While HSA is not the only extracellular chaperone active in plasma, as transferrin, clusterin and possibly other globular proteins have been shown to inhibit amyloid formation, HSA is likely to elicit the most significant net chaperoning effect given its abundance. As a result, the amyloid-inhibitory properties of HSA open new opportunities in the management of amyloidopathies in general and especially neurodegenerative diseases in the context of the peripheral sink hypothesis.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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