Molecular Mechanism for the Suppression of Alpha Synuclein Membrane Toxicity by an Unconventional Extracellular Chaperone


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ABSTRACT: Alpha synuclein (αS) oligomers are a key component of Lewy bodies implicated in Parkinson’s disease (PD). Although primarily intracellular, extracellular αS exocytosed from neurons also contributes to PD pathogenesis through a prion-like transmission mechanism. Here, we show at progressive degrees of resolution that the most abundantly expressed extracellular protein, human serum albumin (HSA), inhibits αS oligomer (αS_n) toxicity through a three-pronged mechanism. First, endogenous HSA targets αS_n with sub-μM affinity via solvent-exposed hydrophobic sites, breaking the catalytic cycle that promotes αS self-association. Second, HSA remodels αS oligomers and high-MW fibrils into chimeric intermediates with reduced toxicity. Third, HSA unexpectedly suppresses membrane interactions with the N-terminal and central αS regions. Overall, our findings suggest that the extracellular proteostasis network may regulate αS cell-to-cell transmission not only by reducing the populations of membrane-binding competent αS oligomers but possibly also by shielding the membrane interface from residual toxic species.

INTRODUCTION

The aggregation of alpha synuclein (αS) into Lewy bodies (LBs) is a clinical hallmark of Parkinson’s disease (PD).1 It is widely accepted that β-sheet-rich αS oligomers contribute to the neurodegeneration observed in PD.2 Indeed, genetic mutations of familial PD are observed exclusively in the αS encoding gene (SNCA), and such mutations enhance the accumulation of toxic αS oligomers.3 Moreover, sporadic forms of PD also result in αS accumulation.4

While LBs observed in PD are intracellular, emerging evidence suggests that extracellular αS also exists and contributes to PD pathology via a prion-like cell-to-cell transmission mechanism.5,6 LBs expand through anatomically connected regions of the brain from the peripheral tissues of the olfactory bulb to the cerebral cortex,6 suggesting a prion-like transmission hypothesis. Moreover, monomeric and oligomeric forms of αS have been detected in blood plasma7 and cerebrospinal fluid (CSF).8 Notably, grafting fetal mesencephalic neurons in the neostriatum of PD patient brains caused the development of intracellular LBs in the exogenously introduced cells.9,10 Similar observations have also been reported in cell culture and mouse model studies,11 which collectively showed that αS is secreted from cells via an unconventional exocytosis pathway independent of the endoplasmic reticulum and Golgi apparatus.12 The extracellular αS is able to subsequently enter into recipient cells, with αS aggregates exhibiting enhanced propensity to internalize compared to monomeric forms.13,14

While a substantial body of evidence now points to the presence of extracellular αS species, the specific interactions of such species with the extracellular proteome and its role in...
regulating αS internalization into cells are poorly understood. The extracellular milieu thus remains an untapped potential that could be harnessed for therapeutic interventions in PD. As a first step toward tapping the translational potential of the extracellular milieu, here we focus on the most abundantly expressed protein in human blood plasma and CSF, i.e., human serum albumin (HSA). HSA is not only the endogenous transporter for a broad range of serum solutes, such as fatty acids and exogenous ligands, but also the most potent plasma inhibitor of self-association for numerous unstructured polypeptides prone to form cytotoxic oligomers, such as the prototypical amyloidogenic peptide Ap[1]25-35 and more recently αS.23-25 Hence, the extracellular chaperone HSA26 serves as an ideal candidate to explore the regulatory capacity of the extracellular proteome in the context of cell-to-cell transmission in PD.

Several open questions remain about the mechanism through which HSA regulates the conformations of extracellular αS species and their capacity to internalize into cells. First, it is currently unclear whether HSA interacts with αS oligomers, which are the predominant internalized species and, if so, what are the determinants of such interactions. Second, it is unknown whether HSA perturbs the interactions and subsequent internalization of αS into cells. Addressing these questions is critical to understanding the intercellular transmission of αS and the role of the extracellular proteome in this process.

Here, we have examined the interactions of defatted (rHSA) and nondefatted, endogenous (gHSA) HSA extracted from blood plasma with both monomeric and oligomeric αS. We also evaluated how such interactions influence the association of αS with membranes. By combining both solution and solid-state NMR with extrinsic fluorescence, dynamic light scattering (DLS), size exclusion chromatography with multiangle light scattering (SEC-MALS), transmission electron microscopy (TEM), biolayer interferometry (BLI), and wide-angle X-ray diffraction (WAXD) we show that HSA binds αS oligomers with sub-μM affinity through multiple solvent-exposed hydrophobic surfaces. The αS oligomer–HSA interactions result not only in the inhibition of αS self-association but also in the remodeling of existing low molecular weight (LMW) and high molecular weight (HMW) αS aggregates into chimeric intermediates that are thermodynamically stable and exhibit reduced toxicity. Unexpectedly, HSA also inhibits the interactions of the αS N-terminal and non amyloid-β component (NAC) regions with lipid membranes, pointing to a role of HSA in suppressing membrane damage by αS. These two HSA-induced effects, i.e., the αS oligomer remodeling and the preservation of membrane integrity, provide a viable mechanism to explain how HSA, and possibly the extracellular proteome, suppresses cell-to-cell transmission of αS.

■ RESULTS

Both Defatted and Nondefatted HSA Reduce the Cytotoxicity of αS Oligomers and Bind αS Oligomers with Sub-μM Affinity. Our working definition of “oligomer” refers to any pre fibril lar species with diameters less than 1000 nm and aggregated MW < 1 M Da.27 Three different oligomer preparation protocols are used in this work (Experimental Section), each of which is optimized for a different experimental technique. The size distributions of the αS oligomers prepared according to these protocols were characterized by SEC-MALS (Table 1, Figure 1a–c). Despite originating from different protocols, the size distributions of the αS species are similar for all three preparations (Table 1, Figure 1a–c).

Table 1. Molecular Weights (kDa) of αS Species as Determined by SEC-MALS

<table>
<thead>
<tr>
<th>sample</th>
<th>monomer</th>
<th>LMW oligomer</th>
<th>intermediate MW oligomer</th>
<th>HMW aggregates</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 μM, 2 d, 37 °C</td>
<td>14.1 ± 0.9</td>
<td>31.8 ± 6.9</td>
<td>122 ± 10</td>
<td>731 ± 12</td>
</tr>
<tr>
<td>800 μM, 1 d, 37 °C</td>
<td>15.1 ± 0.1</td>
<td>37.7 ± 0.5</td>
<td>112 ± 2</td>
<td>835 ± 1</td>
</tr>
<tr>
<td>800 μM, 2 d, 37 °C</td>
<td>14.1 ± 0.1</td>
<td>32.5 ± 0.9</td>
<td>132 ± 6</td>
<td>767 ± 2</td>
</tr>
</tbody>
</table>

We first analyzed whether HSA suppresses the cytotoxicity of αS oligomers and if this capacity is dependent on the fatty acid load of HSA. To this end, we used defatted and nondefatted HSA extracted from pooled blood plasma, denoted here as rHSA and gHSA, respectively. Under our conditions, the two highest-affinity sites of gHSA are occupied by long-chain fatty acids (FAs) (A and B, Figure S1). We measured the αS oligomer cytotoxicity in the absence and presence of rHSA and gHSA using a Presto Blue assay. As seen in Figure 1de, both albumins significantly recover the loss of cellular viability induced by αS oligomers to comparable levels. These effects are consistently observed in both retinal pigment epithelial (RPE1) (Figure 1d, Table S1) and SHSY5Y neuroblastoma (Figure 1e, Table S1) cell lines, pointing to a detoxification mechanism that appears to be largely independent of cell type and presence of fatty acid.

As a first step toward understanding the mechanism by which HSA detoxifies αS oligomers, we measured the affinity of αS oligomers for both rHSA and gHSA using biolayer interferometry. Our BLI analyses (Figure 1fg) reveal that both rHSA and gHSA bind to αS oligomers with comparable affinities of 0.37 ± 0.07 and 0.37 ± 0.05 μM, respectively, consistent with their equivalent detoxification capacities. While these results consistently point to similar efficacy of defatted and nondefatted HSA, they do not offer any insight on the drivers of the HSA–αS oligomer interactions. Hence, we relied on competitive binding experiments with well-known HSA binders, such as 13C-oleic Acid (OA),28 8-anilinonaphthalene-1-sulfonic acid (ANS), and dansyl- L-phenylalanine (Dan F),29 to probe the determinants of HSA–αS oligomer interactions.

αS Oligomers Target Multiple Solvent-Exposed Hydrophobic Sites in HSA. OA occupies nine binding sites scattered throughout the three domains of HSA, starting from the three high-affinity sites at low FA:HSA stoichiometric ratios and progressing to lower affinity loci occupied at higher FA:HSA ratios (Figure S1a). Of the six low-affinity loci, two are commonly occupied by drugs, Sudlow sites I and II, located in domains 2 and 3, respectively (Figure S1a). The 1H–13C methyl HSQC spectra of 13C-OA in the presence of HSA provide a comprehensive map of the occupancy of these HSA sites by long-chain fatty acids (LCFAs) such as OA (Figure S1a,b). Moreover, both ANS and Dan F exhibit comparable affinities for HSA and occupy HSA Sudlow sites I and II, as revealed by 1H–13C HSQC spectra showing the competition between ANS/Dan F with 13C-OA bound to HSA at these sites (Figure S1c–f). However, unlike Dan F, ANS
Figure 1. Both defatted and nondefatted HSA suppress αS oligomer cell toxicity and bind αS oligomers with sub-μM affinity. (a–c) SEC-MALS characterization of the αS oligomers. A zoomed-in chromogram is shown in the upper panel of (c) to more clearly display lowly populated oligomers. (d) Cellular viability of retinal pigment epithelial (RPE1) cells after treatment with αS oligomers in the absence and presence of defatted (rHSA) and nondefatted (gHSA) HSA, as monitored by the reduction of Resazurin using the Presto Blue assay. The data reported show the mean and standard deviation of technical replicates. One-way ANOVA and subsequent Tukey posthoc test was used to determine statistical significance between treatments and mock (PBS delivery solution), with *, **, and **** representing p-values of ≤0.05, ≤0.001, and ≤0.0001, respectively. (e) As (d) except measured for SHSY5Y neuroblastoma cells. (f, g) Biolayer interferometry (BLI) analysis of the (f) rHSA and (g) gHSA binding to αS oligomers, respectively. (h, i) Fluorescence intensities of ANS and Dan F bound to rHSA in the absence (dark purple) and presence (light purple) of two different αS oligomer preparations, normalized to the rHSA alone state. The data reported show the mean and standard deviation of technical replicates. Fluorescence contributions arising from ANS and Dan F binding each αS oligomer are accounted for. Two-way ANOVA and subsequent Sidak’s multiple comparison tests were used to determine statistical significance between the HSA or rHSA with αS oligomer samples in ANS vs Dan F competition experiments. Cartoons in (h) and (i) depict the protocols for preparation of the αS oligomers.

bonds to additional sites on HSA that do not overlap with 13C-OA, consistent with the substantially smaller decrease in ANS fluorescence relative to Dan F in gHSA vs rHSA (Figure S1g). Overall, the combination of 13C-OA, ANS, and Dan F competition experiments provides an informative spectroscopic handle to probe the determinants of HSA–αS oligomer interactions.

In order to separate the contributions from αS monomers vs αS oligomers to the ANS and Dan F fluorescence, we prepared two variations of αS oligomers with progressively larger populations of HMW aggregates and reduced populations of residual monomers (Figure 1c vs b, Table 1). Subsequently, we added these preformed αS oligomers to ANS or Dan F-bound rHSA and observed changes in the ANS or Dan F fluorescence to assess the relative degree of competition. As the population of HMW species increases and monomer population decreases, a significantly larger decrease in ANS relative to Dan F fluorescence is observed (Figure 1h,i). For example, αS oligomers prepared at a concentration of 100 μM through a two-day incubation at 37 °C lead to a substantially larger Dan F vs ANS fluorescence reduction (Figure 1h). However, when the population of αS oligomers is further promoted (monomer population further decreased) through higher αS concentrations, αS oligomer addition leads to comparable fluorescence losses for ANS and Dan F (Figure 1i). These results are further confirmed for a substantially aggregated αS sample with no residual monomers (Figure S2). Overall, these results show that αS oligomers preferentially bind to solvent-exposed hydrophobic sites in HSA, which are distinct from the Sudlow site I and II fatty-acid-binding loci. Nevertheless, these results do not explain how the binding of HSA to αS oligomers leads to a reduction in cytotoxicity. To address this question, we monitored through 1H NMR and TEM/DLS how HSA remodels the distribution of αS monomers and HMW species.

HSA Remodels αS Aggregates into Thermodynamically Stable Chimeric Intermediates. To understand how the αS oligomer–HSA interactions remodel αS oligomer species, we first recorded DLS data for αS oligomers prepared in the absence and presence of rHSA and gHSA (Figure 2a). Figure 2a shows that albumin shifts the populations of LMW (<10 nm) and HMW (>100 nm) αS species into intermediate assemblies with a hydrodynamic radius of ~100 nm, resulting in a distribution more uniform in size compared to that in the absence of HSA. Such size remodeling effect is observed for both rHSA and gHSA to a comparable extent (Figure 2a), and it is also largely independent of whether albumin is added prior to or after αS aggregation (Figure 2a vs b). These observations suggest that the albumin-induced remodeling of αS oligomers is independent of fatty acid binding to the high-affinity sites and is under thermodynamic rather than kinetic control. Further inspection of the albumin-induced remodeling of αS oligomers through TEM imaging corroborates that albumin converts LMW spherical oligomers and HMW αS fibrils into intermediate assemblies (Figures 2c and S3a).

Residue-resolution insight into the structural changes occurring upon HSA-induced remodeling of αS oligomers was gained through 13C–13C dipolar assisted rotational resonance (DARR) solid-state NMR (ssNMR) experiments (Figure 2d). The 13C–13C DARR spectra were assigned based on previous assignments of αS oligomers 32 and fibrils. 33 The DARR spectrum acquired in the absence of HSA (Figure 2d, dark purple) shows good overlay with αS fibrils simulated using the chemical shifts reported by Cornellias and colleagues 33 (Figure 2d, light violet), confirming that our αS oligomers form a rigid β-sheet rich core centered around the NAC region, as previously shown. 32 However, relative to the fibrils far fewer DARR cross-peaks are observed for αS oligomers, indicating an overall less rigid structure. The αS
Figure 2. HSA remodels pre-existing αS oligomers, redirects aggregation pathways toward intermediate chimeric structures, and inhibits the conversion of NMR-visible αS species into NMR-invisible αS oligomers. (a) Dynamic light scattering (DLS) intensity measurements of αS oligomers prepared in the absence and presence of 50 μM rHSA and gHSA starting from an essentially monomeric state. Error bars are based on standard deviation of technical replicates. (b) As (a) except rHSA and gHSA were added to preaggregated αS oligomers. (c) Negative stain transmission electron microscopy images of the preformed αS oligomers in the absence (dark purple) and presence (blue) of 50 μM gHSA at various temperatures, normalized to the intensities at 283 K. The Hb protons used for HSA addition. (i) Average of three well-resolved αS oligomer populations of αS oligomers with and without rHSA or gHSA (Figures 2i,j, red bars), possibly arising from gHSA—αS monomer binding, which is expected to be weaker (vide infra).^2^ These concentration-dependent contributions are somewhat greater in the case of rHSA (Figure S3c,d, red bars). While these results consistently show that the archetypal extracellular protein, HSA, remodels and suppresses the formation of toxic αS oligomers, they do not provide insight into whether these αS species retain the capacity to associate with and insert into the membrane, a process known to underlie the neurotoxicity of several amyloidogenic proteins. Hence, we evaluated whether HSA perturbs the interactions of αS with membranes.

HSA Perturbs Interactions of αS with Membranes through a Dual Mechanism. To probe how albumin modulates αS–membrane interactions, we measured the leakage of the aqueous internal compartment of large unilamellar vesicles (LUVs) in the absence and presence of αS oligomers with and without rHSA or gHSA (Figure 3a). Interestingly, in the absence of αS oligomers, both rHSA and gHSA reduce membrane permeabilization, pointing to direct interactions of albumin with lipid membranes. Similarly, the protective effect of both albumins is preserved in the presence of αS oligomers, significantly reducing αS oligomer-induced membrane permeabilization. We complemented these membrane permeability results with wide-angle X-ray diffraction (WAXD) experiments, which probe the αS/HSA–membrane interactions from the perspective of the lipids (Figure 3b–g). Stacks of membranes in the absence and presence of αS oligomers with and without rHSA or gHSA were subjected to oligomer–fibril continuum thus serves as an effective tool to evaluate the effect of HSA addition to αS oligomers. Notably, addition of substoichiometric amounts of gHSA shifts the populations of αS oligomers to more rigid fibril-like states, as evidenced by the appearance of new DARR cross-peaks that overlay with αS fibrils (Figure 2d–f). These observations are further supported by the loss of DARR resonances that are more unique to αS oligomers (Figure 2d, g,h blue vs dark purple), indicating a shift away from the oligomeric states. The high-resolution ssNMR experiments are in good agreement with our low-resolution DLS and TEM data, both of which suggest that HSA remodels preformed αS aggregates into chimeric structural assemblies that are reminiscent of both oligomers and fibrils.

HSA Inhibits the Heat-Induced Self-Assembly of αS Monomers by Binding Primarily to αS Oligomers. In order to further explore how the albumin-induced αS oligomer remodeling affects the self-association of αS, we monitored how albumin perturbs the transition from NMR-visible monomers to NMR-invisible αS oligomers induced by heating. For this purpose, we measured how the NMR intensity of Hb and other side chain αS protons is reduced upon heating in the absence and presence of gHSA and rHSA (Figures 2i,j and S3c,d). gHSA at concentrations 2 orders of magnitude above the K_diss for αS oligomer binding, i.e., conditions under which αS oligomers are saturated with gHSA, is effective at reducing the heat-induced NMR intensity losses (Figure 2i,j, orange bars), indicating that αS oligomer binding is sufficient to inhibit αS self-association. A similar pattern is observed for rHSA (Figure S3c,d, orange bars), consistent with the αS oligomer–HSA interactions being largely independent of fatty acid binding to the first two high-affinity sites. However, when the gHSA concentration is increased by an additional order of magnitude, further inhibition of αS self-association is detected (Figure 2i,j, red bars), possibly arising from gHSA–αS monomer binding, which is expected to be weaker (vide infra).^2^ These concentration-dependent contributions are somewhat greater in the case of rHSA (Figure S3c,d, red bars). While these results consistently show that the archetypal extracellular protein, HSA, remodels and suppresses the formation of toxic αS oligomers, they do not provide insight into whether these αS species retain the capacity to associate with and insert into the membrane, a process known to underlie the neurotoxicity of several amyloidogenic proteins. Hence, we evaluated whether HSA perturbs the interactions of αS with membranes.
HSA perturbs αS–membrane interactions independent of the fatty acid load. (a) Normalized calcein dye leakage from LUVs composed of 5:3:2 DOPE/DOPS/DOPC lipids treated with αS oligomers in the absence and presence of rHSA and gHSA. Negative (positive) values indicate reduced (enhanced) leakage compared to buffer. (b) Absolute out-of-plane reflectivity of 1:1 POPC/POPG bilayers in the absence and presence of HSA, αS oligomers, and HSA-remodeled αS oligomers. (c) Number of Bragg peaks for the samples shown in (b). (d) Peak intensity of the first Bragg peak for the samples shown in (b). (e) Calculated lamellar spacing \(d_i\) for the samples shown in (b). (f) Electron density maps for POPC/POPG bilayers in the absence and presence of gHSA, αS oligomers, and αS oligomers in the presence of gHSA. Arrows indicate the shifts in the electron density maxima upon addition of αS oligomers (yellow) and gHSA (violet). (g) As (f), except with rHSA (blue) replacing gHSA. WAXD measurements. Out-of-plane diffraction \(q_z\) is shown in Figure 3b. The observed Bragg peaks are the result of multilamellar membranes stacking. A decrease in the number of peaks is indicative of a less well-ordered lamellar phase. Addition of r/gHSA preserves the number of observed Bragg peaks but reduces their intensities (Figure 3b–d), once again pointing to the interaction of both defatted and nondefatted albumin with membranes.

Compared to albumin, a significantly greater loss of intensity and fewer Bragg peaks are observed upon addition of αS oligomers (Figure 3b–d), suggesting a marked loss of membrane integrity. Interestingly, the effects of albumin and αS oligomers on the membrane are nonadditive, with the presence of both species resulting in an effect intermediate to either protein alone (Figure 3b–d). These results are consistent with the hypothesis that albumin alters the effect of αS oligomers on lipid membranes. In further support of these results is the effective lamellar spacing between membrane bilayers (Figure 3e), which is enhanced in the presence of αS oligomers alone, consistent with αS being embedded or on the surface of the membranes, but reduced in the presence of r/gHSA (Figure 3e). A similar effect is observed for the electron density profiles (Figure 3f,g), which are sensitive to the position of the molecules in the membrane. A shift toward the bilayer center is observed in the presence of αS oligomers, consistent with their partitioning in and interacting with the membrane (Figure 3g).

However, addition of both r/gHSA and αS oligomers results in an electron density profile more similar to the unperturbed membrane (Figure 3g), indicating a reduced interaction. Overall, Figure 3 shows that both defatted and nondefatted HSA perturb the interactions of αS oligomers with membranes, through directly interacting with the membrane and/or the αS oligomers themselves.

**HSA Inhibits the Interactions of the αS N-Terminal and NAC Regions with Membranes.** To complement the WAXD and membrane permeability data and to gain residue-resolution information about the modulation of αS–membrane interactions by HSA, we acquired \(^{15}\)N-dark state exchange saturation transfer (DEST) NMR experiments of \(^{15}\)N-labeled αS prepared under four different conditions: αS alone or in the presence of unlabeled lipids, unlabeled gHSA, or both lipids and gHSA (Figure 4). The \(^{15}\)N–DEST experiment probes the interaction of αS with HMW species, such as membranes, αS oligomers, and their complexes, through the lens of NMR-visible αS monomers.\(^{21,39–41}\) As expected, addition of the lipids in the absence of albumin results in a major DEST reduction that is more pronounced for the N-terminal amphipathic and the NAC regions than the acidic C-terminus (Figure 4f, black vs blue traces). Addition of gHSA in the absence of lipids also leads to a significant DEST reduction, but now the DEST losses are more uniform across the αS sequence (Figure 4f, black vs orange traces).

Interestingly, the DEST reduction observed upon addition of gHSA in the absence of lipids is not observed when gHSA is added in the presence of lipids (Figure 4f, black–orange vs blue–red; Figure 4h green vs pink), suggesting that the lipids interfere with the gHSA–αS interactions. Similarly, the marked DEST losses caused by the addition of lipids in the absence of gHSA (Figure 4f, black vs blue traces) are significantly reduced when lipids are added in the presence of gHSA (Figure 4f, orange vs red traces; Figure 4g, yellow vs violet), revealing that gHSA weakens the lipid–αS interactions. These conclusions are independent of the DEST offsets utilized to monitor the binding of αS to lipids and gHSA (Figure S8g) and are in agreement with changes in HSQC intensities (Figure S6). Similar DEST variations are also observed when gHSA is replaced with rHSA (Figure S5a–f), corroborating that the inhibition of lipid–αS interactions by albumin is largely independent of fatty acids binding to the high-affinity sites of HSA.

Overall, our NMR data consistently point to albumin detuning the interactions of the αS N-terminal and NAC regions with lipids and vice versa (Figure 4g,h), explaining how albumin counters the loss of membrane integrity caused by αS oligomers. However, in addition to the albumin–αS oligomer interactions, binding to αS monomers may further influence the formation and cytotoxicity of αS oligomers, as suggested by the further recovery of αS NMR signal in the presence of higher [HSA] in heating-induced aggregation assays (Figures 2h and S3c,d, red vs orange).

**HSA Binds αS Monomers with mM to Sub-mM Affinity Targeting Both the N- and C-Termini of αS.** To measure the affinity of monomeric αS for both rHSA and gHSA, we titrated unlabeled rHSA and gHSA into \(^{15}\)N-labeled αS monomers and monitored the titration through 1D-STD-HSQC NMR (Figure S7a,b). The resulting binding isotherms are shown in Figure 5a. Based on Hill-like fitting of the STD data and the HSQC intensities (Figure S6), the following binding constants were obtained for rHSA and gHSA: K_MG = 2.3 mM and K_MG = 570 μM, respectively.
data (Figure 5a), monomeric αS binds rHSA in the sub-mM range ($K_D = 520 \pm 12 \mu M$) and gHSA in the supra-mM range ($K_D \gg 1 \text{ mM}$), indicating that the affinity of monomeric αS for defatted albumin is comparable to the physiological concentration of albumin in plasma, but it is reduced when HSA binds fatty acids. These results are further confirmed through BLI (Figure S8g,f). To map the binding sites for HSA within monomeric αS, we also acquired 2D-STD-HSQC spectra for 15N-labeled αS monomers in the presence and absence of excess rHSA or gHSA (Figure 5b; Figure S7c–h). The 2D-STD-HSQC spectra show that HSA targets not only the acidic C-terminal region of αS (residues >110) but also select residues of the N-terminal amphipathic segment (Figure 5b, gray highlights). The central NAC core and adjacent residues remain largely unaffected by saturation transfer from albumin. Interestingly, the interactions with the acidic C-terminus are almost completely suppressed on going from the defatted rHSA to the nondefatted gHSA (Figure 5b, red vs black circles). These results are independently corroborated by the relative HSQC intensity profiles (Figure S8), which indicate significant albumin-induced intensity losses at both C- and N-termini, with the C-terminal intensity reduction more pronounced than the N-terminal but also more dramatically suppressed by the fatty acids bound to gHSA (Figure S8c).

Moreover, the interaction of the αS C-terminus with HSA is electrostatically driven, as addition of 150 mM NaCl completely recovers HSQC signal losses at the C-terminus but not the N-terminus (Figure S8d,e). Overall, the HSQC intensity, BLI, and STD-HSQC data consistently indicate that the interactions of monomeric αS with HSA are partially compromised in gHSA vs rHSA, wherein binding of about two fatty acid equivalents is sufficient to perturb the electrostatic interactions at the C-terminus.

**αS Monomers Target Multiple Fatty Acid Dependent Sites within HSA.** The fatty acid dependence of the αS monomer–albumin affinity suggests that monomeric αS competes with fatty acid binding at high-affinity sites. In order to test this hypothesis, we acquired methyl 1H–13C HSQC spectra of 13C-labeled OA bound to albumin in the presence and absence of excess monomeric αS (Figure 5c). This spectrum reveals that αS monomer binding displaces OA from the two highest-affinity sites (i.e., A and B, Figure 5c,d), causing the displaced OA to relocate to other albumin loci and result in increased HSQC intensities (i.e., Figure 5c–e). As a further means to probe the competition between αS monomers and fatty acids, we also acquired fluorescence spectra for the ANS and Dan F fluorophores. Interestingly, when αS monomers bind rHSA, a loss of both ANS and Dan F fluorescence is observed (Figure Sf), revealing that monomeric αS competes with binding of fatty acids at multiple albumin

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**Figure 4.** Double-ligand DEST analyses reveal how HSA perturbs the αS NAC and N-terminal regions that interact with membranes. (a–d) Normalized 15N–DEST cross-peak intensities as a function of offset and residue number (see Experimental Section) measured for (a) 300 μM αS, (b) 300 μM αS + 300 μM gHSA, (c) 300 μM αS + 1.5 mM DOPE/DOPS/DOPC, and (d) 300 μM αS + 300 μM gHSA + 1.5 mM DOPE/DOPS/DOPC. (e) Double-ligand cycle for the interaction of αS with HSA and lipids. Color coding is preserved in the following panels. (f) Residue-specific 15N–DEST profiles computed using off-resonance and far off-resonance saturation frequencies of 14 and 28 kHz, respectively, from the 15N–DEST profiles shown in (a)–(d). The DEST profile was smoothed by averaging the $I_{14\text{kHz}}/I_{28\text{kHz}}$ values for each residue and the two residues directly adjacent to it, when available. (g, h) Differences between the residue-specific 15N–DEST profiles shown in (f) according to the double-ligand cycle shown in (e).
loci including not only high-affinity sites but also the lower-affinity Sudlow sites I and II.

**DISCUSSION**

We have investigated how the prototypical extracellular protein HSA interacts with αS monomers and oligomers and how these interactions modulate the association and insertion of αS into the membrane. Our results are summarized in Figure 6 and provide a foundation to explain the mechanism by which HAS inhibits the cell-to-cell transmission of extracellular αS species. HSA binds αS oligomers with an affinity ~3 orders of magnitude greater than monomers (K\(D_{\text{app oligomer}} \approx \sim 0.4 \mu M\) vs K\(D_{\text{monomer}} \approx 500 \mu M\); Figures 1f,g, S5a, S6a,b). Whereas the binding of αS monomers to the C-terminus is electrostatically driven and fatty acid dependent, the N-terminal binding is more resilient to the presence of salt or fatty acids (Figures S5a, 6a, S8d,e). These results suggest that the αS monomer N- and C-terminal regions serve as two distinct HSA binding sites. However, it is also possible that transient interactions between the N- and C-terminal regions of αS oligomers may be relevant for HSA binding. Unlike the fatty-acid-dependent interaction with αS monomers, the defatted rHSA and the endogenous gHSA exhibit similar affinities for the αS oligomers (Figure 1f vs g). Hence, the HSA−αS oligomer interactions are physiologically relevant not only in plasma but also in CSF where HAS is diluted to ~3 μM.15

The binding of HSA to αS oligomers is sufficient to suppress αS-associated toxicity (Figure 1d,e). A viable explanation of the detoxifying effect of albumin is that HSA functions through three distinct but concurrent mechanisms (Figure 6c,d,e). First, HSA remodels the distribution of LMW oligomers and HMW fibrils into intermediate-MW chimeric assemblies (Figures 2a–h, S3a, 6c) by binding to αS oligomers through interactions that are largely hydrophobically driven (Figure 1h,i). These results are observed irrespective of whether HSA is added prior to or after aggregation (Figure 2a vs b), suggesting that the HSA-induced remodeling of αS oligomers is primarily under thermodynamic rather than kinetic control. Moreover, these results suggest that HSA may serve as a viable late-stage intervention strategy when toxic αS oligomers have already formed.

Second, the αS oligomer−HSA interactions also inhibit the conversion of NMR-visible αS monomers into NMR-invisible αS oligomers (Figures 2j and S3c–d orange vs black, Figure 6d). This result is consistent with the reduced αS aggregation kinetics observed in ThT fluorescence experiments in the presence of HSA.23,25 However, here we unambiguously show that the αS oligomer−HSA interactions are the primary driver of inhibition, whereas previously it was unclear whether such inhibition arose from monomer vs oligomer binding. Moreover, here we show that such inhibitory effect is largely independent of the fatty acid load of HSA (Figure 2j, orange, vs Figure S3d, orange), consistent with the similar αS oligomer affinities of rHSA and gHSA. These findings are also in agreement with HSA perturbing the catalytic cycle that generates toxic oligomers, which has been shown previously for another molecular chaperone, Brichos.43
Third, HSA suppresses the association of αS oligomers with membranes, which has been implicated in the pathogenesis of PD. The loss of membrane integrity caused by αS oligomers results in a shift in the morphology of toxic low-MW β-sheet core oligomers and high-MW β-sheet-rich fibrils into less toxic intermediate-MW, thermodynamically stable chimeric assemblies stabilized by HSA. (c) HSA binding of αS oligomers results in a shift in the morphology of toxic low-MW β-sheet core oligomers and high-MW β-sheet-rich fibrils into less toxic intermediate-MW, thermodynamically stable chimeric assemblies stabilized by HSA. (d) HSA perturbs the conversion of NMR-visible αS monomers into NMR-invisible αS oligomers. Addition of low [HSA], wherein HSA binding of αS monomers is negligible, shields the αS oligomers from monomers, resulting in reduced incorporation of monomers into NMR-invisible species. Such inhibition effect is largely independent of the fatty acid load of HSA. Addition of high [HSA], wherein HSA binding of αS monomers is significant under our conditions, a further reduction in monomer conversion into NMR-invisible species is observed relative to low [HSA]. The effect is dependent on the fatty acid load of HSA, as expected given the rHSA vs gHSA differences in K₅ values for αS monomer binding. (e) Both rHSA and gHSA perturb the interactions of the αS N-terminal and NAC regions with negatively charged membranes. The inhibition may arise from either the direct binding of HSA to the membrane, displacing the bound αS and/or binding of HSA to αS, outcompeting membrane interactions. This inhibitory effect is also independent of the fatty acid content of HSA. αS in this panel denotes either monomeric and/or oligomeric species.

Figure 6. Proposed mechanism for the inhibition of αS self-association and toxicity by human serum albumin. (a) HSA binds αS monomers at the N- and C-termini through hydrophobic and electrostatic interactions, respectively. (b) HSA binds αS oligomers with an affinity several orders of magnitude greater than monomers. The binding is largely hydrophobically driven and is independent of the fatty acid load of HSA. (c) HSA binding of αS oligomers results in a shift in the morphology of toxic low-MW β-sheet core oligomers and high-MW β-sheet-rich fibrils into less toxic intermediate-MW, thermodynamically stable chimeric assemblies stabilized by HSA. (d) HSA perturbs the conversion of NMR-visible αS monomers into NMR-invisible αS oligomers. Addition of low [HSA], wherein HSA binding of αS monomers is negligible, shields the αS oligomers from monomers, resulting in reduced incorporation of monomers into NMR-invisible species. Such inhibition effect is largely independent of the fatty acid load of HSA. Addition of high [HSA], wherein HSA binding of αS monomers is significant under our conditions, a further reduction in monomer conversion into NMR-invisible species is observed relative to low [HSA]. The effect is dependent on the fatty acid load of HSA, as expected given the rHSA vs gHSA differences in K₅ values for αS monomer binding. (e) Both rHSA and gHSA perturb the interactions of the αS N-terminal and NAC regions with negatively charged membranes. The inhibition may arise from either the direct binding of HSA to the membrane, displacing the bound αS and/or binding of HSA to αS, outcompeting membrane interactions. This inhibitory effect is also independent of the fatty acid content of HSA. αS in this panel denotes either monomeric and/or oligomeric species.
structure (Figure 3). Moreover, similar observations have been reported for HSA in the context of liposomal trafficking, wherein the direct interactions of HSA with liposomes interfere with the delivery of solutes into cell membranes.\textsuperscript{46,47} Taken together, we show that the ameliorative effect of HSA is multifaceted, operating not only at the level of remodeling the αS oligomer distribution but also directly at the membrane.

Overall, our comparative analyses of the αS-HSA interactions at progressive degrees of resolution uncover an unprecedented mechanism by which a model extracellular chaperone inhibits the toxicity of PD-associated αS oligomers. On the same grounds, our results point to the notion that the extracellular proteostasis network may play a critical role in regulating the cell-to-cell transmission of αS.

Our work thus underscores the importance of evaluating how these control mechanisms are dysregulated in diseased states.

Unexpectedly, the data obtained here lend support to the idea that chaperones not only assist in the folding and assembly of a protein into nontoxic species but also prevent the interactions of toxic oligomers with membranes. The latter are known to promote the formation of toxic intermediates and enhance neuronal dysfunction.\textsuperscript{32} Lastly, the results presented here illustrate the effectiveness of our integrated experimental strategy to comprehensively probe at multiple length scales protein–protein interactions involving a heterogeneous and transient amyloidogenic system.

## Experimental Section

### Alpha Synuclein Expression and Purification

Alpha synuclein was expressed in *E. coli* BL21(DE3) cells using the pT7-7 plasmid harboring the αS sequence, as described previously.\textsuperscript{38} Briefly, the bacteria were grown at 37 °C in isotope-enriched M9 minimal media containing 1 g/L of \(^{15}\)N-ammonium chloride, 3 g/L of \(^{13}\)C-glucose, and 100 μg/mL of ampicillin. At an OD \(_{600}\) of ~0.6–0.8, αS was overexpressed with 100 μM isopropyl β-D-thiogalactopyranoside (IPTG) at 37 °C for 4 h, and the cells were subsequently harvested by centrifugation at 9800 × g. The cell pellets were resuspended in lysis buffer (10 mM Tris-HCl pH 8, 1 mM EDTA, and 1 mM AEBSF protease inhibitor) and lysed by three cycles of freeze–thawing followed by sonication. The cell lysate was heated for 20 min at >100 °C and centrifuged at 19500g for 1 h. Subsequently, streptomycin sulfate was added to the supernatant to a final concentration of 10 mg/mL. The mixture was stirred for 15 min at 4 °C followed by further centrifugation at 19500g. Next, ammonium sulfate was added to the supernatant to a concentration of 360 mg/mL in order to precipitate the protein. The solution was stirred for 30 min at 4 °C and centrifuged again at 19500g. The resulting pellet was resuspended in 25 mM Tris-HCl, pH 7.7, and loaded onto an anion exchange column (HiTrap Q Sepharose high performance, GE Healthcare) and eluted with a 0–600 mM NaCl step gradient. The eluted fraction (~300 mM NaCl) containing purified αS was dialyzed into ddH2O, filtered through a 3.2 mm rotor, and loaded into a 50 kDa Amicon filter to remove monomers, and subsequently split into two equal aliquots. One solution was added gHSA to a molar ratio of 10:1 (αS/gHSA) and to the other an equivalent volume of PBS. The mixtures were incubated for a further 2 h at 37 °C to allow gHSA remodeling of αS oligomers, subsequently washed frozen using liquid nitrogen. A 200 μL aliquot of each sample was loaded onto a Superdex 200 Increase 10/300 GL SEC column for characterization of their relative size distributions (Figure S4a).

### Human Serum Albumin Stock Preparation

Fatty acid and globulin free human serum albumin (rHSA; Sigma-Aldrich A3782) and globulin free human serum albumin (gHSA; Sigma-Aldrich A8763) were purchased from Sigma-Aldrich as lyophilized powders. The lyophilized powders were resuspended in PBS pH 7.4 or 50 mM HEPES pH 7.4 to match alpha synuclein conditions. The concentration of αS (monomer equivalent) was determined by A280 measurements using an extinction coefficient of 5600 M\(^{-1}\) cm\(^{-1}\).\textsuperscript{51}

### Cellular Viability Probed through Presto Blue Assay

SH-SY5Y cells in 1:1 Dulbecco’s modified eagle medium (DMEM)/F12 medium supplemented with 10% fetal bovine serum (FBS) and RPE-1 cells in 1:1 DMEM/F12 medium, 10% FBS, and 0.01 mg/mL hygromycin were seeded onto a 96-well plate (10 000 cells in 180 μL) and incubated for 24 h at 37 °C, 5% CO\(_2\). The media was then aspirated, and cells were treated with preformed αS oligomers (5 μM final concentration), mock (PBS delivery solution), and αS oligomers in the presence of rHSA/gHSA (10 μM final concentration) dissolved in fresh media. The cells were then incubated for 48 h at 37 °C and 5% CO\(_2\). The media was aspirated and replaced with fresh media containing the Presto Blue reagent (resazurin), and the plate was incubated for a further 2 h at 37 °C, 5% CO\(_2\). Fluorescence measurements were acquired using excitation and emission wavelengths of 560 and 590 nm, respectively, using a Biotek Cytation 5 plate reader. The error on these measurements was estimated through the standard error of five and seven technical replicates for RPE1 and SH-SY5Y cells, respectively.

### Biolayer Interferometry for the Assessment of HSA Binding Affinities to αS Monomers and Oligomers

The binding affinities of αS monomers and oligomers for rHSA and gHSA were assessed by biolayer interferometry measurements (Octet Red 96, ForteBio). Biotinylation of αS significantly perturbed αS aggregation,\textsuperscript{19} and hence prolonged incubation at 37 °C was used to form αS oligomers. Specifically, 800 μM biotinylated αS monomers in PBS pH 7.4 were incubated at 37 °C for 1 month to form oligomers. In contrast, freshly prepared biotinylated αS was used for monomer binding experiments. The αS monomers and oligomers were immobilized on Streptavidin (SA) biosensors (ForteBio) by dipping the sensor into a solution of 100 μM biotinylated αS (monomers or oligomers) for 300 s. Excess nonimmobilized αS was washed off by dipping the sensor into PBS pH 7.4 for 120 s. The SA biosensor was subsequently dipped into
solutions of rHSA or gHSA in PBS pH 7.4 at varying concentrations ranging from 0.25 μM to 600 μM for αS monomers and 0.25 μM to 10 μM for αS oligomers for 900 s to allow for association. Dissociation was subsequently monitored by dipping the biosensor in PBS pH 7.4 for 900 s. The association and dissociation curves were fit against a heterogeneous ligand binding model, assuming multiple independent ligand binding sites, as found in the Octet analysis program provided by ForteBio to derive the effective $K_{\text{app}}$ values of the complexes formed by the αS (monomer and oligomers) with rHSA and gHSA.

15C-Oleic Acid Competition. 15C-Methyl-labeled OA was purchased from Cambridge Isotope Laboratories as hydrolyzed powder and resuspended in 100% DMSO-d6 to a final concentration of 100 mM. The 100 mM 15C-OA stock was incubated at 50 °C for 5 min prior to co-incubation with rHSA. Similarly, the rHSA was preincubated for 30 min at 37 °C. Following this preincubation period, the 15C-OA and rHSA solutions were mixed to a final concentration of 250 μM rHSA and 1.5 mM 15C-OA and further incubated for 2 h at 37 °C. To probe the competition of ANS and Dan F with 15C-OA, the two fluorophores were added from their DMSO-d6 stocks to two separate 15C-OA-containing mixtures to a final concentration of 500 μM prior to the 2 h incubation period at 37 °C. At the end of the 2 h incubation period, 1H−15C HSQC NMR spectra were acquired for the three samples, i.e., rHSA/13C-OA, rHSA/15C-OA/ANS, and rHSA/15C-OA/Dan F, to assess the degree and sites of competition (further details in the NMR section below).

To probe the competition of αS monomers with 15C-OA, a similar protocol to the ANS and Dan F fluorescence was used with a few exceptions, which are detailed below. Given that the affinity of αS monomers is significantly weaker compared to ANS and Dan F, a reduced concentration of rHSA and 15C-OA was used, while keeping the molar ratio of rHSA/15C-OA constant at 1:6, i.e., 125 μM rHSA and 750 μM 15C-OA. The alpha synuclein monomer was then added as hydrolyzed powder to the rHSA/15C-OA sample to a final concentration of 500 μM prior to further incubation for 2 h at 37 °C. Prior to NMR data acquisition. Importantly, a 1H−15C HSQC spectrum was acquired for the rHSA/15C-OA sample prior to αS monomer addition to serve as the reference spectrum for the competition.

15C-Oleic Acid for the NMR-Based Assessment of Albumin-Bound LCFA Concentration (CONFAB). Similar to above, the 100 mM 15C-OA stock was incubated at 50 °C for 5 min prior to co-incubation with gHSA. Similarly, the gHSA was preincubated for 30 min at 37 °C. Following this preincubation period, the 15C-OA and gHSA samples were mixed to a final concentration of 500 μM gHSA and 500 μM 15C-OA and further incubated for 2 h at 37 °C. The [15C-FA]/gHSA ratio ($r$) was determined according to eq 1:

$$r = (\alpha - \Delta v_{AB})/\beta$$

where $\alpha = 71.28\pm 0.235$ and $\beta = 1.8697 \pm 0.1057$ and $\Delta v_{AB}$ is the 1C chemical shift difference in Hz between 1C-OA HSA-bound peaks A and B scaled to 700 MHz (i.e., $\Delta v_{AB,700\,\text{MHz}} = \Delta v_{AB,850\,\text{MHz}} \times 700/850$). Further details of the CONFAB method have been described previously.

1-Anilino-8-naphthalenesulfonate and Dansyl Phenylalanine Fluorescence Competition. ANS and Dan F were diluted from their DMSO stock solutions into 50 μM rHSA in PBS pH 7.4 to a final concentration of 50 μM. These concentrations are greater than the $K_v$ of each fluorophore for rHSA, and hence a significant fraction is expected to be bound. The final concentrations of DMSO in the samples were less than 0.1% and had no appreciable effect on the measurements (data not shown). Fluorescence measurements were taken using a Biotek Cytation 5 plate reader using excitation and emission wavelengths of 400, 465, and 370, 480 nm for ANS and Dan F, respectively. Fluorescence competition was assessed by the relative reduction in fluorescence intensity upon addition of either freshly dissolved αS monomer or preformed αS oligomers. The final concentration of αS monomers was 500 μM, and the concentration of αS oligomers (monomer equivalent) was 50 μM for type I (100 μM αS, 48 h at 37 °C) and 200 μM for type II (800 μM αS, 24 h at 37 °C). The final concentration of aggregated αS (800 μM αS, 240 h at 37 °C) was 200 μM. Fluorescence reduction arising from the direct binding of ANS or Dan F to αS monomers or oligomers is accounted for by subtracting the fluorescence intensity in concentration-matched αS monomer or oligomer samples in the presence of ANS or Dan F.

Dynamic Light Scattering. The samples used for DLS were prepared using two protocols. First, hydrolyzed αS powder was resuspended in buffer or in the presence of 50 μM rHSA or gHSA to a final concentration of 100 μM. The resulting mixtures were then incubated for 48 h at 37 °C to initiate oligomer formation. In the second protocol, the 100 μM αS was preincubated for 48 h at 37 °C to form the oligomers. rHSA, gHSA, or an equivalent volume of buffer to account for dilution by HSA was then added to three aliquots of this preincubated oligomeric sample and allowed to further incubate for 24 h at 4 °C. The αS alone and αS/rHSA and αS/gHSA samples prepared from the two protocols were then subjected to DLS measurements. DLS was performed using a Zetasizer Nano ZS Instrument (Malvern Instruments, Malvern, UK). Autocorrelation functions were accumulated for 2 min at 10 °C with an angle θ of 173° and a 4 mW He−Ne laser operating at a wavelength of 633 nm. All measurements were performed using a 40 μL (ZEN0040) plastic cuvette. The particle diameter detection limit was 0.6−6 μm. The viscosity value for water was used in the analysis of all measurements. All the samples were centrifuged for 10 min at 13 000 rpm prior to DLS measurements.

Negative Stain Transmission Electron Microscopy. The samples used for TEM matched those used for the postaggregation DLS protocol above. The DLS reaction mixtures were diluted 100-fold with ddH2O. Copper EM grids (400-mesh), which had been freshly coated with a continuous layer of amorphous carbon, were glow discharged with a 5 mA current for 15 s, and shortly afterward the grids were floated on 3 μL drops of the diluted assembly reaction mixtures for 2 min. Excess of sample was blotted off with filter paper, and the grids were stained with 1% uranyl acetate for 30 s. Grids were loaded in a room-temperature holder and introduced into a JEOL 1200-EX electron microscope operated at 80 kV. All images were acquired with an AMT XR-41 side-mount cooled 4 megapixel CCD camera.

Preparation of DOPE/DOPS/DOPC Lipid Films. SUVs were prepared as described before. Briefly, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-sn-glycero-3-phospho-l-serine (DOPS), and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) were purchased from Avanti Polar Lipids. The lipids were stored at −20 °C, under argon. Solutions in chloroform were prepared from the lipids and were mixed to result in a 5:3:2 lipid molar ratio. The solvent was then evaporated under a stream of nitrogen gas, and the sample was dried thoroughly under vacuum to yield a thin lipid film on the wall of a glass test tube.

Preparation of DOPE/DOPS/DOPC Small Unilamellar Vesicles (SUVs). The thin film was rehydrated with 50 mM HEPES, 1.5 mM Tris pH 6.8, and 10% D2O at a concentration of 15 mg/mL and subjected to vortex mixing at room temperature to form multilamellar vesicles (MLVs). The MLVs were subsequently sonicated in a Cole-Palmer bath type ultrasonic cleaner until a clear solution was obtained, indicating the formation of SUVs. The concentration of total phospholipids was confirmed by measuring the amount of inorganic phosphate released after digestion.

Membrane Permeabilization Measurements. Lipid films were suspended in a calcine solution (50 mM calcine, 20 mM HEPES, pH 7.4) by vigorous vortexing to yield MLVs. In order to form LUVs, the MLVs were then extruded by 11 passages through two stacked polycarbonate membranes (100 nm pore size; Nucleopore Filtration Products, Pleasanton, CA, USA) in a barrel extruder (Lipex Biomembranes, Vancouver, BC). External calcine was removed in a CL-2B Sepharose column by elution with 20 mM HEPES, pH 7.4, and 370 mM NaCl, which had the osmositivity matching the calcine solution as measured by a cryo-osmometer (Advanced Model 3 Plus micro-osmometer, Advanced Instruments Inc., Norwood, MA, USA). The calcine-loaded LUVs were collected in the column void volume, had their concentration determined as above, and were kept at 4 °C until use. Alpha synuclein oligomer-induced calcine dye leakage was...
measured in a Biotek Cytation 5 plate-reader at 37 °C using calcein fluorescence excitation and emission wavelengths of 490 and 525 nm, respectively. Lipid concentration of calcein-containing LUVs was kept constant at 20 μM. Mock (PBS pH 7.4) and 1% (v/v) Triton X-100 were used as negative and positive controls, respectively. The liposomes were treated with 40 μM rHSA, 40 μM gHSA, rS αs oligomers (20 μM final concentration), and rS αs oligomers in the presence of rHSA/gHSA (20 μM rS αs oligomers and 40 μM HSA final concentration).

Liposome permeabilization was quantified using eq 2:

$$\text{Permeabilization} = \frac{(F_0 - F_T)}{(F_T - F_B)} \times 100$$

where $F_0$, $F_B$, and $F_T$ are respectively the fluorescence intensity after addition of treatment, buffer negative control, and Triton X-100 positive control.

**Wide-Angle X-ray Diffraction.** Solutions of 800 μM αs were aggregated in PBS buffer pH 7.4 in the absence or presence of 1600 μM rHSA or gHSA over 48 h. Membranes were prepared with 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-sn-glycerol-3-phospho-(1′-rac-glycerol) (POPG) at a 1:1 molar ratio, ensuring a net negative membrane charge while retaining a fluid structure. The αs and αs/HSA solutions were then mixed with the membranes to ensure a protein/lipid ratio of 1:50.

Polished silicon wafers (1 x 1 cm)2 were incubated in piranha solution, H2SO4/H2O2 (7:3, vol/vol %), to prepare a hydrophilic surface. The solutions were then deposited on the wafers and annealed at 37 °C for 2 h in an orbital shaker to ensure full coverage of the wafers and formation of highly oriented membranes on the surfaces. The samples were then hydrated at 97% RH in a humidity chamber for 48 h prior to acquisition of the X-ray diffraction data at a temperature of 30 °C.

Out-of-plane X-ray scattering data were obtained using BLADE at McMaster University. BLADE uses a 9 kW (45 kV, 200 mA) Cu Kα rotating anode at a wavelength of 1.5418 Å. Both source and detector are mounted on moveable arms such that the membranes stay horizontal during measurements. Focusing, multilayer optics provide a high-intensity collimated, 200 μm sized beam with monochromatic X-ray intensities up to 108 counts/s. Scattering was detected using a Rigaku HyPix-3000 2D semiconductor detector with an area of 3000 mm2 and 100 μm pixel size.51

Electron density profiles were determined from specular reflectivity. The relative electron density was calculated as previously described.52 The membrane electron densities were then normalized to the peak phosphate density, while holding the density of the bilayer center fixed, as previously described.53

**Size Exclusion Chromatography Coupled with Multitangle Light Scattering.** SEC-MALS measurements were conducted on a Wyatt miniDAWN MALS detector coupled to a Wyatt Optilab rEX online refractive index detector. αs samples (100 μL injection volume), prepared as described above, were resolved using a Superdex 200 increase 10/300 analytical gel filtration column (GE Healthcare) running at 0.5 mL/min in PBS buffer before passing through the light scattering and refractive index detectors in a standard SEC-MALS format.

Protein concentration was determined from the excess diﬀusion coefficient of the wafers and formation of highly oriented membranes on the surfaces. The samples were then hydrated at 97% RH in a humidity chamber for 48 h prior to acquisition of the X-ray diffraction data at a temperature of 30 °C.

**General Solution NMR Spectroscopy.** All solution NMR spectra were recorded using either a Bruker AV 700 spectrometer equipped with a TCI cryo-probe or a Bruker 800 HD spectrometer equipped with a TXI room-temperature probe. All spectra were analyzed with TopSpin 3.2.1, NMRpipe, and Sparky using Gaussian line-fitting. Additional details are discussed below.

**H NMR to Probe Alpha Synuclein Self-Association.** αs was freshly dissolved in 50 mM HEPES pH 7.4/10% D2O to a final concentration of 200 μM in the absence and presence of 50 or 400 μM rHSA and gHSA. The samples were then subjected to a temperature gradient ranging from 10 to 30 °C at 5 °C intervals with a 30 h incubation at each temperature. 1H NMR spectra were acquired at both the beginning and end of the 30 h incubation period. 1H NMR spectra were recorded with 128 scans, 64K points, and a spectral width of 20.00 ppm. A 30 ms spinlock was introduced to minimize contributions arising from HSA. The normalized average intensities of three protons in the H8 region (shown in Figure S3b) were used to evaluate the monomer population at each temperature relative to the starting temperature of 10 °C. We selected the NMR signals arising from H8 protons, because for the NH protons the intensity losses at increasing temperatures are amplified by the exchange with water, while for the methyls the intensity losses upon heating are minimized by increased dynamics (Figure S3b).

**H-15N HSQC to Probe Competition with 13C-OA Binding Sites in HSA.** The samples for the 13C-OA competition experiments, prepared as described above, were monitored by H-15N HSQC NMR. Two-dimensional NMR spectra were recorded with a recycle delay of 1.4 s, 32 scans, and 2K (t1) and 200 (t2) complex points for spectral widths of 13.9 ppm (1H) and 31.8 ppm (15N), respectively. NMR experiments were acquired at 298 K. Chemical shift and intensity changes were measured through Gaussian fitting of the peaks in Sparky.

**H-15N HSQC to Probe HSA and Membrane Binding Sites in Alpha Synuclein.** HSA and membrane binding to αs was probed by losses in H-15N HSQC intensities upon addition of HSA or membrane relative to a sample of αs alone. H-15N HSQC experiments were recorded at 283 K with a recycle delay of 1.0 s, 16 scans, and 2K (t1) and 300 (t2) complex points for spectral widths of 14.05 ppm (1H) and 31.82 ppm (15N), respectively.

**Methyl STDHSQC to Probe the HSA Binding Sites in Alpha Synuclein Monomers and the Binding Affinity of HSA—Alpha Synuclein Monomer Interactions.** Methyl STDHSQC (MeSTDHSQC) spectra were acquired for alpha synuclein monomers with and without rHSA or gHSA. Briefly, saturation was introduced through methyl spins (resonances ν1 = 33.33 ppm) of adamantane at 25 kHz with a 90% to 100% linear ramp. The spectra were recorded with 64 scans and 2K (t1) and 200 (t2) complex points for spectral widths of 14.1 ppm (1H) and 31.8 ppm (15N), respectively. Reference (STRHSQC) spectra with far-off-resonance saturation (21 000 Hz at 700 MHz) were also recorded but with only 16 scans. The residue-specific STD vs STR ratios were set to 33.33 and 3.33 kHz, respectively. In the DUMAS experiment, during the SIM-CP (simultaneous cross-polarization) preparation period, 13C and 15N RF amplitudes were set to 35 kHz, whereas 60 kHz 1H RF was applied with a 90° to 100% linear ramp. The spectra were processed in an NMR pipe and analyzed using Sparky. Spectral resolution of the DARR spectrum was improved by using TIDE (T,8 weighted deconvolution) covariance processing with customized MATLAB scripts,55 and the spectra are shown in Figure S4b—g. NCA spectra were not reported due to poor resolution. Spectra were externally referenced to the most downfield CH resonance (29.46 ppm) of adamantane at 25 °C.56 The fibril DARR spectrum was simulated from chemical shifts deposited in BMRB (ID: 18207) using an in-house python tool, sim-sparky, freely available at https://github.com/weberdak/sim-sparky. The spectrum was simulated using artificial line widths of 200 Hz and 1k points in the direct and indirect dimension.
then computed to map the HSA binding sites in αS monomers. Residual contributions arising from the direct saturation of the αS monomers were accounted for by subtracting from the αS-HSA MeSTDHSQC/STRHSQC intensity ratios the corresponding MeSTDHSQC/STRHSQC intensity ratios for a sample containing αS alone.

\(^{13}\text{N}−\text{DEST}\) to Probe the Interaction of αS with Membranes, HSA, and Their Complexes. The \(^{13}\text{N}−\text{DEST}\) experiment was implemented as described previously. Briefly, a 900 ms \(^{13}\text{N}\) continuous wave (CW) saturation pulse was applied at 16 different radio-frequency offsets (no saturation, \(-28, -21, -14, -9, -5, -3, -1, 0, 1, 3, 5, 9, 14, 21,\) and 28 kHz) with a field strength of 170 Hz. The experiment was recorded in interleaved mode with 16 scans, 128 dummy scans, a recycle delay of 1.20 s, 200 (t1) and 2K (t2) complex points, and spectral widths of 14.28 ppm (\(^1\text{H}\)) and 31.82 ppm (\(^{15}\text{N}\)). All spectral processing was implemented in TopSpin 3.2.1. and transferred to Sparky for peak intensity measurements. The Gaussian fitting function in Sparky was used to determine the fitted peak heights, and the signal-to-noise ratio was used as a measure of error for the fitted peak heights. The residue-specific \(^{13}\text{N}−\text{DEST}\) ratio was calculated as \((I_{+} + I_{-})/(I_{-28} kHz + I_{28} kHz)\) where I denotes the peak height measured for a given residue at the \(^{15}\text{NC}\) Wo.

### ASSOCIATED CONTENT

**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.0c01894.

ANS and Dan F binding sites in HSA, DARR controls, rHSA DEST profiles, HSQC and STDHSQC controls (PDF)

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