Small-angle neutron scattering reveals the assembly of alpha-Synuclein in lipid membranes

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Abstract

The aggregation of α -synuclein (asyn), an intrinsically disordered protein (IDP), is a hallmark in Parkinson's disease (PD). We investigated the conformational changes that asyn undergoes in the presence of membrane and membrane mimetics using small-angle neutron scattering (SANS). In solution, asyn is monomeric and unfolded assuming an ensemble of conformers spanning extended and compact conformations. Using the contrast variation technique and SANS, the protein scattering signal in the membrane-protein complexes is selectively highlighted in order to monitor its conformational changes in this environment. We showed that in the presence of phospholipid membranes asyn transitions from a monodisperse state to aggregated structures with sizes ranging from 200 – 900 Å coexisting with the monomeric species. Detailed SANS data analysis revealed that asyn aggregates have a hierarchical organization in which clusters of smaller asyn aggregates assemble to form the larger structures. This study provides new insight into the mechanism of asyn aggregation. We propose an aggregation mechanism in which stable asyn aggregates seed the aggregation process and hence the hierarchical assembly of structures. Our findings demonstrate that membrane-induced conformational changes in asyn lead to its heterogeneous aggregation which could be physiologically relevant in its function or in the diseased state.

Introduction

Intrinsically disordered proteins (IDPs) are a unique class of highly flexible proteins. Their dynamic ensemble of interconverting conformations enable them to bind to different binding partners [1]. Most of these proteins present a coupled binding and folding mechanism as they undergo a transition from a disordered state to a folded conformation upon interaction with binding partners [2]. They have gained attention in structure-function studies because they are involved in many disease-related signaling pathways which make them a target for drug design and therapies [3].

α -synuclein (asyn) is a 140-amino acid IDP that is highly abundant in the brain particularly in the presynaptic terminals and in smaller quantities in other tissues [4, 5]. It has a N-terminal region that binds to membranes, a hydrophobic central domain called the non-A β -amyloidogenic component (NAC), which has a critical role in the aggregation process, and a negatively-charged C-terminal region [6, 7]. Although its physiological function is not fully understood, its localization in the presynaptic termini and its association with membranes suggest possible functions in presynaptic signaling, vesicular membrane trafficking, synaptic behavior and oxidative stress regulation [8]. Its accumulation from the cytosol to the nerve terminals during brain development supports its possible role in neuronal development and synaptogenesis. Studies have indicated a physiological neuroprotective function of asyn at the synapse by modulating Soluble *N*-ethylmaleimide-sensitive factor Attachment Protein Receptor (SNARE)-complex assembly [9].

The early onset of Parkinson's disease is a result of missense mutations (A30P, E46K, A53T, G51D, H50Q and A53T), gene duplication and triplication of asyn [10-13]. Insoluble and fibrillar forms of asyn are the major component in the Lewy bodies in PD, in the Alzheimer's

disease senile plaques, in the glial and neuronal inclusions of multiple system atrophy (MSA), and in dementia with Lewy bodies (DLB) [10, 14-16]. The diseases which share the aggregation and deposition of asyn as the pathological feature are collectively known as synucleinopathies [17]. There has been substantial controversy about comparative cytotoxic effects of different asyn species that range from oligomers to mature fibrils. *In vivo* and *in vitro*, different sizes of oligomers were observed to coexist with the monomers and fibrils but there has been no clear explanation on how the oligomeric species are formed [18]. Although there is evidence showing that the fibrillar form of asyn constitutes the toxic species in these neurodegenerative diseases, numerous studies have also pointed to the existence of pre-fibrillar oligomeric asyn as the main toxic species [19, 20]. There is speculation that the oligomeric species act as intermediates between the monomeric soluble protein and the fibrils [20, 21].

In the presynaptic termini, asyn exists free and in plasma membrane or vesicle-bound forms [22]. asyn has been found to interact strongly with crude brain vesicles, cellular membranes and synthetic anionic phospholipid vesicles [22-24]. The most compelling evidence of asyn binding to membranes is its conformational change from an unfolded state to predominantly α -helical conformation [25, 26]. Nuclear Magnetic Resonance (NMR) and Electron Paramagnetic Resonance (EPR) spectroscopy indicate that the N-terminal region of asyn associated with micelles or membrane is an amphipathic helix, while the C-terminal remains unstructured [25, 27-30]. Neutron reflectometry and fluorescence studies show the penetration of asyn in the outer leaf of the phospholipid head groups to approximately 9-14 Å [31]. Hellstrand and co-workers reported the co-aggregation of asyn with anionic lipid membranes [32] while Varkey and co-workers reported the remodeling of vesicles and the formation of lipoprotein nanoparticles with asyn [33]. All these studies suggest that membrane

binding is relevant to the physiological function of asyn. In order to understand the function or pathology of asyn, it is important to elucidate the molecular events that occur when it associates with membranes and the factors that either stabilize asyn or increase its propensity to aggregate in the membrane.

We investigated how asyn transitions from a monodisperse disordered protein in solution to folded, aggregated structures when it is exposed to membrane-like environments using small-angle neutron scattering (SANS). Detailed analysis of the SANS data reveals a hierarchical assembly of asyn in membrane-like environments in which monomeric asyn species coexist with heterogeneous, oligomeric lipid-bound aggregates. We present a model of membrane-induced asyn aggregation based on these findings.

Materials and Methods:

Sample preparation and characterization

1-palmitoyl-2-oleoyl-sn-glycero-3-phosphate (POPA, 110616P), and 1-hexadecanoyl-2--(9Z-octadecenoyl)-sn-glycero-3-phospho-L-serine (POPS, 840034P) were purchased from Avanti Polar Lipids, Inc. D₂O (99.8%) was obtained from Cambridge Isotopes and deuterated sodium dodecyl sulfate (SDS d-25, 451851) was purchased from Sigma-Aldrich. All reagents were used as received. Expression and purification of recombinant asyn in *E. coli* BL21(DE3) was performed as previously described [34] with the addition of gel filtration in the final purification step. The protein was identified by Western blot analysis using mouse monoclonal anti- α -syn antibody 4D6 (Santa Cruz Biotechnology) as the primary antibody and horse radish peroxidase-conjugated anti-mouse IgG as the secondary antibody (Santa Cruz Biotechnology). Chemiluminescence detection was carried out using ChemiDoc XRS system (GE Healthcare). A

MALDI-TOF mass spectrometer (Bruker Daltonics Autoflex II) was used to determine the molecular weight of the purified protein. The purity of the protein was determined by SDS-polyacrylamide gel electrophoresis (PAGE). Amicon Ultra-4 centrifugal filter units (Millipore, 3 kDa MWCO) were used to concentrate the protein and H₂O/D₂O buffer exchange was carried out by dialysis using slide-a-lyzer mini dialysis devices (Thermo Scientific, 3.5 kDa MWCO). Protein concentration was determined spectrophotometrically using the extinction coefficient, $\epsilon_{280\text{nm}}=5120 \text{ M}^{-1} \text{ cm}^{-1}$ [35] using a Shimadzu UV-2700 UV-visible spectrophotometer. Analysis of the secondary structure of asyn was determined using a JASCO J-810 spectropolarimeter. The spectra were recorded with a scan speed of 100 nm/min, 0.25 s response time, and a bandwidth of 1nm from 190–250 nm. Each spectrum presented is an average of 5 scans. The secondary structure content of the samples was quantified using the CD Pro program with the CONTIN method and STR43 basis set [36]. In addition, the spectra were also analyzed using the following equation;

$$f_h = \frac{[\theta]_{222,\text{meas}} - [\theta]_{222,\text{c}}}{[\theta]_{222,\text{h}} - [\theta]_{222,\text{c}}} \quad (1)$$

where f_h is the helical content, $[\theta]_{222,\text{meas}}$ is the measured mean residue ellipticity at 222 nm; $[\theta]_{222,\text{c}} = 3000 \text{ deg cm}^2 \text{ dmol}^{-1}$, for a fully random coil, and $[\theta]_{222,\text{h}} = -36,000 \text{ deg cm}^2 \text{ dmol}^{-1}$, for fully α -helical conformation [37].

Thioflavin-T (ThT) fluorescence measurements were performed using the FluoroLog-3 (Jobin Yvon) spectrofluorometer. Emission spectra were obtained by excitation at 440 nm and recording the emission from 460-600 nm with excitation and emission slit widths, 2.5 nm and 5.0 nm, respectively. Lipid vesicles were prepared from 30-50 mM stock solutions of 1:1 molar POPA:POPS. The lipid films were briefly hydrated in 20 mM NaHPO₄, 100 mM NaCl, pH 7.2 and vortexed before undergoing several freeze-thaw cycles between -80 °C and 40 °C. The small

unilamellar vesicles (SUVs) of 60-70 nm radius were prepared using an Avanti mini-extruder fitted with a 100 nm diameter pore size polycarbonate filter. Dynamic light scattering using DynaPro NanoStar (Wyatt) was used to confirm the hydrodynamic sizes of the vesicles. Appropriate solutions of the protein and lipid vesicles were mixed to achieve the desired protein to lipid ratios at 15% (v/v) D₂O. Asyn fibrils were formed by incubation of 274 μ M asyn at 37 °C with stirring (450 rpm) in a Thermomixer C (Eppendorf). Fibril formation was monitored using circular dichroism (CD) spectroscopy.

Small angle neutron scattering

SANS measurements were conducted at the Bio-SANS instrument at the High-Flux Isotope Reactor, Oak Ridge National Laboratory (Oak Ridge, TN) [38]. The three different instrument configurations employed to cover the range, $0.003 < q \text{ (\AA}^{-1}\text{)} < 0.7$, of scattering vectors with sufficient overlap were sample-to-detector distances of 1.13 m, 6.83 m and 15.33 m at a wavelength of 6 $\text{\AA}$. The scattering vector q ($q=4\pi\sin\theta/\lambda$) describes the relation of q to λ (neutron wavelength), and 2θ , the scattering angle. The center of the moveable area detector (1 m x 1 m GE-Reuter Stokes Tube Detector) was offset by 350 mm from the beam center to cover the required q range. The wavelength spread $\Delta\lambda/\lambda$ was set to 0.15 by a neutron velocity selector. The scattering intensity profiles $I(q)$ versus q , were obtained by azimuthally averaging the processed 2D images, which were normalized to incident beam monitor counts, and corrected for detector dark current, pixel sensitivity and scattering from the quartz cell. High concentration asyn samples (274 μ M) were measured in 1-mm path length cylindrical quartz cuvettes (Model# 120-QS 1.0 mm, Hellma USA) while low concentration samples (56 μ M) were measured in 5-mm path cells (Model# 120-QS 5.0 mm, Hellma USA) at 22 °C to achieve the same effective

amount of protein in the beam. Contrast match experiments were performed at buffers of 15% D₂O and 100% D₂O the contrast match points for hydrogenated lipids [39] and deuterated SDS, respectively [40]. Samples of asyn in solution were measured in 100% D₂O buffer. The final scattering curves were background corrected using the corresponding buffer solutions. The experiments were conducted at 22 °C with each measurement for 2 hours at 2 instrument configurations (4 hours total for each sample).

Data fitting and analysis:

The Debye equation and Ensemble Optimization Method (EOM) from ATSAS suite [41] were used for the determination of the radius of gyration, R_g values. The form factor for evaluating Gaussian chain has been known to be more accurate for flexible molecules such as IDPs [42] which is given by the Debye scattering function [43, 44]:

$$\langle g_D(x) \rangle = \frac{2}{x^2} [e^{-x} - (1-x)] \quad (2)$$

where, $x = q^2 R_g^2$. For a random Gaussian coil chain conformation,

$$R_g^2 = \frac{Nl_p^2}{3} = \frac{Ll_p}{3} \quad (3)$$

where, the R_g , was evaluated from the Debye-Bueche fitted to the SANS curve, . Since the total length of the fully extended protein chain with 140 amino acids, $L = 140 \times 3.63 = 508.2 \text{ \AA}$ [45], is fixed, $R_g \sim \sqrt{l_p}$, provides a methodology to be able to evaluate an average statistical step length, called the persistence length, l_p , as [46]:

$$l_p = \frac{3R_g^2}{L} \quad (4)$$

The average number of amino acid residues in each persistent length, n_p , in each step is given by:

$$n_p = \frac{l_p}{l} \quad . \quad (5)$$

where, $l = 3.63\text{\AA}$ is the length of a single amino acid. The persistence length can be viewed as a quantitative measure of the extent of chain folding where a longer persistence length may be inferred as a less folded protein. n_p can also be viewed as a measure of chain folding which means that a lower n_p reflects a comparatively folded protein structure.

In addition to the determination of R_g , EOM is also used to evaluate the maximum distance (D_{max}) of asyn in solution. Kratky plot ($q^2 I$ vs q) is commonly used to infer the presence of proteins in disordered state. Typically, folded proteins exhibit a bell-shaped curve in Kratky. For IDPs, a monotonic increase is expected with the absence of a clear maximum.

For the protein-SDS and protein-lipid complexes, the Unified Fit model was used to fit the scattering intensity using three structural levels [47-52].

$$I(q) = \frac{1}{q^3} \left\{ G_3 e^{-\left(q^2 R_{g3}^2\right)/3} + B_3 e^{-\left(q^2 R_{g2}^2\right)/3} \left(q_3^*\right)^{-4} \right\} + \frac{1}{q^3} \left\{ G_2 e^{-\left(q^2 R_{g2}^2\right)/3} + B_2 e^{-\left(q^2 R_{g1}^2\right)/3} \left(q_2^*\right)^{-d_{f2}} \right\} + \frac{1}{q^3} \left\{ G_1 e^{-\left(q^2 R_{g1}^2\right)/3} + B_{f1} \left(q_1^*\right)^{-d_{f1}} \right\} \quad (6)$$

$q_i^* = q / \left\{ \text{erf} \left(q k_{sc} R_{g,i} / \sqrt{6} \right) \right\}^3$, $k_{sc} \gg 1.06$, and erf is the error function. The terms in the first curved bracket with subscript, 3, represent the aggregate regime with smooth surfaces, the second bracket with subscript, 2, represent the second fractal scaling regime and the third bracket with subscript, 1, represents folded asyn cluster regime. G_i 's are the prefactors for the Guinier regimes in each structural level and they correspond to the I_0 , in the Guinier law. B_i 's are the

power law prefactors for each level. R_{gi} 's are the radii of gyration for each level. The number of smaller sized particles from lower, $(i - 1)^{th}$ level, in an average successive larger structure (higher i^{th} level) is determined by the ratio of successive Guinier prefactors, $z_i = G_i/G_{i-1}$ [48, 49].

Results

Helical conformation of asyn increases significantly with lipid and SDS

Recombinant asyn was over-expressed in *E. coli* and purified with minor modification to a previously described procedure [34]. Size exclusion chromatography (SEC) was used to remove high molecular weight contaminants and the lyophilization step was omitted because preliminary SANS measurements indicated that after lyophilization the reconstituted protein showed signs of aggregation (see Fig. S1). In subsequent measurements, freshly prepared protein was used for SANS measurements. The mass of the purified protein was ~44 kDa, as determined using SEC, which is consistent with other studies and has been ascribed to its natively unfolded conformation in physiological buffer [53]. The addition of chemical denaturants or boiling of asyn samples did not change asyn migration in SEC [33]. The single band in the SDS-PAGE gel that corresponded to asyn migrated more slowly than expected resulting in a calculated mass of 18 kDa (Fig. 1a). This is consistent with previous studies [5] and with other studies of disordered proteins [54]. Western blot analysis confirmed that the purified protein was asyn (Fig. 1b).

Figure 1. (A) SDS-PAGE of purified asyn at different concentrations; (B) Western blot analysis of the purified asyn; (C) The far UV-CD spectra of 274 μ M asyn (blue), with SDS (red), with POPA-POPS (green) and asyn fibrils (yellow) at pH 7.2.

The far-UV CD was used to monitor the secondary changes in asyn upon binding with SDS and lipid membranes. In solution, asyn shows a spectrum typical for an unfolded protein that has a characteristic minimum around 196 nm (Fig. 1c) indicating a random coil secondary structure. The CD spectrum changes in the presence of SDS or with POPA-POPS. asyn folds into an α -helical conformation characterized by the presence of two minima around 208 nm and at 222 nm in the spectra. The α -helical content was estimated based on the ellipticity at 222 nm, (θ_{222}), using a method previously described for both globular proteins and IDPs [37]. In the sample with asyn only, the α -helical content is estimated to be approximately 14%. The α -helical contents increase to 51.4% in asyn-lipid and 54.1% in asyn-SDS samples, which is consistent with what has been reported in the NMR structure (PDB ID: 1XQ8) (Table 1) [29]. The CONTIN program [36], shows similar results with the α -helical content of asyn at 7.1%, asyn-lipid at 40.4% and asyn-SDS at 42.1% (Table 1). This indicates that asyn underwent major conformational changes in the presence of SDS and the lipid vesicles. No discernable

differences were observed between the CD spectra of asyn/ lipid or SDS complexes measured before and after the SANS experiments (see Fig. **S5**).

Thioflavin T (ThT) binding analysis is a common technique used to identify amyloid fibrils [55]. An increase in the ThT fluorescence along with a red shift in the maximum emission indicates binding of ThT to amyloid fibrils. The addition of ThT to asyn fibrils with predominantly β -sheet conformation (see Figure 2) resulted in a 24 fold increase in fluorescence intensity compared to purified asyn in aqueous buffer. In the case of the asyn-lipid complexes and asyn-SDS complexes, a 1.4 fold and 3.3 fold increase in fluorescence emission was observed, respectively, indicating binding of ThT but to a lesser extent compared to that observed for asyn fibrils. No changes in the ThT fluorescence were observed in samples measured before and after SANS measurements (Fig. **S6**).

Figure 2. The fluorescence emission spectra of asyn in the presence of ThT: ThT (gray), asyn (blue), asyn-SDS (red), asyn-POPA/POPS (green) and asyn fibrils (yellow) at pH 7.2.

Asyn is highly flexible and monomeric in solution

The solution structure of asyn in aqueous buffer was studied using SANS. The SANS intensity profiles for asyn measured at 56 μM and 274 μM are shown in Figure 3A. The data was fitted well with a Gaussian chain for flexible molecules using equation (2), yielding an R_g of 38 ± 1 Å and 34.7 ± 0.8 Å for the low and high concentration samples, respectively. The expected R_g for a 140-residue protein in random coil conformation is ~ 39 Å [56]. The R_g values that we obtained provide further evidence of the unstructured nature of the protein and are consistent with previously reported values of R_g of 40 ± 1 Å at neutral pH using SAXS [57, 58] and 39 ± 1 Å using SANS in solution [59]. Using equation (4), an average statistical persistence length, l_p , of 8.6 ± 0.6 Å and 7.1 ± 0.3 Å were evaluated for the 56 μM and 274 μM solutions, respectively, suggesting that the protein is in a slightly less folded state at lower concentration but nevertheless both values are in the range typical for a disordered protein [60]. Furthermore, the average number of amino acid subunits, n_p , in each length step given by equation (5), were 2.4 ± 0.2 and 1.96 ± 0.09 for the 56 μM and 274 μM solutions, respectively.

Table 1. Secondary structure analysis of asyn

Sample	Helical content (%)	
	A	B
asyn	14	7.1
asyn-SDS	54	42
asyn-lipid	51	40

Notes A[32], B[31]

It can be inferred from these evaluations that an average of 2.4 amino acids form a rigid step chain for 56 μM solution while the average number of amino acids in 274 μM is ~ 2 . In addition, the profile of the Kratky plot (q^2I vs q), as shown in Fig. 3B is consistent with an unstructured protein lacking characteristic feature of a maximum for a folded protein [61].

Figure 3. (A) SANS profiles of asyn at 56 μM and 274 μM concentrations. The data points at 56 μM are shown in open red triangles and 274 μM data points in open blue circles. The Debye fits are in red and blue lines for the 56 and 274 μM , respectively. (B) The Kratky plots of 56 μM (red, data offset by 0.1) and 274 μM (blue) asyn.

The unstructured nature of asyn in solution allows it to assume many different conformations, making it difficult to determine its structural parameters directly from the SANS profiles. EOM is widely used to obtain such structural parameters, R_g and D_{max} , from SANS and SAXS profiles of highly flexible and disordered proteins [62]. In this approach, a pool of random protein models is generated for which theoretical scattering curves are calculated. Theoretical scattering curves are compared to the experimental data to produce an ensemble of structures that results in a weighted average scattering curve that best fits the experimental data. Both concentrations of asyn gave good fits against the scattering data. For the low concentration asyn, the EOM profile exhibits a distribution of R_g with an average value of 33.1 Å (Fig. 4A) and a D_{max} average of 101 Å (Fig. 4D), which is reasonably close to the evaluated R_g value obtained from the Debye fit (38 ± 1 Å). These results were obtained from 50 conformers in the selected ensemble that give the best fit with a normalized chi value 0.971. The high concentration sample displayed a similar EOM profile (Fig. 4b). The average R_g was 33.2 Å (Fig. 4C) and average D_{max} was 104 Å (Fig. 4D). These values were obtained from 50 selected conformers with a fit that gives a normalized chi value of 0.976. The average R_g and D_{max} values for each profile fit are given in Table 2.

Table 2. Structural fit parameters of asyn in solution

Asyn	[56 μM]	[274 μM]
Guinier R_g ($\text{\AA}$) I_0 (cm^{-1})	38.1 ± 2.04 0.0097	37.9 ± 2.44 0.008
Gaussian R_g ($\text{\AA}$)	43.3 ± 1.7	41.8 ± 1.3
EOM R_g ($\text{\AA}$) EOM D_{max} ($\text{\AA}$)	45.4 136	41.8 133

There is a narrow distribution of the R_g and D_{max} values in both samples that are shifted towards more compact structures compared to the starting pool of conformation. A unimodal distribution indicates that there is little structural heterogeneity in the conformers. The R_g distribution of the asyn conformers are within the range of R_g values that Stultz and co-workers have reported using Replica Exchange Molecular Dynamics to analyze the Bayes ensemble of asyn in solution [63]. Overall, the EOM results confirm the disordered nature of asyn. Figure 5 shows the three structural models from the selected ensemble for the 274 μ M asyn.

Figure 4. EOM analysis of asyn in solution. Panels A and B show the R_g distribution of the selected ensemble (solid red) compared to the generated pool (dashed black) at low (A) and high (B) concentrations. Panels (C) and (D) show the size distribution (D_{max}) of the low and high concentration samples, respectively.

Asyn forms a hierarchical assembly in the presence of lipid or SDS

SANS was used to investigate the effect of detergents and lipids on the structure of asyn. This technique is ideally suited to studying multicomponent systems because it is possible to examine the structure of individual components in a mixture using the contrast match technique [64]. Figure 6A shows the scattering profile of asyn in the presence of SDS at 100% D_2O , the contrast match point of deuterated SDS (Fig. S4) [40]. Under these conditions the scattering profile is dominated by the protein signal. The protein to detergent molar ratio used was 1:70

because, it was previously reported that all asyn is bound to SDS under this condition [29, 65]

Figure 5. Representative structures of asyn in solution (274 μ M) obtained from EOM analysis; Purple (R_g 26.4 $\text{\AA}$, D_{max} 80.8 $\text{\AA}$), orange (R_g 32.4 $\text{\AA}$, D_{max} 101 $\text{\AA}$), and cyan (R_g 34.5 $\text{\AA}$, D_{max} 113 $\text{\AA}$).

The SANS profile of the asyn-SDS complex shows clear deviations from asyn only in solution (Fig. 6A). There is a monotonic increase in scattering intensity with decreasing q values, which indicates the presence of a large scattering particle that would be consistent with aggregated proteins. In addition, there is a ‘knee’ in the mid- q region (~ 0.006 - $0.015 \text{ \AA}^{-1}$) of the scattering profile, which suggests a mixture of different sized scattering objects in the system. The Unified fit model [21] was used to interpret the structural details of the sample on three different length scales. This approach is suited to interpreting the small-angle scattering profiles of hierarchical structures and has been applied extensively for analyzing materials exhibiting hierarchical structure [66, 67]. For each level, the Guinier regime accounts for the size of the structure while the fractal dimension, d_f , from the power law associated with it provides an estimate of the mass

density of structure within that size. Branched polymeric chains are expected to have a fractal dimensions between 1-3 [67]. It is also noted that there is uncertainty in the determination of the R_g in the low- q region because of the limited q range in this region while the power law features (high- q) are limited by statistics and incoherent background scattering. The fit at the very low q region ($q < 0.07 \text{ \AA}^{-1}$) is represented by a gray dashed line and was fit to a power law scattering exponent of -4 which represents scattering from a smooth surface. This scattering profile in low- q was a good fit for Porod's law (slope of -4) using equation (6) and corresponds to the presence of aggregates that are packed with smooth surfaces with an R_g of $820 \pm 40 \text{ \AA}$, fitted within the limited q -range available at low q . The fit to the middle q region ($0.07 < q < 0.018$) is shown as blue dashed-dotted line which represents dense mass fractals of $R_g \sim 300 \pm 40 \text{ \AA}$ and a power law exponent of 2.86 ± 0.88 . This is consistent with the power law exponent for protein fractals that has previously reported at 2.7 [68]. The fit at the high q (> 0.02) region is represented by a red dotted line that gives a R_g of $46 \pm 4 \text{ \AA}$ and is similar to the R_g calculated from EOM and the Gaussian fit for flexible polymers for asyn in solution within error limits.

The interaction of asyn with lipid vesicles was also studied to investigate the influence of the membranes or lipid interfaces on the folding and aggregation of asyn. SANS measurements were carried out in 15% D₂O to contrast match the hydrogenated lipids and selectively highlight the scattering contribution of the protein only [39]. Figure 6B shows the scattering profile and the Unified fit of the asyn-lipid sample. A model with three length scales best describes the asyn in the membrane as monomers that form clusters with smooth surfaces, which then assemble into aggregates. Similar to the asyn-SDS sample, there is a monotonic increase in the scattering intensity at low- q that indicates the presence of scattering objects larger than the length scales accessible in the experiment. The fit to this region is represented by a gray dashed line which

Figure 6. SANS data of 1:70 molar asyn-SDS in 100% D₂O (black circles) in (A) and in 1:70 molar asyn:POPA/POPS in 15% D₂O (B). The Unified fit is shown is solid black line while the three fit levels are represented by the red dotted line, blue dotted-dashed line and dashed gray line.

yields an approximate R_g of 920 ± 60 Å. The power law exponent was fitted to a fixed exponent of -4, to be able to approximate R_g , which is indicative of aggregates with smooth surfaces. The fit to the mid- q region is shown as a blue dashed-dotted line and has a power law exponent of -2.98 ± 0.96 and R_g of 190 ± 20 Å. The power law exponent of this region suggests the presence of dense fractal aggregates that result from the aggregation of asyn monomers. At the high q level, the fit is shown in red dotted line with a R_g of 44 ± 17 Å obtained from the Guinier region. In general, the larger error in the Guinier regime originates from the statistical limitation of the high- q data, which has weaker scattering from the protein aggregates. At the same time, the power laws for higher- q structures are limited by incoherent background and therefore the values are not being reported. The fit parameters are summarized in Table 3.

asyn in both SDS and lipid environments showed α -helical conformation in CD. The power law exponents for each hierarchical level for both samples as well as the R_g values suggest that the asyn clusters and aggregates formed have similar packing and structure. We have also measured the SANS profile of asyn at 34 μ M and observed a similar scattering profile (Fig. S2 and S3), suggesting that aggregation of asyn in the presence of lipids or detergents is not concentration dependent within the concentration range in this study.

Table 3. Structural fit parameters of asyn in SDS and in POPA/POPS lipid vesicles from the Unified Fit Model

Sample	Structural level	Guinier prefactor, G1	Power law Prefactor, B1	R_g (Å)	Power law exponent (P)
asyn-SDS	1	$53 \pm 9 \times 10^{-5}$	$3 \pm 1 \times 10^{-7}$	46 ± 4	-
	2	$12 \pm 6 \times 10^{-3}$	$9 \pm 9 \times 10^{-10}$	300 ± 40	2.86 ± 0.88
	3	$95 \pm 14 \times 10^{-2}$	$10 \pm 2 \times 10^{-11}$	820 ± 40	4
asyn-lipid	1	$17 \pm 7 \times 10^{-5}$	$7 \pm 1 \times 10^{-8}$	44 ± 17	-
	2	$4 \pm 1 \times 10^{-3}$		190 ± 20	2.98 ± 0.96
	3	$10 \pm 2 \times 10^{-1}$		920 ± 60	4

Discussion:

We investigated the structural properties of asyn in membrane-like environments in this study. The physiological functions of asyn have been proposed to be membrane-related, such as vesicular trafficking, synaptic vesicle stabilization, and neuronal plasticity [23, 69, 70]. A commonly observed feature of asyn pathogenicity in Parkinson's disease is asyn-membrane interactions that result in damage to the mitochondria and the disruption of cellular membranes [8, 15, 71-73]. Thus, understanding the molecular mechanisms involved in asyn-membrane interactions is crucial not only in probing its function but also for gaining insights into its role in the pathogenesis of the diseases in which it has been implicated. The lack of structural characterization of asyn in a membrane-like environment is a major obstacle to correlate asyn membrane binding with its mechanism of action and toxicity. There are limited techniques

available to access such a system at the molecular level. In this work, SANS affords us the ability to study the structure of asyn both in solution and in a complex with detergent and with lipid bilayers without the need for bulky exogeneous labels. Differences in the scattering length densities of protein, lipid, and deuterated SDS makes it possible to use the contrast match technique to selectively study the structural properties of asyn by adjusting the D₂O/H₂O ratio of the solvent such that it matches the scattering length density of the lipid or detergent thus eliminating their contribution to the scattering profiles. This approach enables us to examine the conformation of asyn in membranes without chemically modifying or altering its amino acid sequence. SANS analysis of asyn in solution clearly demonstrates its disordered nature and monodispersity under the conditions studied. This is consistent with MD simulations that show asyn backbone is solvent exposed 60% of the time with minimal secondary structure [74]. The persistence length which describes the flexibility of a chain based on the Debye approximation for a Gaussian coil is comparable to the values for other disordered proteins [60]. The R_g values that we obtained from the Debye fit and EOM analysis are consistent with literature using SAXS and MD simulations [63, 74, 75].

More intriguingly, our study provides an in-depth insight into the molecular-level organization of asyn when it associates with the membrane, showing that there is a hierarchical order of the aggregated system. The particle sizes observed can be related to asyn monomers, aggregates and agglomerates of asyn aggregates. Our model of aggregation (Fig. 7) illustrates that approximately 30 monomers form clusters with $R_g \sim 300$ Å and an approximately 80 of these clusters assemble to form larger aggregates with $R_g \sim 820$ Å. Taken together, this suggests that the system is relatively dynamic containing monomeric conformers existing in equilibrium with aggregated conformers. We can propose an aggregation mechanism model whereby interaction

of asyn with the membrane induces a conformational change in the protein that increases its propensity to aggregate. The individual protein molecules serve as the seed for aggregation and aggregation growth occurs not in random but by the addition of aggregates which form the much larger agglomerate structures. Although the aggregates are primarily α -helical, the ThT binding assay suggests that some β -sheet content starts to form in the aggregates, which could nucleate to serve as protofibrils.

Membrane-induced conformational changes of asyn are critical whether they directly or indirectly affect amyloid formation. The factors that can play roles in these interactions include the chemical and physical properties of the lipid membranes such as surface charge, bilayer

Figure 7. The schematic illustration of asyn aggregation in membrane environment. The monomeric asyn in solution that are folded into α -helical conformation are shown in panel (A). The smallest cluster of aggregates (B) formed from the monomers which then formed into larger aggregates (C).

curvature, hydrophobic and electrostatic interactions that drive adsorption or penetration of the proteins in the membrane, and the dynamics and fluidity of the membrane [76, 77]. For example, POPS used in this study comprises $\sim 30\%$ of the lipid content in the cellular membranes.

Furthermore, asyn has been shown to preferentially bind to vesicles with negatively-charged head groups such as those that contain POPA and POPG in order to effect the disordered to helical transition [78]. Comparing POPA-POPC and POPC-POPS in a 1:1 molar ratio, we observed the largest secondary structural change of asyn from a random coil to α -helix in POPA-POPS vesicles based on far-UV CD analysis. We chose to study POPA-POPS lipid composition because POPA comprises 0-2% and POPS 7-12% of the total lipid synaptic vesicles where asyn localizes [79, 80]. The conformational change observed in SDS micelles is similar in magnitude to that observed in POPA-POPS liposomes. We noticed that the size of the large cluster is about the size of the lipid vesicle in the study. It suggests that the lipid membrane may provide a surface to facilitate the clustering of asyn. Recent studies have provided new insights into the initial events that occur when asyn interacts with membranes and propose that the N-terminal region serves as the anchor for binding to the membrane resulting in the NAC and C-terminal regions becoming exposed and enabling them to interact with binding partners [81, 82]. Furthermore, the NAC region is thought to play a role in modulating [83] how the asyn partitions between being bound to the membrane or free in solution. The results of this SANS study indicate that after asyn binds to the membrane, it has tendency to aggregate. This is supported by previous studies that report that asyn forms aggregates in the size range of 250 – 1500 Å in the presence of SUVs and on supported lipid bilayers [84-86]. In addition, it has also been shown that other amyloid proteins favor the aggregated state over the monomeric state in lipid membranes [82, 87, 88]. We can postulate that the formation of hierarchical aggregates of asyn could cause extensive membrane structure remodeling if not destruction and hence contribute to the pathogenic effects of this protein.

Conclusion:

It is crucial to understand the molecular basis for the association of asyn with membranes in order to gain insight into its pathology in the disease state. While there has been progress on characterizing the molecular mechanism and structures involved in amyloid fibril formation, there is not much known about which conformers or species of asyn are most relevant to its function or its toxicity. In this work we studied the transition of asyn from its disordered state to a folded but aggregated structure when it is associated with membranes. Our SANS analysis shows that in a membrane-like environment, the aggregation of asyn does not proceed in a completely random manner. Rather, the individual monomers form α -helical aggregates that in turn assemble into larger structures. This provides new insights into the mechanism of aggregation of asyn. Even though the aggregates are amorphous in nature, it is possible that they represent a prefibrillar state of asyn where asyn is sequestered in a membrane-bound complex. These aggregates may be key in understanding the mechanism of asyn toxicity and in developing strategies for therapeutic intervention.

Acknowledgments:

The authors would like to thank Dr. Dung Vu of Los Alamos National Laboratory for the asyn plasmid. We also thank Dr. Sai Venkatesh Pingali for valuable discussions on SANS data analysis. The Center for Structural Molecular Biology supports the Bio-SANS instrument and laboratories used for this research. It is funded by the Office of Biological and Environmental Research of the U.S. Department of Energy. The High Flux Isotope Reactor is sponsored by the Scientific User Facilities Division, Office of Basic Energy Sciences, U.S. Department of Energy. Oak Ridge National Laboratory is managed by UT-Battelle, LLC, for the U. S. DOE under Contract No. DE-AC05-00OR22725.

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