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Interplay between α -synuclein amyloid formation and membrane structure

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Abstract

Amyloid formation is a pathological hallmark of many neurodegenerative diseases, including Alzheimer’s, Parkinson’s, and Huntington’s. While it is unknown how these disorders are initiated, *in vitro* and cellular experiments confirm the importance of membranes. Ubiquitous *in vivo*, membranes induce conformational changes in amyloidogenic proteins and in some cases, facilitate aggregation. Reciprocally, perturbations in the bilayer structure can be induced by amyloid formation. Here, we review studies in the last 10 years describing α -synuclein (α -syn) and its interactions with membranes, detailing the roles of anionic and zwitterionic lipids in aggregation and their contribution to Parkinson’s disease. We summarize the impact of α -syn α comparing monomeric, oligomeric, and fibrillar forms — on membrane structure, and the effect of membrane remodeling on amyloid formation. Finally, perspective on future studies investigating the interplay between α -syn aggregation and membranes are discussed. This article is part of a Special Issue entitled: Lipid-protein interactions in amyloid formation.

Keywords

Parkinson’s disease; thioflavin T; protein-lipid interaction; aggregation; membrane remodeling; tabulation

1. Introduction

Neurodegenerative diseases such as Alzheimer’s, Parkinson’s and Huntington’s are broadly characterized by progressive cell death and the presence of amyloid fibrils. While the sequences of these amyloidogenic proteins differ, they share the ability to self-polymerize into highly structured, β -sheet rich conformations, indicating that there may be common pathological mechanisms involving protein misfolding and aggregation [1]. Interestingly, amyloid formation has been shown to be influenced by membranes [2]. Although membranes have proven to be important in disease pathology, a consensus has not been

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reached on the precise effect, which is likely due to differences in experimental aggregation conditions (*e.g.* lipid composition, lipid-to-protein (L/P) ratio, and type of membrane mimic) [2]. Furthermore, it remains to be determined where amyloid formation begins within a cell. Understanding fundamental protein-lipid interactions will help pinpoint which cellular compartments are the most probable culprits. In addition, elucidation of how functional, soluble proteins transform into insoluble, aggregated fibrils in a cellular environment will aid the development of diagnostic tools and novel therapeutic approaches to treat these disorders [1].

In this review, we focus on α -synuclein (α -syn) and its association with Parkinson's disease (PD) to discuss the dynamic relationship between protein aggregation and membrane structure both *in vivo* and *in vitro*, concentrating primarily on studies published in the last 10 years. First, we will discuss α -syn and its involvement in PD, highlighting the importance of membranes in disease pathology. Next, we will describe the heterogeneous lipid environment found inside mammalian cells and explain how α -syn changes its conformation when interacting with these membranes. Then, aggregation of α -syn in the presence of anionic lipids will be detailed, outlining two different aggregation trends. The impact of monomeric, oligomeric, and fibrillar α -syn on membrane structure will then be summarized for both anionic and zwitterionic membranes. Lastly, we provide our perspective on the gaps in the current knowledge about α -syn-membrane interactions and propose future studies that would benefit our understanding of α -syn amyloid formation inside a cell.

2. Linking α -Syn-membrane interactions to PD

By 2030, it is estimated that PD will affect up to 9 million people worldwide [3]. This devastating neurodegenerative movement disorder is characterized by the loss of dopaminergic neurons in the *substantia nigra* [4]. Cytoplasmic inclusions called Lewy bodies are pathological hallmarks of PD, and the protein α -syn comprises the major component [5]. Notably, overexpression of human α -syn in a triple knockout mouse of endogenous α -, β -, and γ -synuclein resulted in the formation of Lewy bodies *in vivo* [6]. High levels of α -syn through duplication and triplication of the *SNCA* gene have also been linked to early-onset PD [7, 8], and elevated levels of α -syn mRNA have been observed [9]. Taken together, it is undeniable that α -syn is connected to the disease etiology of PD.

While the mechanism of cell death is unclear in PD, α -syn-membrane interactions have been implicated [2, 10]. Not only are lipids also a major component of Lewy bodies [11], but membrane disruption and organelle dysfunction of the lysosome [12], Golgi [13], and mitochondria [14] have been documented in PD patients. Moreover, the eight currently known missense mutations of α -syn associated with autosomal dominant PD (A18T, A29S, A30P, E46K, H50Q, G51D, A53T, and A53E) [15–17] are all located in the N-terminal membrane-binding region of the protein [18]. Different aggregation trends have been reported for the disease-related mutants compared to the WT protein. Some mutants (E46K, H50Q, G51D, and A53T) accelerate amyloid formation [19–24] and even promote oligomer formation [25, 26], whereas others (A30P and A53E) slow α -syn aggregation *in vitro* [27, 28]. Differences in the lipid binding affinity of the PD-mutants, as well as their abilities to cause membrane perturbations [29–31] reinforce the connection between membranes and

PD. Therefore, studying the relationship between α -syn and membranes is essential to gain mechanistic insight into the aggregation process.

2.1 Biophysical characterizations of α -syn conformations

Although there is debate regarding whether the dominant soluble form of α -syn is a tetramer [32, 33], numerous studies have shown that α -syn exists as an intrinsically disordered, largely cytosolic monomer in living cells [34–36]. α -Syn is composed of three partially overlapping regions: the N-terminal lipid-binding domain (residues 1–100), the hydrophobic non-amyloid core (NAC) region (residues 61–95), and the acidic C-terminal tail (residues 100–140) [37]. The N-terminus contains 7 imperfect amphipathic 11-amino acid repeats (consensus sequence KXKEGV), which adopts an α -helical conformation in the presence of lipids [18, 37]. Depending on the curvature of the membrane, α -syn can form either a bent helix [38], characterized by two anti-parallel α -helices connected by a linker, or a single extended α -helix on larger membrane surfaces [39]. However, interconversion between these two helical states has also been observed [40].

Membranes are not only associated with the monomeric form of α -syn, but also have been demonstrated to enhance the aggregation process by acting as a 2-dimensional surface that increases the local protein concentration [41]. The NAC region, named for its original identification in Alzheimer's disease plaques [42], is essential for the self-polymerization of α -syn. α -Syn amyloid fibrils have a complex, highly structured cross- β conformation, which has been characterized using X-ray diffraction [43], cryo-EM [44–46], and solid-state NMR [47]. α -Syn amyloid fibrils have been shown to be polymorphic [44], and a variety of structures may be possible since the final fibril conformation is likely dependent on the aggregation conditions [48]. While amyloid fibrils are found in Lewy bodies, α -syn oligomeric intermediates, which can be purified using size-exclusion chromatography (SEC) [14, 25, 49–52], have also been reported to be toxic to cells [53].

Membranes induce conformational changes in α -syn, and reciprocally, all forms of α -syn – including the monomer, oligomer, and fibril – have been associated with membrane binding and remodeling [54–62]. While membrane curvature generation is essential for healthy cellular function [63], membrane remodeling has also been associated with the disruption of membrane integrity [56, 58, 59, 64, 65], the formation of nanoparticles [54, 55, 62], membrane tubulation [30, 61, 66–68], and vesicle budding [14, 61]. In the following sections, the interplay between protein and membrane structure will be discussed in the context of α -syn aggregation in the presence of anionic or zwitterionic lipids.

3. The heterogenous cellular lipid environment

α -Syn is a cytosolic protein that comprises about 1% of the total protein found in brain tissue [69], and about 15% of α -syn is membrane bound [41]. In a typical mammalian cell, there are over 1000 different types of phospholipids [70]; however, ~40–50% of the total phospholipids found in membranes are zwitterionic phosphatidylcholine (PC) lipids [70–72] (Figure 1). Although most PC lipids are *cis*-monounsaturated [71], resulting in a largely fluid, electrostatically neutral lipid bilayer, cellular membrane dynamics cannot be described

accurately by the fluid mosaic model due to factors such as variable membrane thickness and protein density on and in the membrane [73].

The second most common lipid in mammalian cells is phosphatidylethanolamine (PE) (20 – 50%, depending on the tissue), which is also zwitterionic but has a smaller, conical head group leading to more packing defects [70, 71]. Sphingolipids are another major lipid type, made up primarily of sphingomyelin (SM) (5 – 10%) and glycosphingolipids, which have a tall, cylindrical geometry, resulting in enhanced lipid packing and association with cholesterol (Chol) to form ordered domains [71, 72]. It is important to note that anionic lipids such as phosphatidylinositol (PI) (8%) and phosphatidylserine (PS) (2 – 10%) comprise only a small percentage of the overall membrane composition in a cell [72] (Figure 1). Though, as the secretory pathway progresses from the plasma membrane to the lysosome, the membrane becomes more charged as levels of PS and bis(monoacylglycero)phosphate (BMP) increase [71, 77].

3.1 Plasma membrane

The plasma membrane, like other membranous organelles, has a distinct membrane asymmetry, with specific lipid compositions for the inner and outer leaflets (Figure 1). A high percentage of PC, SM, and Chol exist on the outer leaflet, promoting the formation of lipid rafts, which has been observed both *in vivo* and recapitulated *in vitro* [71, 74, 78]. Lipid rafts or microdomains are characterized by the phase separation of lipid mixtures into liquid-ordered and fluid phases, allowing for the local enrichment and assembly of proteins [71]. In fact, α -syn has been shown to bind to lipid rafts [79] and lipid raft-like mixtures [80]. In addition, α -syn is proposed to concentrate, and perhaps aggregate, on the inner plasma membrane due to an increase in concentration of lipids such as PS and PE [70–72].

3.2 Mitochondria

α -Syn also localizes to the inner membrane of the mitochondria *in vivo* due to the presence of a 32-residue targeting sequence in the N-terminus of the protein [81]. Accumulation of α -syn in the mitochondria has been linked to a decrease in the activity of mitochondrial complex I and thus the production of reactive oxygen species (ROS) [81]. Protein build up in the mitochondria was exacerbated for the disease mutant A53T compared to WT, suggesting that the mitochondria is a key organelle linked to PD [81]. The mitochondrial inner membrane also drives α -syn membrane binding since it has a high concentration (~10 – 25% [71]) of cardiolipin (CL) (Figure 1), an anionic phospholipid with a double negative charge. Due to its small headgroup, cardiolipin can facilitate tight packing. On the other hand, it also has four unsaturated acyl chains which can enhance fluidity of the bilayer [82]. Further, it is suggested that cardiolipin promotes the formation of highly-curved, nonlamellar membrane structures [82]. These surfaces are prime candidates for α -synuclein binding, which could result in mitochondrial membrane permeabilization and fragmentation [83–85]. A high amount of PE has also been reported in the inner membrane, which supports α -syn binding by the packing defect model [70, 71].

3.3 Lysosome

The lysosome is in charge of the removal and recycling of lipids and proteins, including α -syn through chaperone-mediated autophagy [86]. Disruptions in the function of the lysosome have been linked to diminished α -syn turnover in PD [87]. Cysteine proteases – namely cathepsin B (CtsB) and cathepsin L (CtsL) – are responsible for the degradation of α -syn in the lysosome [88]. Further, CtsL is capable of digesting α -syn fibrils first by truncating the exposed C-terminus and then proceeding to cut at the more central region [89]. Interestingly, the previously considered essential lysosomal protease, cathepsin D (CtsD), requires the anionic phospholipid BMP to cut within the α -syn amyloidogenic core [88]. BMP is found almost exclusively in the lysosome, enriched specifically in the inner membrane [75] (Figure 1), and is required for the recruitment of cationic hydrolases and sphingolipid activator proteins, which are necessary for the breakdown of lipids [90]. Since BMP is anionic, it also promotes α -syn membrane binding *via* electrostatic interactions [88].

3.4 Synaptic vesicles

Currently, α -syn is thought to play a role in regulating neurotransmission and synaptic structure, as well as maintaining the homeostasis of dopamine in neurons [91]. Triple knockout mice of endogenous synuclein presented with impaired synaptic transmission and abnormalities in synapse ultrastructure [92]. Interestingly, synaptic vesicles isolated from rat brains and characterized by Takamori *et al.* were shown by mass spectrometry analysis to have over 80 integral membrane proteins [76]. Together with a large number of associated peripheral proteins, it is clear that a very high density of proteins exist on or in the membrane, resulting in increased membrane rigidity [76]. This high concentration of protein limits the accessible area for interaction between α -syn and the membrane. Lipidomic analysis of isolated synaptic vesicles show a high proportion of PC and PE (~59–78%), moderate levels of PS (~12%), and low levels of SM (~5–7%) [76, 93] (Figure 1). Moreover, about 40% of the total lipid is cholesterol, making the ratio of phospholipids to cholesterol around 1:0.8 [76]. Taken together, it is remarkable that α -syn is able to bind to synaptic vesicles despite the reduction of total surface area for binding.

4. α -Syn aggregation in the presence of anionic lipids

α -Syn amyloid formation has been widely studied *in vitro* since α -syn fibrils were discovered in Lewy bodies [5], and the impact of anionic lipids has been evaluated using a wide range of lipid compositions (*e.g.* PS, phosphatidic acid (PA), phosphatidylglycerol (PG), CL, and gangliosides) and different membrane mimics (*e.g.* small unilamellar vesicles (SUVs), large unilamellar vesicles (LUVs), giant unilamellar vesicles (GUVs), and supported lipid bilayers (SLBs)) [2, 10]. α -Syn aggregation kinetics are typically monitored using thioflavin T (ThT) which increases in fluorescence intensity in the presence of amyloid [94]. While widely used and considered a “gold standard”, ThT fluorescence intensity varies between different amyloids and fibril polymorphs, thus comparisons based solely on ThT fluorescence is not recommended. For example, amyloid fibrils formed from the prion-domain (residues 218–289) of the fungal protein Het-s (Het-s_{218–289}) give little to no ThT response [95] and polymorphs of β -amyloid peptide exhibit intensity differences [96]. Furthermore, the presence of lipid vesicles and membrane mimics can add further

complications such as competing for ThT binding. Therefore, amyloid structure should always be verified using other methods such as microscopic visualization by TEM or AFM and secondary structure determination by circular dichroism (CD), FTIR, or Raman spectroscopy, in addition to ThT.

Despite differences in aggregation conditions, the consensus in the field is that α -syn amyloid formation can be enhanced by anionic lipids, as seen by shorter lag times [2, 10]. Stimulation is thought to be mechanistically driven by high concentrations of the monomer on the membrane, leading to an increase in the rate of primary nucleation [1, 41]. Lipid-enhanced aggregation has not been consistently observed, however, likely due to differences in the many variables that affect protein aggregation (*i.e.* temperature, pH, quiescent or shaking, air-water interface, and *etc.*) [10, 97]. Here, aggregation experiments with anionic lipids are divided into two groups: one in which stimulation is only seen at low L/P ratios (< 8) and the other where it is observed at L/P ratios up to 50 (Table 1).

4.1. Stimulation of aggregation at low L/P ratios

The delicate balance between membrane-bound and free monomeric α -syn and its effect on amyloid fibril formation was demonstrated by Galvagnion *et al.* and Burre *et al.* [100, 104]. Using 100% 1,2-dimyristoyl-*sn*-glycero-3-phospho-L-serine (DMPS) SUVs, Galvagnion *et al.* showed that binding by α -syn is required for the acceleration of aggregation kinetics under quiescent conditions, and inhibiting its binding affinity through the addition of sodium chloride slowed amyloid formation in a concentration-dependent manner [100]. Free monomeric protein, however, is also necessary for polymerization to occur [25]. Enhanced rates of aggregation were only observed for L/P = 10 (with a maximum rate at L/P = 8), with lag times lengthening for L/P = 15 and near complete inhibition at L/P = 40 [100]. Similarly, Bartels *et al.* showed complete inhibition of α -syn aggregation in the presence of POPC/GM1 (80:20) at L/P = 10 [105], and Burre *et al.* observed no amyloid formation in the presence of excess L- α -phosphatidylserine/L- α -phosphatidylcholine (Brain PC/PS) (70:30) SUVs at L/P = 20 due to the lack of free monomer [104]. When α -syn binding was impaired by mutating two residues in the membrane-binding domain, aggregation was detected [104], demonstrating that blocking membrane binding at high L/P ratios aids in amyloid formation.

Furthermore, using 100% DMPS and 100% 1,2-dilauroyl-*sn*-glycero-3-phospho-L-serine (DLPS) SUVs, Galvagnion *et al.* showed that aggregation was also promoted by short-chain lipids [98]. In agreement with previously described trends, as the L/P increased from 1 to 5, lag times increased, suggesting that the pool of free monomer was limited [98]. Likewise, Grey *et al.* observed progressive inhibition of α -syn aggregation kinetics as the L/P increased to 7 for the following lipid compositions: 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC)/CL (94:6), DOPC/D-*erythro*-sphingosine (sphingosine) (85:15), and DOPC/1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (DOPS) (70:30) [101]. In agreement, Zhu *et al.* observed inhibited α -syn fibrillization at L/P = 5 for SUVs composed of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) in a 1:1 ratio with either 1,2-dipalmitoyl-*sn*-glycero-3-phosphate (DPPA), 1,2-dipalmitoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (DPPG), or 1,2-dipalmitoyl-*sn*-glycero-3-phospho-L-serine (DPPS) [102, 106]. In

summary, populations of both membrane-bound and free α -syn are essential for the stimulation of amyloid formation by anionic lipids.

4.2 Stimulation of α -syn aggregation at both low and high L/P ratios

In this section, we highlight α -syn aggregation experiments that demonstrated enhanced kinetics at both low and high lipid concentrations, contrary to the previous set of experiments outlined in section 4.1. Grey *et al.* purified exosomes from mouse neuroblastoma cells and showed that the small vesicles reduced the time until half maximal ThT intensity by more than a third [101]. Further, α -Syn aggregation was enhanced at L/P ratios as high as 33 using isolated exosomes [101]. Based on mass spectrometry analysis, the lipid composition of exosomes was determined to contain a mixture of PC, PS, PE, PI, and gangliosides GM2 and GM3 [101]. Interestingly, synthetic DOPC SUVs with either 10% GM1 or GM3 ganglioside resulted in progressively shorter lag times as the L/P increased from 3 to 17 [101].

Enhanced α -syn aggregation kinetics were also observed using equimolar 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphate (POPC/POPA) SUVs [57]. Amyloid formation was not only observed, but strikingly accelerated at L/P = 50 compared to the protein in buffer [57]. These results were corroborated by Comellas *et al.* through the use of magic-angle spinning solid-state NMR, which confirmed the formation of α -syn fibrils with POPC/POPA (3:1) vesicles at L/P = 50 [99]. Lastly, synaptosomes isolated from rat brains were also shown to support amyloid formation at high lipid concentrations (L/P = 20) [103]. It should be noted that the L/P ratio for lipid membranes derived from biological sources (*i.e.* rat synaptosomes and mouse exosomes) may be significantly smaller than described, as there are likely numerous proteins in the isolated native membrane, limiting the surface area available for α -syn binding. Nevertheless, these sets of aggregation experiments demonstrate that stimulation of α -syn amyloid formation is possible at high L/P ratios, suggesting that the aggregation mechanism proposed in section 4.1 may be more nuanced.

5. Remodeling of anionic membranes by α -syn and effect on amyloid formation

Membranes not only have a clear impact on α -syn aggregation, but α -syn can also alter lipid bilayer structure. This reciprocal relationship between protein and membrane structure is exemplified using 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (POPG) vesicles [58]. Monomeric α -syn was shown to rapidly remodel SUVs (Figure 2A) into thin micellar tubules (diameter (d) ~ 7 nm) at L/P = 1 (Figure 2B) and thick bilayer tubes (d ~ 30 nm) at L/P = 50 (Figure 2C). After three days of incubation, the micellar tubules present at L/P = 1 disappeared and long, straight filaments resembling α -syn fibrils were present (Figure 2E). The filaments were confirmed to be amyloid based on the presence of β -sheet secondary structure using CD spectroscopy (Figure 2H). Visualization by TEM and solid-state NMR analysis also revealed that POPG lipids were incorporated into the fibrillar structure. Conversely, the presumed bilayer tubes (L/P = 50) were stable upon incubation and no amyloid formation was observed (Figure 2F). The monomer was proposed to be

stabilized in an α -helical conformation on the membrane (Figure 2F), limiting the amount of free monomer available for self-association. Consistent with these results, aggregation kinetics revealed that micellar tubules shortened the lag phase of α -syn (Figure 2K) and amyloid formation was enhanced at L/P = 1 (Figure 2H) compared to the protein alone. These results suggest that POPG micellar tubules stimulate α -syn fibril formation, thereby illustrating the interplay between α -syn aggregation and membrane remodeling.

Although observations of α -syn-induced membrane deformation are relatively recent, the basis for α -syn as a membrane curvature-sensing protein has long been established [18]. In fact, triple knockout of the synuclein family in mice resulted in the up-regulation of other curvature-sensing and curvature-generating proteins such as endophilin A1, endophilin B2, annexin A5, and synapsin IIb [30], strengthening the assertion that α -syn is a membrane remodeling protein. Changes in membrane curvature have been explained by the wedge model, in which the insertion of an amphipathic α -helix into the bilayer near the phospholipid headgroups induces stress and subsequent lateral lipid re-organization [107]. In the following sections, we will discuss the impact of α -syn monomers (sections 5.1 and 5.2), oligomers (section 5.3), and fibrils (section 5.3) on the structure of membranes composed of anionic lipids.

5.1 Membrane thinning and tubulation by monomeric α -syn

Monomeric α -syn has been shown to thin lipid bilayers upon association for a variety of membrane compositions involving anionic lipids. Ouberaï *et al.* measured the α -syn-induced lateral membrane expansion of SLBs composed of brain total lipid extract by AFM and dual polarization interferometry [108]. Further, measurements using neutron reflectometry showed that α -syn caused membrane thinning of sparsely-tethered POPC/POPA lipid bilayers [109]. The importance of the N-terminus for membrane thinning was verified by Braun *et al.* and Leftin *et al.*, who showed that α -syn variants with C-terminal truncations (1–100 and 1–25, respectively) were able to prompt thinning of POPC/1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine (POPS) (3:1) and POPC/egg SM/cholesterol, correspondingly [110, 111]. Lastly, Shi *et al.* observed membrane thinning and expansion of DOPS and DOPC/1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE)/DOPS (25:30:45) GUVs by the N-terminal acetylated form of α -syn [60]. Thinning was directly correlated with the amount of monomer present on the bilayer, and tubulation of the membrane was observed after bilayer expansion [60].

Formation of thin micellar tubules and thick bilayer tubes were described earlier for POPG; however, tubulation of other anionic membranes, including POPA and POPS, by the monomer has also been reported [58]. In general, the consensus is that membrane tubulation is enhanced when α -syn adopts a highly α -helical structure upon binding to lipids, elicited either by the sequence of the protein (A53T ~ E46K ~ WT > A30P) [30] or by the membrane composition (POPG > POPC/POPG (1:1)) [66]. In addition, chemical properties of the lipid acyl chains were found to influence the amount of tubulation, where increased chain length and unsaturation resulted in more bilayer tubes compared to micellar tubules [67]. In contrast to this paradigm, Pandey *et al.* used epi-fluorescence to show that tubulation of Egg PG SLBs was increased when α -syn had less α -helical content (A30P > WT) [68].

Less lipid tubule formation was observed for WT α -syn as greater amounts of Egg PG were incorporated into Egg PC SLBs [68]. Differences in results may be attributed to the use of different membrane mimics and/or lipid compositions, as Egg PG is composed of a variety of PG lipids including 16:0 and 18:1.

5.2 Vesicle budding and formation of lipoprotein nanoparticles

Dramatic changes in POPG structure were observed by TEM as the L/P decreased, from thick tubes ($d = 30\text{--}40$ nm) at L/P = 40 to thin tubules ($d = 10\text{--}15$ nm) at L/P = 20 and small circular structures at L/P = 10 [61]. Varkey *et al.* and Drescher *et al.* monitored the transformation of large POPG vesicles into smaller vesicles by monomeric α -syn through fluorescence microscopy and double electron-electron resonance spectroscopy, respectively [61, 112]. Budding of small vesicles was also observed in DOPC/CL vesicles made *in vitro* to mimic mitochondrial membranes [14].

POPG vesicles were not only converted into tubules and small vesicles by α -syn, but also into lipoprotein nanoparticles [62, 67]. POPG nanoparticles were isolated *via* centrifugation and SEC and characterized by cryo-EM and DLS [62]. Ellipsoidal nanoparticles (7 – 10 nm) with a L/P ratio of 20–25 were formed by α -syn, which wrapped around the periphery of the nanodisc in a broken-helix conformation [62]. Remodeling of POPC/CL and mitochondrial mixture POPC/1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE)/POPS/PI/CL/SM/Chol (39:34:1:5:18:1:2) also resulted in nanoparticle formation, albeit at a lower abundance [62]. α -Syn monomer was also shown to assemble DOPS and POPS lipids into ellipsoidal, 400 kDa nanodiscs ($d \sim 23$ nm) with a L/P ratio of 8–10 using a different method [54]. Similar to POPG nanoparticles, α -syn wrapped around the lipids in a helical conformation using residues 1–100 [54].

In summary, monomeric α -syn can alter membrane morphology in a variety of ways depending on the L/P ratio, lipid composition, and type of membrane mimic. These changes in membrane structure may be beneficial for the function of α -syn in synaptic transmission [91, 92], or damaging and linked to PD pathology [12–14].

5.3 α -Syn oligomers and the process of aggregation disrupt membrane integrity

Beginning with WT α -syn oligomers, membrane disruption through dye leakage assays has been reported for POPG [51, 52, 65], DOPG [51, 64], 1,2-dioleoyl-*sn*-glycero-3-phosphate (DOPA) [51], DOPS [52], PI [52], and Egg PG vesicles [51]. Interestingly, A53T oligomers were discovered to permeabilize Egg PG vesicles to a greater extent than WT oligomers [51], implicating oligomer membrane disturbances in the disease mechanism of PD. Vesicle disruption by oligomers was found to be sensitive to the charge of the lipids in some cases; for example, no change in membrane integrity was observed for DOPG/DOPC (1:1) and DOPG/DOPE (1:1) [51]. However, leakage of calcein and AFM images confirmed membrane damage of DPPC/DPPA (1:1) and DPPC/DPPG (1:1) SLBs by both oligomeric and fibrillar α -syn [106]. Differences may be due to the use of saturated *vs.* unsaturated lipids. Oligomers have also been shown to kill dopaminergic neurons in rats, specifically in the *substantia nigra* where substantial cell death occurs in PD patients [113]. Changes in vesicle integrity by α -syn oligomers were also observed using mitochondria and lysosomes

isolated from HeLa cells [53], indicating that membrane remodeling is translatable to biological membranes. Indeed, prefibrillar α -syn species induced permeabilization of isolated mitochondrial membranes and prompted the release of cytochrome *c* [85]. It should be noted that while monomeric α -syn can induce membrane disruption, the outcome is either less pronounced [106] or requires high concentrations of protein [64].

Membrane damage is also caused by the process of aggregation, in which monomeric α -syn aggregates on the membrane surface and destroys the bilayer through lipid extraction [56, 59, 114]. Using confocal microscopy, membrane integrity was compromised as α -syn formed ThT-positive aggregates on POPC/POPG (1:1 and 3:1) SLBs [114]. Binding of α -syn alone did not disrupt the integrity of the membrane as an α -syn deletion mutant (71–82), that could bind membranes but not aggregate, did not alter membrane structure [114]. Lipid extraction was also observed for DOPS and DOPC/DOPS (7:3) vesicles using polarization transfer solid-state NMR, with the greatest uptake seen for pure DOPS SUVs [56], indicating a higher affinity of anionic lipids for α -syn fibrils. Lastly, aggregation of WT α -syn on DOPC/DOPS (65:35) SLBs prompted lipid extraction and clustering using super critical angle microscopy and fluorescence resonance energy transfer [59]. Notably, the disease-related mutants A53T and E46K disrupted the membrane more than WT α -syn [59], and pre-formed fibrils had no effect on membrane integrity [56, 59], indicating that it is the aggregation process itself that is responsible for membrane perturbation.

6. Remodeling of zwitterionic membranes by α -syn and effect on amyloid formation

An overwhelming proportion of studies on α -syn aggregation have focused on anionic lipids. While negatively charged phospholipids increase during aging [115] and are therefore biologically relevant, research on α -syn-membrane interactions with zwitterionic lipids has lagged far behind that of anionic lipids, in part due to their weaker binding to α -syn. Many have reported little or no association with zwitterionic lipids, including DPPC [49, 102, 106] and DOPC [108]. While remodeling of anionic lipid membranes by α -syn has been discussed and reviewed in detail [116], changes in the structure of zwitterionic membranes have largely been neglected. No remodeling of POPC [52, 61, 62] or DOPC [14, 56, 64] was observed by some groups; however, in the following sections, we will describe the few studies that have reported aggregation and/or remodeling of zwitterionic lipids by α -syn, including sphingomyelin and POPC.

6.1. Sphingomyelin

Brain sphingomyelin is a *trans*-monounsaturated lipid with a PC head group that is found largely in lipid rafts on the plasma membrane [70–72, 78], and interestingly in Lewy bodies [11]. Eichmann *et al.* revealed that α -syn is able to assemble SM lipids into high-density lipoprotein-like (HDL-like) particles with the help of detergents, albeit less efficiently than DOPS or POPS [54]. The study was later expanded to show that all human synucleins and disease variants (A30P, E46K, H50Q, G51D, and A53T) remodel SM into nanodiscs [55]. Using TEM, the HDL-like particles were characterized to be circular with $d \sim 25$ nm [55]. Notably, the amount of α -helical content of α -syn was correlated to the efficacy of

nanoparticle formation; for example, N-acetyl α -syn increased nanodisc formation by about 14% compared to the unmodified form [55]. While HDL-like particles were formed from biologically relevant SM lipids, Eichmann *et al.* proposed that membrane remodeling is unlikely related to PD pathology, as both healthy and disease-related forms of α -syn are implicated [55].

6.2. POPC

PC is the major type of lipid found in mammalian membranes with the majority containing *cis*-monounsaturated acyl chains [70–72], making POPC an appropriate lipid for mimicking cellular membranes. Previously, TEM was used to show α -syn remodeling of POPC vesicles into lipid tubules ($d \sim 20$ nm) in a concentration dependent manner [57]. In contrast to experiments employing anionic membranes, α -syn in the presence of POPC vesicles did not adopt a defined secondary structure based on CD measurements, and only weak, non-specific interactions with the membrane were detected by tryptophan intensity and time-resolved fluorescence anisotropy experiments [57]. Furthermore, membrane fluidity was necessary for tubulation, as the addition of cholesterol made the vesicles more rigid and less prone to remodeling [57]. Interestingly, POPC inhibited α -syn amyloid formation as a function of increasing vesicles, as seen by overall decreasing ThT intensity and protracted lag and growth phases (Figure 3A). The reduction in the levels of amyloid fibrils formed was corroborated by CD spectra (Figure 3B) and TEM images (Figure 3C, D, and E). The co-existence of α -syn fibrils and membrane tubules was observed, with more tubulation as the L/P increased (Figure 3E), suggesting a competitive binding mechanism between membrane remodeling and self-association. Changes in membrane structure were attributed to a protein crowding mechanism [117], as pre-formed fibrils had no effect on membrane structure; however, involvement of oligomers could not be ruled out [57]. Interestingly, similar protrusions from Egg PC vesicles were seen by TEM when α -syn oligomers were added to the membrane; however, the protrusions were interpreted as radiating amyloid fibrils, not membrane tubules [53]. Oligomers, verified through CD and staining with CongoRed and ThT, were found to strip away lipids from the membrane, which were then incorporated into the protein structure, resulting in membrane disruption and calcium release [53]. Evidence of membrane remodeling by α -syn oligomers was also observed in POPC/Chol membranes, where Chol promoted the formation of lipid-protein aggregates [118].

7. Perspective on future studies

While much has been learned about α -syn aggregation in the context of membranes, most of the work has utilized synthetic lipids and lipid mixtures. The majority of studies use high proportions of anionic lipids (Table 1), which was also reviewed recently by Galvagnion [10]. Upon scrutiny of the lipid compositions observed to accelerate α -syn aggregation, we propose that membrane fluidity may be responsible for the different aggregation trends outlined in Table 1. At high L/P ratios (> 50), all membranes were composed of either unsaturated synthetic lipids (DOPC/GM1 (90:10) [101], DOPC/GM3 (90:10) [101], and POPC/POPA (1:1 or 3:1) [57, 99]) or biologically-derived lipids (mouse exosomes [101] and rat synaptosomes [103]). In contrast, the membranes that stimulated amyloid formation at only low L/P ratios (< 8) were largely composed of saturated lipids (DLPS [98], DMPS [98,

100], DPPC/DPPA (1:1), DPPC/DPPG (1:1), and DPPC/DPPA (1:1) [106]) or mixtures with ordered lipids (Brain PC/PS (70:30) [104] and DOPC/Sphingosine (18:15) [101]). Although, there were some exceptions (DOPC/DOPS (70:30) and DOPC/CL (94:6) [101]), we assert that membrane fluidity plays a role in α -syn aggregation by regulating binding of the monomer, and that lipid packing is an important characteristic of the bilayer *in vivo*.

Considering lipid compositions found inside a cell (section 3), it is clear that PC and PE lipids dominate; therefore, we propose that more work on zwitterionic phospholipids is needed. Furthermore, not only should electrostatically neutral membranes be used, but bilayers with leaflet composition asymmetry also need to be included in α -syn-membrane interaction studies. The cell expends large amounts of energy to maintain asymmetry across the membrane, which is closely linked to cellular function and signaling processes [70–72, 78]. An additional problem when using purely lipid-containing vesicles is that they lack the protein density found on and within the bilayer. Notably, synaptic vesicles isolated from rat brain were found to have over 80 integral membrane proteins in the lipid bilayer [76], which affect the curvature of the membrane and overall area available for protein binding [63]. Because α -syn has been shown to localize to synaptic vesicles *in vivo* [91], it is useful to evaluate binding and aggregation by using membrane mimics that recapitulate the lipid environment in a cell.

One strategy is to reconstitute biological membranes and proteins into GUVs. GUVs are appropriate models for cells because of their size (few tens of microns), which is advantageous since they can be easily tracked using light microscopy, as opposed to SUVs which are restricted to electron microscopy [119]. Since GUV preparation by electroformation was first developed in 1986 [120], changes in the protocol have expanded the types of lipids and proteins that can be incorporated [119]. The observation of phase separation in GUVs containing a mixture of saturated and unsaturated lipids with cholesterol has been achieved using fluorescence microscopy [121]. In addition, protocols that induce asymmetry into synthetic lipid vesicles using cyclodextrin to promote lipid exchange have been developed [122]. The embedding of proteins into lipid vesicles has also been demonstrated [119], including the incorporation of SNARE proteins into GUVs with phase-separated domains [123]. Furthermore, it is possible to make GUVs out of native cellular membranes [124, 125]. For example, Montes *et al.* developed a protocol that reconstitutes red blood cell membranes extracted from human blood into GUVs, remarkably maintaining the integrity of the membrane throughout the electroformation process and therefore preserving the asymmetry of membrane proteins and glycosphingolipids in the bilayer [125]. Furthermore, de la Serna *et al.* formed GUVs made from pulmonary surfactant, the material that lines the surface of lung tissue [124].

We consider the effort towards using membranes obtained from living cells as especially important when taking into account the changes in lipid composition that occur during the process of aging. Studies have reported a reduction in polyunsaturated lipids and an increase in anionic lipids (namely PG, PS, and PA) as a function of age [115]. Lipidomic studies have also shown that cell membranes become more rigid in PD patients compared to controls, with an increase in saturated fatty acids (16:0 and 18:0) and a decrease in polyunsaturated fatty acids [126]. As PD is an age-related disease, the use of membranes extracted from aged

mice and human tissue is important to further our understanding of α -syn-membrane interactions, aggregation, and remodeling in a biological context.

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Highlights

- Membranes influence the conformation and aggregation mechanism of α -syn
- Stimulation or inhibition by anionic lipids depends on relative bound/free protein
- Phosphatidylcholine lipids suppress α -syn amyloid formation despite weak binding
- α -Syn remodels phospholipid membranes independent of surface charge
- An intimate relationship between membrane remodeling and amyloid formation exists

Figure 1.

TEM image of N27 rat dopaminergic cell. Lipid compositions (% mol of total phospholipid) of the inner and outer plasma membrane [74], mitochondria [71], lysosomes/late endosomes [75], and synaptic vesicles [76]. Lipids are arranged in a counter-clockwise order starting with phosphatidylcholine (PC, red), and followed by phosphatidylethanolamine (PE, orange), phosphatidylserine (PS, light green), phosphatidylinositol (PI, black), phosphatidic acid (PA, blue), sphingomyelin (SM, cyan), bis(monoacylglycero)phosphate (BMP, lavender), cardiolipin (CL, green), cholesterol (Chol, magenta), and other lipids (Other, gray).

Figure 2.

α-Syn remodeling of POPG SUVs and effect on amyloid formation. Representative TEM images of POPG vesicles alone (A), α-syn + POPG SUVs at L/P = 1 (B) and L/P = 50 (C) before (T_0) agitation and fibrils formed by α-syn alone (D) and by α-syn + POPG SUVs at L/P = 1 (E) and L/P = 50 (F) after (T_{end}) agitation (70 μM α-syn in 20 mM MOPS, 100 mM NaCl, pH 7, 37 °C). Length of scale bar is 100 nm. Far-UV CD spectra of α-syn alone (G), L/P = 1 (H), and L/P = 50 (J) at T_0 (black) and T_{end} (red). (K) Aggregation kinetics of α-syn in the absence (red) or presence (green, L/P = 1; black, L/P = 50) of POPG SUVs monitored by ThT fluorescence.

Figure 3.

α -Syn aggregation in the presence of POPC SUVs. **(A)** Representative aggregation kinetics monitored by ThT fluorescence of α -syn (70 μ M in 20 mM MOPS, 100 mM NaCl, pH 7) in the absence (black) or presence of POPC ((L/P = 1 (red), L/P = 10 (blue), L/P = 50 (green)) at 37 °C with agitation. **(B)** Far-UV CD spectra of α -syn before (lines) and after (symbols) aggregation in buffer (black) or with POPC at L/P = 50 (green). TEM images of α -syn after aggregation with POPC at L/P = 1 (**C**), L/P = 10 (**D**), and L/P = 50 (**E**), with orange and yellow arrows denoting α -syn fibrils and membrane tubules, respectively. Length of scale bar is 100 nm.

Table 1.Compilation of α -syn aggregation data with anionic lipid membranes.

Stimulation at only low L/P (< 8)				Stimulation at low and high L/P (50)			
<i>Lipid composition</i>	<i>L/P</i>	<i>Aggregatio conditions</i>	<i>Ref</i>	<i>Lipid composition</i>	<i>L/P</i>	<i>Aggregation conditions</i>	<i>Ref</i>
DLPS	<5	20 mM NaPi, pH 6.5, 30 °C, quiescent	[98]	POPC/POPA (1:1 or 3:1)	50	20 mM MOPS buffer, 100 mM NaCl, pH 7, shaking, glass beads	[57]
					50	50 mM NaPi buffer, pH 7.4, 37 °C, shaking	[99]
DMPS	<8		[98, 100]	DOPC/GM1 (90:10)	17	10 mM MES buffer, 140 mM NaCl, pH 5.5, 37 °C, quiescent and shaking	[101]
DPPC/DPPA (1:1)	<1	20 mM Tris-HCl buffer, 0.1 M NaCl, pH 7.5, 37 °C, shaking	[102]	DOPC/GM3 (90:10)	17		
DPPC/DPPG (1:1)				Mouse exosomes	33		
DPPC/DPPA (1:1)				Rat synaptosomes	20	PBS buffer, pH 7.4, stirring	[103]
Brain PC/PS (70:30)	<20	PBS buffer, 1 M NaCl, pH 8.45, 37 °C, shaking	[104]				
DOPC/Sphingosine (18:15)	<7	10 mM MES buffer, 140 mM NaCl, pH 5.5, 37 °C, shaking	[101]				