Disruptive membrane interactions of alpha-synuclein aggregates

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A B S T R A C T

Alpha synuclein (αS) is a ~14 kDa intrinsically disordered protein. Decades of research have increased our knowledge on αS yet its physiological function remains largely elusive. The conversion of monomeric αS into oligomers and amyloid fibrils is believed to play a central role of the pathology of Parkinson’s disease (PD). It is becoming increasingly clear that the interactions of αS with cellular membranes are important for both αS’s functional and pathogenic actions. Therefore, understanding interactions of αS with membranes seems critical to uncover functional or pathological mechanisms. This review summarizes our current knowledge of how physicochemical properties of phospholipid membranes affect the binding and aggregation of αS species and gives an overview of how post-translational modifications and point mutations in αS affect phospholipid membrane binding and protein aggregation. We discuss the disruptive effects resulting from the interaction of αS aggregate species with membranes and highlight current approaches and hypotheses that seek to understand the pathogenic and/or protective role of αS in PD.

1. From generic amyloids to amyloids of alpha synuclein

1.1. A time capsule; the discovery of alpha synuclein (αS) amyloids

The term “amyloid” as used today in the biochemistry and biophysics community, refers to fibrillar protein structures with a typical width of 5–10 nm that have a characteristic cross β-sheet secondary structure. Deposits of these fibrils in or outside cells are also called plaques and exhibit positive birefringence under polarized light. Less than two centuries ago, the normal starch-like constituent in plants that can be visualized using iodine staining was referred as amyloid [1]. Sixteen years later, intracellular deposits in brain tissues stained positively by iodine were therefore thought be carbohydrates and their relevance to disease was believed to be circumstantial [2]. It was in 1859 when Friedrich and Kekulé showed that amyloid plaques mainly contained proteins, that the research attention shifted to the study of amyloids as protein aggregates [3]. Subsequently, the presence of amyloids was thought to be a consequence of aging and disease conditions including cancer and many auto-immune diseases rather than a cause of disease.

In 1912, Friedrich Lewy described proteinaceous inclusion bodies in neurons of patients suffering from Parkinson’s disease (PD) [4]. These spherical and thread like inclusions in neuronal bodies, now called Lewy bodies (LBs) and Lewy Neurites [5], would later be recognized as a pathological hallmark of PD. > 4 decades later, Cohen and Calkins showed, using electron microscopy, that the proteins in these inclusions had a characteristic fibrillar ultra-structure. The dimensions of the fibrils ranged between 5 and 12 nm in width and were referred to as amyloid fibrils [6]. Further studies showed that the protein fibrils, irrespective of their origin, were composed of even thinner structures which were named protofibrils [7,8]. The following year, the basic structure of the protein fibrils was shown to be a β-pleated sheet [9]. We shall henceforth refer to such fibrils as amyloids. Since then, numerous reports, using high resolution techniques like cryo-electron microscopy (Cryo-EM), solid state nuclear magnetic resonance (ssNMR), magic angle spinning nuclear magnetic resonance (MAS-NMR), x-ray fiber diffraction (XRD) and two-dimensional infra-red spectroscopy (2D-IR), have fueled the structural understanding of the amyloid state of numerous proteins (Fig. 1). It is now known that amyloid formation is not a rare phenomenon associated merely with diseases but rather it defines a structurally and thermodynamically stable form of proteins. The amyloid fibril is a low energy alternative to the native state, which can in principle be adopted by many, if not all, polypeptide sequences [10]. There are now about 50 known disorders with widely disparate symptoms, each of which involve the conversion of normally soluble and functional peptides/proteins that possess either a distinct secondary structure or are intrinsically disordered into amyloid fibrils [11]. If, and how, the transition to the amyloid state is a

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consequence or a cause of these diseases is still debated. Here we will review the possible causative relation between the formation of amyloid aggregates of the protein alpha-synuclein (αS) and the development of Parkinson's disease. αS is a soluble intrinsically disordered protein (IDP) that is abundantly present in neurons. The protein is associated with intracellular membranes and membrane binding is one of its major putative functional roles. Membranes are however also implicated as the main target of toxic interactions. In reviewing the role of αS aggregates in the development of PD, we will therefore focus on the possible membrane damage caused by amyloid aggregation or aggregates.

The main protein found within amyloid deposits in LBs of Parkinson's disease patients is αS. This protein was initially found in the synapse and in the nuclear envelope of *Torpedo californica* [12] in 1988.

**Fig. 1.** Timeline of selected events relating to αS and amyloids.
The connection of αS to neurodegenerative disorders was not established until the discovery of a distinct peptide component in the amyloid plaques in the brains of Alzheimer’s disease (AD) patients [13]. This ~35 amino acid peptide component was referred to as the non-Αβ component (NAC). The NAC was generated from the proteolytic cleavage of a 140 amino-acid protein called NAC precursor protein, NACP. NACP was later shown to be a homologue of human αS [14,15]. The NACP peptide itself turned out to be highly amyloidogenic and antibodies raised against synthetic NAC peptides recognized amyloid fibrils in AD plaques [13,16]. NACP was subsequently described as a natively unfolded protein [17] that loosely associates with synaptic vesicles [15,18] and expresses abnormally in the presynaptic terminals of neuronal cells of the central nervous system in patients afflicted with AD [15,19] (Fig. 1).

The link between αS/NACP and PD was established in 1997, when a point mutation (A53T) in the αS gene was identified in families with autosomal dominant PD [20]. This observation was followed by the seminal discovery of αS as the major component of LBs in brain tissues of sporadic PD cases [21] and the positive immunostaining of these LBs with anti-NACP antibodies [22]. The following year, it was shown that αS was present in LBs as 5–10 nm thick fibrils. In these fibrils αS monomers organized in β-strands that are oriented parallel to the fibrillar axis [23]. These discoveries triggered tremendous scientific interest in αS and the possible causality of αS aggregation and the development of PD. The following year, reports appeared showing that disease-related familial mutants of αS accelerated fibril formation. The accelerated formation of fibrils suggested a direct link between aggregation of αS and the development of early onset PD [24]. Triplication of the αS gene was also shown to cause early onset PD [25] and mRNA levels of αS were found to be consistently elevated in brains of both early onset familial PD [26] and idiopathic PD patients [27]. αS amyloid deposits were also detected in several other neurodegenerative diseases, including multiple system atrophy (MSA), amyotrophic lateral sclerosis (ALS), dementia with Lewy bodies (DLB) and Hallervorden-Spatz syndrome. These diseases are now collectively referred to as synucleinopathies [28]. It is worthwhile to note that often neurodegenerative disorders consist of a continuum of amyloid-related proteinopathies i.e. besides amyloid deposits of αS other amyloidogenic proteins also accumulate amyloid aggregates [29].

1.2. Alpha-synuclein

The synuclein family consists of α-synuclein, β-synuclein, and γ-synuclein. These small highly conserved proteins consist of an amino-terminal domain with a variable number of 11-residue repeats, range from 127 to 140 amino acids in length and are 55–62% identical in sequence. Full length monomeric αS is a 140 amino-acid protein. Its sequence can be divided into three major regions: the N-terminal region, the NAC region and the C-terminal region. The N-terminal region comprises of amino acid residues 1–60 and can organize into a membrane binding amphipathic helix. The hydrophobic NAC region comprises residues 61–94 and can organize into cross β-sheets. The NAC region is therefore required for aggregation into amyloid fibrils. Part of the NAC region is also involved in membrane binding and seems to define the affinity of αS for lipid membranes [30]. The negatively charged C-terminal region comprises residues 95–140 and is highly unstructured and weakly hydrophobic. It experiences weak and transient interactions, if any, with model lipid membranes [30] (Fig. 2). The C-terminal region remains unstructured in the amyloid fibril and truncations of this region have been shown to modulate aggregation of αS into amyloids [31–33]. The C-terminal region contains sites that can be post-translationally modified by e.g. nitration and phosphorylation [34–36]. Whether these modifications are related to function or result in functional disorders is not known. It is however clear that they affect the net charge of the C-terminal region and have profound impact on the αS aggregation and membrane binding as discussed in the later section. β- and γ-synucleins differ from α-synuclein by virtue of the deletion of 11 amino acids in the NAC region and a shorter C-terminal domain respectively. The β- and γ-synuclein proteins are not found in LBs, but both are associated with hippocampal axon pathology in Parkinson’s disease and dementia with LBs [37].

Till today, 5 additional point mutations, besides the aforementioned A53T, in the αS gene have been identified that lead to protein variants found in familial forms of PD: A30P [38], E46K [39], A53E [40], H50Q [41] and G51D [42]. Interestingly, all known disease-linked point mutations reside in the membrane binding N-terminal region of αS suggesting that changes in the membrane interactions of monomeric αS are relevant to PD. Although there seems a clear link between mutations in αS and the onset of PD, the role of αS in the disease etiology is constantly debated and finding the mechanism(s) responsible for cellular damage in PD remains a holy grail.

In PD, αS assembles into oligomeric aggregates and amyloid fibrils. Oligomeric protein aggregates have been suggested to play a pivotal role in PD and are often referred to as the most toxic aggregate species responsible for cell death [43–46]. Oligomeric αS was first observed in vitro studies on the aggregation of recombinantly produced αS [24]. Subsequently, αS oligomers were reported to be present in postmortem brain of patients with PD [47], cell line cultures [48–50] and neurons [51]. High oligomer concentrations have been associated with disease, but the toxic mechanism responsible for oligomer induced cell death is still under debate [47,52]. The observation that different types of oligomers exist and that they possibly each contribute differently to toxicity in PD complicates this debate [53]. The best characterized αS oligomer species in vitro studies appears in simple buffer solution without aggregation stimulating additives such as dopamine metabolites, fatty acids, or heavy metal cations (reviewed in [54]). This oligomer consists of approximately 30 monomers [55–57]. The oligomer contains some β-sheet structure [58–60], and the C-terminal region of the protein remains flexible and solvent exposed [60,61]. The β-sheet structure of oligomeric αS species has been reported to be distinct from that of fibrils; oligomers were observed to contain antiparallel β-sheets whereas in αS fibrils monomers are typically organized parallel β-sheets [62]. The N-terminal region of the protein in the oligomers retains some of its membrane binding properties [63]. For many of the proposed toxic mechanisms that involve oligomers, the ability to interact with membranes seems to be essential. These mechanisms include membrane thinning [43], pore formation [46], enhanced lipid flip-flop [64] and vesicle clustering [65].

High resolution structures of αS fibrils have been obtained from micro electron diffraction (microED) studies on αS segments [66], solid-state NMR studies [67–69] and more recently the atomic structure of the N-terminally acetylated αS full length and a truncation variant comprising of residues 1–121 was determined from Cryo-EM images [70,71]. However, whether the Greek-key topology reported in the aforementioned studies, is also adopted by αS in in vivo formed amyloid fibrils remains to be investigated. Structural characterization of αS amyloid fibrils that are formed in vivo or in vitro is complicated by the existence of different fibril polymorphs and the sensitivity of the fibril structure to the aggregation conditions used [72,73]. Structural polymorphs of αS fibrils have been shown to result in significantly different toxicities in neuronal cell cultures [68,74]. A recent study has shown that αS fibrils can be internalized, bind plasma membranes of both neuroblastoma cell lines and hippocampal primary neurons and induce cell death when besides fibrils αS monomers are present [75]. The proposed mechanisms of amyloid fibril-mediated membrane damage include membrane deformations and lipid extraction [76] and the activation of apoptotic cell-death pathways [75].
Not only the role of αS in PD but also the physiological function of αS still eludes us. However, several decades of research have resulted in important progress towards understanding its function as enumerated in the next section. It is now known that αS is predominantly found in the presynaptic terminals of neurons in the human brain. Lower in the next section. It is now known that important progress towards understanding its function as enumerated

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**Fig. 2.** Sequence encoded physicochemical properties of αS. A) Primary amino acid sequence with acidic (green), lysine (red) and aromatic (light blue) residues highlighted. B) Schematic representation of the main (functional) regions of αS. The amphipathic repeats housed in the membrane binding region are indicated in red, the Non-Amyloid β Component (NAC) region in blue and the acidic region in green. The right panel shows a pictorial representation of a disordered conformation of the protein.

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2. αS and phospholipid membrane interactions

αS binds model phospholipid bilayers with equilibrium dissociation constants (K_d) in the micromolar range [89]. This relatively low affinity of αS to model membranes may seem counter-intuitive to a large fraction of the membrane binding N-terminal region of αS consists of imperfect 11-amino acid residue repeats that resemble those found in strongly membrane binding apolipoproteins. These repeats contain a K (A/T)KEGV consensus that is consistent with the capacity to fold into an amphipathic helix [90] with a periodicity of 11 amino acids per 3 turns [91,92]. However, in contrast to what is observed for αS, the conformational energy landscape of apolipoproteins is characterized by a well-defined energy minimum that represents the folded state. The conformational energy landscape of IDPs such as αS is much more continuous, this means that αS loses conformational entropy upon binding. This loss of entropy decreases the free energy gain upon binding and thus increases K_d. In brain tissue and cell model systems, it is proposed that the relatively low affinity of αS for membranes allows for regulation and control over the distribution between the membrane bound and unbound form of the protein [93,94]. The possibility to reverse binding may for instance be important in regulating the lipid vesicle pool at the synapse [84,95,96]. Förster resonance energy transfer (FRET) studies indicate that there is a clear difference in conformation between cytosolic and membrane bound αS [97]. From experiments on brain homogenates, it was estimated that approximately 15% of αS in brains is associated with membranes [98,99]. This membrane associated αS is visible as distinct high-intensity puncta in fluorescence microscopy images [97]. The high intensity puncta represent αS bound to small vesicles. Photobleaching studies on αS-GFP expressing differentiated SH-SYSY cells show that approximately 70 αS molecules are associated with each vesicle [100]. The number of αS per vesicle is high enough for a direct role in curvature generation and membrane remodeling [101,102]. However, this number is also strikingly similar to the number of synaptobrevins, a putative interaction partner of αS per vesicle [103]. This suggests that membrane bound αS has a dual role, it may both directly contribute to generating curvature and act as a non-classical chaperone for SNARE-complex assembly [87].

Association of the unstructured monomeric αS with model phospholipid membranes is accompanied by a dramatic increase in the helical content of the protein from 3% to ~80% [90]. In a seminal report by Eliezer and colleagues in 2001, membrane bound-αS was shown to assume a bipartite structure with residues 1–102 bound to SDS micelles while the remaining residues stay disordered [104]. The amphipathic helix of membrane bound αS is oriented parallel to the membrane surface. The conformation of the membrane-bound helical segment of αS has been a matter of debate as to whether it is a fully extended helix [91,105,106], a broken helix [107,108] or co-existence of both as shown in vitro [109] and in vivo [97]. It seems that all these helical architectures are possible, probably due to variable positions of the break in the helix [105]. The distribution of αS over the extended and broken helix conformations may depend on the lipid composition of the membrane. Besides the lipid composition, the surface concentration of the protein seems to have an effect on the conformation. It has been shown by NMR that αS binds to lipid bilayers via distinct binding modes [110] that can be tuned by changing the lipid-to-protein ratio. Nucleation of αS aggregation may be assisted by membranes that bind the monomeric form of the protein. The critical aggregation concentration of αS in solution has been reported to be ~10–30 μM [111,112]. In the presence of negatively charged lipid bilayers aggregation is however...
observed at much lower concentrations, far below the concentrations reported for αS in cells [113–115].

2.1. Physicochemical properties of lipids aiding αS membrane interaction

There is now strong evidence that the equilibrium dissociation constant and thus the population of the membrane-bound state of αS is not only affected by the sequence encoded (structural) properties of αS but also by the physicochemical properties of the phospholipid bilayer, such as anionic charge density, curvature and packing defects, phase state and degree of hydration [90,107,116–118] [119]. Below we will address how the binding of αS monomers, oligomers and fibrils is affected by physicochemical membrane properties. In reviewing the interaction of oligomers with lipid bilayers we will focus on oligomers prepared in simple buffer solutions in the absence of additives. For these types of oligomers, the relation between oligomer interactions and the physicochemical membrane properties of membranes is relatively well characterized.

2.1.1. Membrane charge

2.1.1.1. Monomers. Although net negatively charged, monomeric αS binds membranes of anionic phospholipids with much higher affinity than membranes composed of zwitterionic ones [89,120]. The preferential binding of αS to negatively charged membranes in comparison to net neutral membranes is attributed to attractive electrostatic interactions between the membrane surface and multiple positively charged lysine residues found in the N-terminal region of αS [121]. In the membrane bound conformation, these lysines are lined up at the boundary between the hydrophobic and hydrophilic part of the amphipathic helix. The higher affinity for negatively charged membranes thus likely originates from the free energy gain resulting from both helix insertion and attractive electrostatic interactions. The involvement of attractive electrostatic interactions between αS and negatively charged membranes is corroborated by studies showing reduced αS binding to anionic lipid vesicles with increasing ionic strength [122]. The increased ionic strength not only screens the membrane charge but also changes the conformational space probed by the protein. The changes in preferred protein conformations with ionic strength are a possible cause for the reduced binding affinity. The binding of monomers seems to be very sensitive to the membrane surface charge density. The binding of αS to bilayers of phosphatidic acid (PA) and phosphatidylinositol(P) from bovine liver lipids that have a slightly higher negative charge at neutral pH, is high compared to binding to bilayers of phosphatidylserine (PS) and phosphatidylglycerol (PG) [89,118,122,123]. For membranes composed of mixtures of zwitterionic- and anionic phospholipid the affinity for αS decreases with increasing fraction of zwitterionic lipids [89,120].

2.1.1.2. Oligomers. Like monomers, the binding of αS oligomers to membranes depends strongly on the presence of negatively charged lipids. The accumulated data from several experiments indicates that for binding to occur to giant unilamellar vesicle (GUV) or large unilamellar vesicle (LUV) membranes at least 20% of the lipids in the membrane must carry a net negative charge [58,124,125]. The ability of the negatively charged oligomers to specifically bind net negatively charged lipid bilayers suggests that, like observed for monomers, oligomer binding is mediated by the lysine residues that reside at the membrane interface when the N-terminal region of the protein is organized into an amphipathic α-helix. The organization of the N-terminal residues of (part of the) monomers in the oligomers into membrane bound amphipathic helices is supported by several experimental findings. In the presence of small unilamellar vesicles (SUVs), the helical content of αS oligomers has been observed to increase [126,127]. Tryptophan fluorescence experiments on single tryptophan mutants of αS indicate that upon membrane binding the environment experienced by residues in the N-terminal region of the protein becomes more hydrophobic [61]. Studies on N-terminal deletion mutants also point at the importance of the N-terminal region of αS for the binding of αS oligomers to membranes [63]. Membrane leakage experiments indicate that, as expected for the binding of negatively charged oligomers to negatively charged membranes, oligomer binding is facilitated at higher ionic strengths [59].

2.1.1.3. Fibrils. Part of the membrane binding N-terminal region of αS remains unstructured and solvent exposed when the protein is organized in amyloid fibrils. Both in vitro and in vivo experiments suggest that this N-terminal region of αS might retain its membrane binding properties in the fibril state [59,128,129]. Solid-state nuclear magnetic resonance (ssNMR) studies on αS fibrils formed in the presence of negatively charged phospholipid vesicles show that the overall fold of the αS amyloid fibril is not affected by the presence of anionic phospholipid vesicles. However, there are major structural differences between the N-terminal domains of αS in fibrils formed in the absence or presence of vesicles [130,131]. The ability of fibrils to bind vesicle membranes may however depend on the assembly mechanism and the associated differences in fibril polymorphs that are formed. The interaction of pre-formed fibrils with phospholipid vesicles seems to differ from the interaction of fibrils that were assembled in the presence of liposomes. In vitro studies indicate that pre-formed αS amyloid fibrils do not bind vesicles of the zwitterionic lipid POPC and show only weak adherence to negatively charged vesicles (50% POPG/50% POPC). However aggregation of αS in the presence of the same POPG/POPC vesicles results in fibrils that strongly adhere to the membrane and deform GUVs [76]. Besides deforming vesicles, the fibrils growing on the anionic lipid bilayer surface can also extract lipids from the bilayer resulting in protein/lipid coaggregates [76,129,132]. The lipid composition dependent interactions between αS fibrils and membranes is reflected in the membrane damage caused by fibrils that appear at the membrane surface. Calcein leakage measurements show that the growth of αS fibrils in the presence of vesicles does not impair the integrity of phospholipid vesicles that have a low affinity for αS [132], while fibril growth does cause dye leakage with increasing fraction of anionic lipids in membrane [59,133]. A recent study has shown that αS fibrils can bind plasma membranes of both neuroblastoma cell lines and hippocampal primary neurons and induce cell death when αS monomers are additionally present [134].

2.1.2. Membrane curvature

2.1.2.1. Monomers. Membrane binding of αS involves a conformational transition from a disordered state to an amphipathic α-helix and the insertion of this helix into the lipid bilayer. Binding of αS to membranes therefore depends on membrane tension and the presence of packing defects [90]. The sensitivity to the presence of packing defects is probably responsible for the curvature sensitivity of αS binding. αS has been observed to bind SUVs with much higher affinity than larger vesicles. Moreover, whereas αS does not bind LUVs and GUVs of zwitterionic lipids it does bind SUVs of zwitterionic phospholipids [88]. In SUVs with a diameter of 25–40 nm the vesicle diameter starts to approach the lipid bilayer thickness which results in an imperfect packing of lipids and the formation of membrane defects. These packing defects become even more pronounced when the membrane of these SUVs is in the liquid-ordered or gel phase. The exposed hydrophobic surface at defects is probably responsible for the observed higher binding affinity of SUVs compared to LUVs and GUVs. Similarly, increasing the fraction of inverted cone-shaped lipids with packing parameter2 $P > 1$, like phosphatidyethanolamine (PE) in anionic lipid vesicles enhances binding of αS [59,122].
Interestingly, αS not only binds preferentially to curved lipid membranes but has also been shown to induce curvature and cause remodelling of lipid membranes [102,135]. The binding of αS has been shown to convert multilamellar vesicles and GUVs but not SUVs of physiologically relevant phospholipid compositions into tubules and smaller vesicles [101]. Separate studies showed that membrane remodelling of supported lipid bilayers by αS strongly decreases with increasing anionic lipid content [136]. The membrane remodelling effect observed in these studies is likely a result of the partial insertion of an amphipathic helix in the outer layer of the membrane [101], although the steric pressure exerted by the solvent exposed C-terminal regions of the protein may also contribute at high surface densities [100,137,138]. Considering the weak binding of monomeric αS the observed increase in the tubulation of zwitterionic supported lipid bilayers is puzzling [136].

2.1.2.2. Oligomers. Although the binding of αS oligomers to SUV, LUV and GUV membranes was not directly compared in a single series, binding seems to depend on curvature. Oligomers have been reported to bind DOPC SUVs but not POPC GUVs [124,139]. This may however not result from the ability of oligomers to sense curvature. The inability of αS oligomers to bind PE SUVs [124,139] suggests that binding to SUVs of zwitterionic lipid bilayers results from packing defects and depends on the exposure of hydrophobic surface. In some, but not all cases the binding of oligomers results in impaired membrane integrity. Whereas αS oligomer binding requires the presence of negatively charged phospholipids, disruption of the lipid bilayer depends on the accessibility of the bilayer hydrocarbon core [59]. This accessibility of the hydrocarbon core cannot only be modulated by changing the membrane curvature and creating packing defects, it also depends on the packing parameters of the lipids in the bilayer. In a series with increasing acyl chain unsaturation; POPG, DOPG, 18:2 PG, the packing parameter P increases and lowers oligomer concentrations are required for calcine to leak out of vesicles composed of these lipids. The addition of the more cone shaped (P < 1) lysolipids improves the packing of lipids in the bilayer and decreases the vulnerability of the membrane to oligomer binding induced impairment of membrane integrity [59]. The addition of cholesterol also improves the packing of the lipid bilayer and has a similar effect [59,139]. In general, it has been observed that although lipid bilayers of more complex physiologically relevant membrane compositions bind oligomers, such membranes are less vulnerable to binding induced leakage of vesicle content. The dye release caused by αS oligomer binding from vesicles of brain extract [139,140], or vesicles mimicking the composition of the plasma membrane or mitochondrial membrane [127], is low compared to the release from vesicles containing only negatively charged lipids. There are however differences, SUVs mimicking the composition of the inner mitochondrial membrane were more susceptible to permeabilization by αS oligomers than model plasma membranes [127,141]. Whereas the main acidic phospholipid in the plasma membrane is the phosphatidylserine, the inner mitochondrial membrane is enriched in cardiolipin; a diphosphatidylglycerol carrying two negative charges. The sensitivity of oligomer binding to membrane composition and charge density may make the membranes of specific organelles more vulnerable to oligomer induced damage than others [142,143].

2.1.2.3. Fibrils. To the best of our knowledge, membrane curvature dependence of αS fibril binding has not been investigated yet. However it has been shown that when monomeric αS is aggregated in presence of membranes, membrane bound fibrils can deform both SUVs [129] and GUVs [76].

2.1.3. Membrane phase state

2.1.3.1. Monomers. Studies on permeabilized cells from rat brains suggest that αS is associated with lipid rafts in the cellular membrane [144]. These lipid rafts are defined by their resistance against treatment with anionic detergent and are typically enriched in cholesterol, sphingolipids and specific phospholipids. In membrane model systems, these rafts are often mimicked using binary or tertiary lipid mixtures containing phase separated liquid ordered (Ld) domains. In contrast to what has been observed in cellular membranes, αS typically binds the anionic lipids in the liquid disordered (Lo) phase of the Ld phase in these phase-separated systems [120]. However, it is important to keep in mind that there is no direct evidence that the lipid rafts in cells are Lo phases. Cellular membranes are not equilibrium structures and raft formation may result from various internal driving forces rather than from phase separation as discussed in [145].

The affinity for different membrane phase states has been studied in more detail in simpler membrane model systems. The phase state of membranes e.g. Ld, Lo, or gel state, depends on lipid composition and temperature. For anionic membranes, the binding affinity of αS is higher for SUV membranes with lipids in the fluid phase than for membranes in the gel phase [146]. For SUVs and LUVs composed of zwitterionic lipids the effect is reversed, αS dissociates from these vesicles upon the phase transition to the Ld state [89]. The phase transition temperature and therefore binding of αS depends strongly on the length of and degree of saturation in the acyl chain of the lipids. However, also above the Ld to Lo transition temperature the binding of αS is affected by the degree of saturation in the acyl chains of the lipids in the bilayer. Compared to membranes composed of saturated lipids, binding of αS to membranes of unsaturated lipids of the same length is higher [89,120]. The sensitivity of αS binding to the membrane composition and phase state of membranes of zwitterionic lipids indicates that exposed hydrophobic area enhances binding. Defects in membranes below the phase transition temperature and the less tight packing of unsaturated compared to saturated lipids in membranes above the phase transition temperature reduce the screening of the apolar acyl chains in the bilayer. Membrane defects and decreased lipid packing thus both enhance the insertion of the amphipathic helix of αS into the bilayer.

It is important to realize that the phase state and lipid packing not only affect binding of αS but that binding also changes these parameters [107,116,147]. EPR and fluorescence spectroscopy show that binding of αS to SUVs of zwitterionic lipids led to an increased chain melting temperatures and to enhanced cooperativity of the phase transition [148]. By contrast, CD and DSC results suggest that αS binding stabilizes the fluid phase of bilayers of negatively charged lipids. Experiments from our own lab indicate that αS organizes in clusters at protein:lipid ratios higher than 10 [113]. At such higher ratios, all αS binding sites on the membrane surface are occupied, the average distance between two membrane-bound monomers is small and inter-protein collisions result in cluster formation. Clustering of membrane-bound αS at high surface concentration is a consequence of a complex interplay mainly between attractive hydrophobic interactions, resulting from the solvent exposed hydrophobic patches on the membrane-bound αS, and repulsive electrostatic interactions, resulting from the negatively charged unstructured solvent exposed C-terminal region of αS. The correlation between the changes in lipid diffusion and DPH anisotropy, suggests a concerted process where the formation of clusters leads to a closer packing of lipids and a decrease of the effective lipid diffusion [149]. The relation between the effective lipid diffusion and area of the αS clusters (Fig. 3) is nontrivial as it does not result from a direct interaction between proteins and lipids. Although αS mainly binds to anionic lipids, clustering also affects effective lipid diffusion of zwitterionic lipids. This indicates that the clustering of αS on lipid membranes induces ordering of underlying lipids. In the Ld membranes studied, we observed an overall increased lipid packing order with increasing size of the lipid clusters (Fig. 3).

For membranes in the Ld state, the opposite has been reported; αS decreases the packing order in lipid mixtures that form Ld membranes [51]. Cholesterol affects the packing order in lipid membranes in a similar way; it increases the packing of membranes in the Ld state and
makes membranes in the Lo state more fluid. Clustering of proteins and ordering of lipids into membrane microdomains are both known to be involved in protein function and this interplay forms the basis for many observations, that αS may play a role in the regulation of lipid packing in cell membranes.

2.1.3.2. Oligomers. Preferential binding of αS oligomers to membranes in the Lo over the Ld state was studied in GUV membranes with coexisting Ld and Lo domains. In these experiments oligomers preferentially accumulated in the Ld domains [124]. Additionally, vesicles of anionic lipids in the L0 state were not able to bind oligomers [124]. The close packing of lipids in the Lo state probably preferentially accumulated in the Ld domains [124]. Additionally, surface charge is high enough. Considering the sensitivity of oligomers mediated by the formation of micelle like clusters at the surface of αS aggregation is not accelerated in the presence of vesicles of less soluble phospholipids, the low solubility of these lipids cannot prevent lipid extraction when membrane bound αS is incorporated in fibrils [132]. Aggregation of membrane bound αS has been reported to result in extraction of lipids and the appearance of protein/lipid co-aggregates [76,115,129,132,153]. This lipid extraction can consume the complete vesicle bilayer and is therefore disruptive.

2.1.3.3. Fibrils. To the best of our knowledge, the binding of preformed fibrils to vesicles with membranes in different membrane phase states has not been investigated yet. However, when fibrils were formed in the presence of net negatively charged SUVs or GUVs large scale vesicle deformations were observed [76,129]. Fibrils at the membrane surface cause the initially spherical GUVs to become faceted. This faceting may indicate that the binding of fibrils causes the membrane to become more rigid or to develop rigid domains. However, dye leakage studies and the distribution of dyes which preferentially accumulate in Ld over Lo domains indicated that the tight coupling to a mesh work of fibrils, rather than a rigidification of the membrane, is the more likely cause of the observed vesicle shape changes [76].

The aggregation of αS on or in the presence of membranes seems to be affected by the membrane composition. Despite the fact that monomeric αS binds with similar affinities to model membranes composed of different negatively charged phospholipids, it was shown recently that the aggregation of the protein is enhanced only in the presence of membranes composed of lipids with the shortest hydrocarbon chains (Fig. 4) and hence highest solubility [146,150]. Membranes composed of phospholipids with 16 or 18 carbons in each acyl chain including DOPE, DOPC, DOPS, POPE cannot prevent lipid extraction when membrane bound αS is incorporated in fibrils [132]. Aggregation of membrane bound αS has been reported to result in extraction of lipids and the appearance of protein/lipid co-aggregates [76,115,129,132,153]. This lipid extraction can consume the complete vesicle bilayer and is therefore disruptive.
In summary, it seems that the interactions of αS monomers, oligomers and fibrils with phospholipid membranes respond similarly to the membrane’s physicochemical properties. Besides the physicochemical properties of membranes discussed above, interactions between αS and membranes are also influenced by the chemical composition of the membranes and the presence of specific lipids. How the chemical composition of membranes affects the aggregation of αS has recently been reviewed by [153] and will not be addressed here.

Most of the investigations on the relation between the physicochemical properties of membranes and the interactions with different αS species have been performed on the non-acetylated version of αS while in vivo most αS contain this post-translational modification. Do monomeric, oligomeric and fibrillar N-terminally acetylated αS species respond differently to changes in membrane properties? Will N-terminal acetylation of αS proteins containing one of the familial PD substitutions change our view on αS-membrane interactions and αS-mediated pathology in PD? Addressing these questions may bring us a step closer to understanding the role of αS-membrane interactions in PD.

2.2. Post-translational modifications and mutations in αS influencing membrane interactions

In vivo, both the monomeric and fibrillar form of αS have been observed to be post-translationally modified. The reported post-translational modifications (PTMs) include acetylation [34,79,88,154], phosphorylation [34,155–157], methionine oxidation [158], N-terminal 2.2.3. Oxidative modifications: tyrosine nitration and methionine oxidation

Their oxygen consumption rate makes dopaminergic neurons quite susceptible to oxidative stress [177]. Under conditions of oxidative stress all four tyrosines in αS (Fig. 5) can be nitrated in vitro and this nitration has also been observed in LBs from the brains of PD patients [159,178–180]. In vivo nitration of Tyr-39 or the C-terminal tyrosines leads to a decreased binding of αS to membranes composed of anionic lipids [181]. The sensitivity of membrane binding to C-terminal nitration is interesting; the C-terminus is not thought to interact directly with membranes. The sensitivity of αS-membrane interactions to nitration suggests a long-range allosteric communication between the C-
truncation does not indicate that the choice of the model system is critical. Fl
reported that deletion of the \[189\]. Concomitant with the lower binding a
membranes but compared to truncations in the N-terminal region, they
therefore expected to negatively a
membrane interactions. Truncations of the N-terminal region are
found in healthy brain tissue and in cultured cells \[186–188\]. Thus, αS
truncation does not per se result in disease. The N-terminal region of αS
with its imperfect KTKEGV repeat motifs is important for establishing membrane interactions. Truncations of the N-terminal region are
therefore expected to negatively affect the membrane binding affinity of αS. The high number of negatively charged amino acids in the C-
terminal region may modulate interactions with negatively charged membranes but compared to truncations in the N-terminal region, they
are expected to have less effect on membrane binding. Initial studies reported that deletion of the first 10 N-terminal amino acids (residues 2–11) dramatically reduced binding to vesicles and yeast membranes \[189\]. Concomitant with the lower binding affinity, the overexpression of this deletion mutant was observed to be considerably less toxic than overexpression of the full-length protein. However, although less toxic in yeast, a subsequent study by the same group failed to reproduce the reduced membrane binding and toxicity in human neuroblastoma SHSY-5Y cells \[190\]. Differences in lipid composition, membrane fluidity and cytosolic factors between yeast and SH-SY5Y cells likely cause this apparent disparity. The observed differences thus indicate that the choice of the model system is critical.

In vitro studies show that the amino acids 1–25 in the N-terminal region of αS trigger membrane binding and helix folding \[191\]. However, the peptide 1–20 had extremely low affinity for anionic liposomes compared to 1–25 and full-length αS. The low affinity of the 1–20 peptide was attributed to the net zero charge of the peptide. However, experiments with other truncations variants show that a net positive charge is not sufficient for membrane recognition and helix folding. Instead the N-terminal residues impart some kind of conformational selectivity that allows for membrane recognition \[191\].

More recent studies have confirmed the roles of the N- and C-termi
minus in membrane binding. Using a small peptide tagged αS (αS-myc), it was shown that truncations in the unstructured C-terminus of αS have minimal effect on lipid binding in vitro and do not affect the presynaptic localization of αS in cultured cortical mouse neurons \[192\]. The C-terminus, however is essential for synaptobrevin-2 binding and promoting SNARE-complex assembly \[192\]. N-terminal truncations in αS, as expected, decreased membrane binding affinity to artificial lipid membranes and significantly decreased the presynaptic localization of αS in cultured cortical mouse neurons \[192\]. In general, the behavior of N- and C-terminal truncation variants to membrane binding are comparable in vitro and in vivo.

2.2.3.1. Truncation. Up to 15% of the αS in LBs contains N-terminal and C-terminal truncations \[183,184\]. In vitro experiments have shown truncations can have a large effect on amyloid fibril structure and morphology \[185\]. However, these PTMs of αS are also commonly found in healthy brain tissue and in cultured cells \[186–188\]. Thus, αS truncation does not per se result in disease. The N-terminal region of αS with its imperfect KTKEGV repeat motifs is important for establishing membrane interactions. Truncations of the N-terminal region are therefore expected to negatively affect the membrane binding affinity of αS. The high number of negatively charged amino acids in the C-terminal region may modulate interactions with negatively charged membranes but compared to truncations in the N-terminal region, they are expected to have less effect on membrane binding. Initial studies reported that deletion of the first 10 N-terminal amino acids (residues 2–11) dramatically reduced binding to vesicles and yeast membranes \[189\]. Concomitant with the lower binding affinity, the overexpression of this deletion mutant was observed to be considerably less toxic than overexpression of the full-length protein. However, although less toxic in yeast, a subsequent study by the same group failed to reproduce the reduced membrane binding and toxicity in human neuroblastoma SHSY-5Y cells \[190\]. Differences in lipid composition, membrane fluidity and cytosolic factors between yeast and SH-SY5Y cells likely cause this apparent disparity. The observed differences thus indicate that the choice of the model system is critical.

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2.2.3.2. SUMOylation. SUMOylation is a post-translational modification that involves the enzymatic addition of small ubiquitin-related modifier (SUMO) to proteins \[193\]. SUMOylation of αS has been shown to occur in HEK293 cells \[161\] and transgenic mice \[194\]. SUMOylation is thought to regulate protein-protein and protein-DNA interactions, it additionally promotes protein solubility \[195,196\]. SUMOylation has been observed on several lysine residues in αS, the most significant of which are lysines 96 and 102. Although the sorting of αS in extracellular vesicles is regulated by SUMOylation and membrane interactions may thus be important it remains to be investigated if in vivo or in vitro membrane binding of αS is influenced by SUMOylation \[197\].

2.2.3.3. Point mutations. All known familial PD point mutations resulting in single amino acid substitutions in αS (A30P, A53T, H50Q, G51D and E46K) reside in its membrane binding region (residues 1–60). Compared to unmodified αS, these single amino acid substitutions either promote or impede membrane-associations of αS \[110,118,198–200\]. Like observed for unmodified αS, the membrane binding affinities of the disease mutants increase with the fraction of negatively charged lipids in the membrane \[199,200\] or the presence of packing defects in small-sized vesicles \[118\]. Compared to unmodified αS, A30P shows a reduced binding affinity to artificial lipid membranes. This reduced affinity presumably results from the break in the membrane bound amphipatic helix caused by the presence of a Pro residue \[110,118,198,201,202\]. In accordance with this hypothesis, mutations of conserved residues in the membrane binding part of αS to prolines resulted in proteins with a reduced membrane binding affinity, with the exception of G41P \[192\]. For the PD related E46K mutation in αS, an increased affinity for binding phospholipid bilayers is observed which can be attributed to enhanced attractive electrostatic interactions resulting from the presence of an additional Lys residue \[110,118,198,201\]. There is currently some
inconsistency regarding data reported on the binding affinity of the A53T mutant to artificial lipid membranes. Compared with unmodified αS, A53T showed either reduced [199,203] or similar [110,198,201] binding affinities. The H50Q and G51D mutations have not yet been extensively studied. Compared to unmodified αS, the G51D mutation decreases the membrane binding affinity, but promotes the formation of partly helical states [204], while the H50Q mutation does not alter binding affinity ortho fold of the bound-state significantly [205].

Unmodified αS and disease related mutants bind to artificial lipid membranes using a very similar combination of electrostatic and hydrophobic interactions [118,199,200], but the presence of vesicles differently affects their aggregation into amyloid fibrils. In a study performed in the presence of exosomes derived from mouse neuroblastoma cells the fibrillation rates were observed to decrease in the order A53T > A30P > E46K > unmodified αS [206].

So far, all the PTMs in αS have been investigated separately, studies aimed at probing the combined effects of different coexisting PTMs are still lacking. For example, phosphorylation at Y125, ubiquitination at K96 or K102 or C-terminal truncations co-exist with pS129, although the modification sequence is unknown. Although PTMs in αS occur at multiple sites, certain positions can carry different modifications (Fig. 5). The functional/pathogenic consequences of combinations of PTMs also require further investigations.

3. The Janus face of αS

To explain the role of the conformational transition of αS from its disordered state to the oligomeric and fibrillar states in PD etiology, several mechanisms of αS mediated cellular toxicity and death have been postulated. These mechanisms can be grouped into two major classes: they either assume a gain of toxic function or a toxic loss of function. Both gain and loss of function mechanisms result in failure of the ubiquitin-proteasome system (UPS), oxidative stress, impaired axonal transport and mitochondrial damage [28,98,207–219]. Although not established, gain and loss of function mechanisms may not be mutually exclusive and possibly act synergistically. The interaction between different cellular processes, makes it difficult to pinpoint one single intracellular location or pathway that is affected in the early stages of PD and eventually results in neuronal cell death. Possibly many different pathways contribute to cell death in PD.

In current literature, the roles attributed to αS in PD come in opposites, in this respect the protein seems to resemble the mythological two-faced Roman god Janus. On one hand, in both familial/idiopathic cases of PD [28,74], the failure of cellular processes stems from familial/idiopathic αS point mutations and the overexpression and aggregation of αS into toxic pre-fibrillar, oligomeric and/or fibrillar species [66,68]. In this respect, soluble oligomeric species of αS have been argued to be the most potent toxic species in both in vitro and in vivo systems [53,216–219]. However, the observed transmission of the amyloid fold from one cell to another suggests a critical role for fibrils [220]. When particle instead of equivalent monomer concentrations are compared fibrils seem more toxic than oligomers [139]. The toxicity of fibrils seems to depend on the fibril strain [74] and the toxicity of LB formations on the cellular compartment in which fibrils accumulate [221]. On the other hand, a number of reports suggest that overexpression of αS per se and aggregation into soluble oligomeric (formed in presence of dopamine) and fibrillar species has a neuroprotective role in PD [28,213,222–224]. This neuroprotective role of αS is also supported by the fact that PD and the associated death of dopaminergic neurons can also occur without formation of LBs [225,226]. The neuroprotective role of aggregates and the absence of a clear correlation between the number of LBs and disease raises questions about the precise role of αS in PD. The Janus face of αS also becomes visible in the role the protein may play during oxidative stress. The scarcity of defense mechanisms against oxidative damage and a high oxygen consumption rate make dopaminergic neurons susceptible to insult by oxidative stress [177]. Oxidation of brain lipids, polysaturated fatty acids (PUFAs) and dopamine in particular, can affect normal functioning of cell membranes. These effects of oxidation increase with age and have been linked to PD [227,228]. Several studies have suggested that αS acts on cellular vesicles acts as an anti-oxidant; αS levels are elevated in neurons exposed to chronic oxidative stress and such neurons showed increased resistance to apoptosis [211]. The binding of monomeric αS to the lipid bilayer prevents oxidation of unsaturated lipids in vesicle models [229]. αS has been shown to be protective by interacting with excess dopamine and its oxidized products. In in vitro experiments the interaction of αS with dopamine results in the formation of non-toxic oligomeric intermediates [45,127] αS thereby prevents toxic interactions of lipid molecules with excess dopamine species [213,230]. However, the interactions of αS with dopamine are not only beneficial. Increased levels of dopamine have been shown to result in stabilization of protofibrillar species of αS that have been shown to be toxic to cells [231] [232]. αS plays a role in the regulation of dopamine transporters preventing dopamine accumulation in neuronal cells [213]. Deficiencies in dopamine packing into vesicles in mice results in an increase in cytosolic dopamine and a corresponding accumulation of αS which causes death of mice dopaminergic neurons [230,233]. The absence of αS in such mice correlated with better survival suggesting presence of αS aggravates dopamine mediated toxicity [233]. The Janus face of the protein may be a result of its ability to also interact with many other cellular components besides membranes as exemplified by the contrasting findings on its function and cytotoxicity in mitochondria. One of the proposed physiological functions of αS involves the modulation of mitochondrial complex I activity [232,234]. By controlling activity αS prevents mitochondrial complex I induced apoptosis. Additionally, the interaction of αS with apoptosis-promoting proteins promotes resistance against mitochondrial toxins [235]. Lastly, the absence of αS in mitochondria alters the mitochondrial lipid composition and causes a reduction in the activities of complexes I and III [212]. However, besides a protective role, the interaction of αS with mitochondrial membranes and membrane proteins in neuronal cells has not been reported to be toxic. αS mediated mitochondrial dysfunction due to inhibition of the respiratory chain is thought to be one of the major triggers for both familial and age-dependent PD [236,237]. Inhibition of mitochondrial complexes I and III results in increased oxidative stress and the generation of reactive oxygen species. The inhibition of the oxidative phosphorylation chain and generation of ROS has consequences for active downstream processes including SNARE mediated exocytosis [238]. Other possible mechanisms by which αS binding can result in mitochondrial damage include the release of cytochrome c, increase of mitochondrial calcium and concomitant apoptosis [237]. Besides cytotoxic mechanisms that involve oxidative stress, αS aggregates have also been reported to induce fragmentation of mitochondrial membranes [239,240]. This fragmentation of mitochondria is often associated with the degradation of dysfunctional organelles.

4. Conclusions and future perspectives

Although the function of αS and its role in PD remain debated, the interaction of αS species with cellular membranes seem to be important for both function and toxicity. In this respect, the interactions with membranes of intracellular vesicles and mitochondria seem to be most significant. Despite the observations that specific intracellular membranes are targeted by αS aggregation or specific αS aggregates, the molecular mechanisms underlying membrane disruption are in many cases not well understood. The inherent compositional complexity of biological membranes and largely unknown physiological functions of αS impede obtaining molecular insights into the mechanisms by which αS aggregates disrupt cellular membranes. Additionally, the impact of relevant combinations of PTM of αS on membrane binding and aggregation have been largely ignored. In spite of this, the studies on different model phospholipid membranes reviewed here indicate that
The αS residues that enable functional monomer-membrane interactions can become disruptive in αS aggregates. How combinations of structural and binding properties of specific aggregate species confer toxicity is still a largely open question.

Membranes have not only been identified as a direct target of toxic interactions with αS species, interactions of αS with the membrane (lipids) also potentially play a role in the aggregation of the protein. If and how enhanced aggregation results from crowding of protein at the membrane surface and/or is a result of interactions with lipids in solution remains to be investigated. In this respect it will be important to carefully reevaluate both the in vitro and in vivo membrane model systems of choice. Opposite effects in membrane binding and toxicity have been observed in different organisms. Although differences in interactions of αS with soluble components in these systems will play a role at least part of this difference in membrane binding can probably be contributed to differences in membrane composition. Difference in membrane composition may not only affect the membrane affinity of αS, in vitro studies have shown that the lipid composition affects the aggregation pathway. Different pathways have been shown to give rise to oligomeric, fibrillar or amorphous aggregates. All of these aggregates have been implicated in toxicity but they are thought to act via different mechanisms. Studies with non-abundant biological lipids or non-physiological membrane compositions may give a wealth of information on interactions and possible toxic mechanisms. However, these mechanisms may have little relevance in vivo. Increasing the complexity of model membranes to more closely mimic the properties of cellular membranes may have little relevance in vitro. The wealth of data obtained in the last two decades points towards a scenario wherein the interaction of αS with membranes can be neuroprotective but at the same time lipid membranes may help but a better characterization of the evolution of membrane bound αS species in vivo is required.

Taken together, the wealth of data obtained in the last two decades points towards a scenario wherein the interaction of αS with membranes can be neuroprotective but at the same time lipid membranes have been implicated in toxicity but they are thought to act via different mechanisms. Studies with non-abundant biological lipids or non-physiological membrane compositions may give a wealth of information on interactions and possible toxic mechanisms. However, these mechanisms may have little relevance in vivo. Increasing the complexity of model membranes to more closely mimic the properties of cellular membranes may help but a better characterization of the evolution of membrane bound αS species in vivo is required.

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A. Stefanovic, Insights into Alpha-Synuclein Oligomer Interactions with Model Membranes, Universite Twente, 2014.


