# Membrane interactions and fibrillization of α-synuclein play an essential role in membrane disruption

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**ABSTRACT**

We studied α-synuclein (αS) aggregation in giant vesicles and observed dramatic membrane disintegration and incorporation of lipids into micrometer-sized suprafibrillar aggregates. In the presence of dye-filled vesicles, dye leakage and fibrillization happen concurrently. However, growing fibrils do not impair the integrity of phospholipid vesicles that have a low affinity for αS. Seeding αS aggregation accelerates dye leakage, indicating that oligomeric species are not required to explain the observed effect. The evolving picture suggests that fibrils that appear in solution bind membranes and recruit membrane-bound monomers resulting in lipid extraction, membrane destabilization and the formation of lipid containing suprafibrillar aggregates.

**KEYWORDS** Amyloid, protein aggregation, vesicles, oligomers, fibrils, phospholipids, suprafibrillar aggregates

**HIGHLIGHTS**

- Binding of α-synuclein is required for fibrillization-induced membrane damage. However, in contrast to oligomer-induced damage, strong binding is not necessary, and membranes with only 30% charged lipids are also vulnerable.
- Aggregation of α-synuclein in the presence of membranes results in complete membrane disruption; lipids and small vesicles end up in large supra-fibrillar amyloid aggregates resembling Lewy bodies.
- Sheds new light on the presence of vesicles and lipid particles in Lewy bodies.

## 1.INTRODUCTION

Misfolding and aggregation of proteins is associated with several diseases, including Alzheimer's, Parkinson's disease and type II diabetes mellitus [1]. A characteristic feature of these diseases is the aggregation of proteins into cross β sheet rich structures [2]. Interaction between aggregated proteins and membranes appears to play a significant role in the pathogenesis of these diseases [3]. The prevailing hypothesis regarding toxicity in amyloid diseases invokes oligomeric protein species as the toxic agent to cells [4-6]. These oligomers are suspected to disrupt membranes [7, 8]. In Alzheimer's disease, oligomeric protein species have been accepted as the cause of cell death [9, 10]. However, it was recently shown that membrane disruption by Aβ is a two-step process, which not only includes pore formation by oligomers, but also membrane fragmentation by fibril elongation [11]. Moreover, when β2-microglobulin amyloid fibrils were incubated with large unilamellar vesicles (LUVs), membrane binding of the fibrils resulted in large membrane deformations [12]. Besides causing mechanical damage to membranes, fibrils were hypothesized to extract lipids from the outer membrane at the points of distortion[12]. In type II diabetes mellitus aggregation or fibrillization of IAPP on the membrane has been observed to damage membranes [13-15]. Although different transient or stable oligomeric species that appear during aggregation are possibly toxic in many protein aggregation diseases, damage induced by fibrils or fibril growth cannot be ruled out.

In Parkinson's disease, aggregation of α-synuclein (αS) leads to the death of dopaminergic neurons. Oligomeric αS species are hypothesized to play an important role in membrane damage [16] and cell death [7]. Although different *in vitro* produced αS oligomers can permeabilize highly negatively charged model membranes [17-19], the effect of protein aggregation and aggregate species on cell membranes and model membranes with more physiologically relevant

compositions and charge densities is still debated [18, 20, 21]. Here we have studied the aggregation of αS inside cell-sized giant unilamellar vesicles (GUVs). This model system provides a cell-sized environment with physiological salt concentration and pH. Confocal microscopy allowed us to validate the encapsulation of αS, and the disintegration of vesicles upon αS aggregation, leading to the appearance of mixed $\mu$m-sized amyloid-lipid aggregates, reminiscent of Lewy bodies that are the pathological hallmark of Parkinson's disease. In experiments with calcein filled LUVs we show that at membrane charge densities where the isolated, *in vitro* produced, oligomers that we have extensively studied [18, 22, 23] do not induce damage, aggregation of membrane-bound protein disrupts the vesicles, possibly by extraction of lipids from the bilayer. Colocalization of fluorescently labeled lipids with preformed αS amyloid fibrils supports this idea.

## 2.MATERIAL AND METHODS

*Expression and purification of αS*

The expression and purification of human wild type alpha synuclein (αS) and 140 cysteine mutant alpha synuclein (140C αS) was performed as previously described [24]. The protein concentration was estimated by measuring absorbance at 276 nm, using molar extinction coefficients of 5600 $M^{-1} cm^{-1}$ for αS and 5745 $M^{-1} cm^{-1}$ for 140C αS respectively, on a Shimadzu spectrophotometer [23, 25].

For more details refer to supporting Information (SI).

## 3. RESULTS

Cell membranes consist of bilayers containing a mixture of anionic and zwitterionic lipids. The affinity of αS for lipid bilayers increases with the density of negatively charged lipids. To investigate the effect of αS aggregation on membrane integrity in a simple model system we chose a mixture of zwitterionic and anionic phospholipids that is known to bind αS well, and prepared GUVs from POPC and POPG in a 1:1 ratio. Incorporation of fluorescently labeled Liss Rhod-PE in the membrane and encapsulation of fluorescently-labeled monomeric αS allowed us to concurrently monitor the fate of both proteins and lipids as a function of time. In the absence of protein, POPC/POPG GUVs remained stable for at least 95 hours (Fig. 1A, B). During this time the vesicles did not change morphology. When 100 $\mu$M αS was encapsulated in the GUVs upon vesicle formation, the protein was initially homogenously distributed over the vesicle volume (Fig. 1C). We did not observe accumulation of protein at the bilayer surface. In time, GUV-encapsulated αS aggregated and the vesicles disappeared from solution. Instead of GUVs, micrometer-sized structures that contained both proteins and lipids were observed 95 hours after vesicle formation (Fig. 1D). To verify that these structures consisted of β sheet-rich amyloid fibrils, the amyloid-specific dye ThT was added to the solution. Confocal microscopy images show that the protein-lipid aggregates are ThT positive and we therefore conclude that they consist of characteristic amyloid structures, most likely of fibrillar form. The colocalization of amyloid and lipid fluorescence suggests that lipids are associated with the amyloid fibrils. At t=160 hours some of the micrometer sized amyloid aggregates observed in solution have a distinct suprafibrillar morphology and are decorated with lipid structures (Fig. 1E, F). The size and heterogeneous distribution of lipid structures suggests that protein aggregation additionally caused bilayer remodeling resulting in small vesicle-like structures. Monomeric αS adopts α-

helical structures on negatively charged membranes [26, 27]. The conformational transition into β sheet fibrils may take place at the membrane surface. This aggregation at the membrane may result in sequestering of lipids and cause the lipids to deposit in amyloid-rich aggregates.

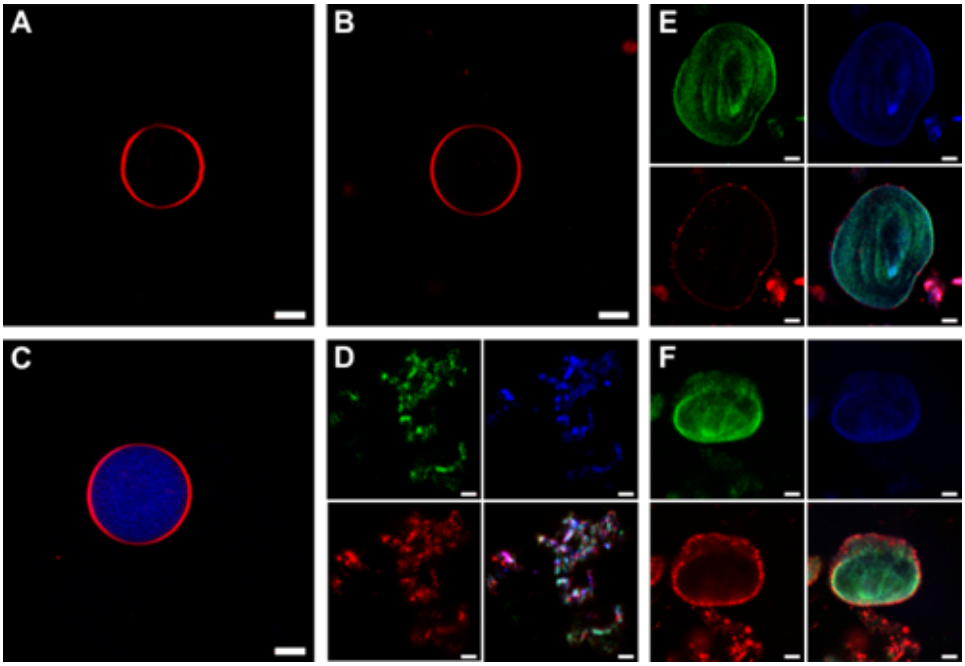


**Figure 1**: αS encapsulation and aggregation at physiological salt conditions. In the absence of protein, POPC/POPG (1/1) Liss Rhod PE labeled GUVs (t=0 hours, **A**) remained intact for at least 95 hours (t=95 h, **B**). Vesicles encapsulating αS 140C-Alexa 647 (**C**) disintegrate into amyloid rich lipid-protein aggregates. After 95 hours these amyloid aggregates were visualized using ThT fluorescence (**D**). After 160 hours the solution additionally contains suprafibrillar aggregates[28] coated with lipids solution (**E, F**). In panels D, E and F lipids are represented in red, αS 140C-Alexa 647 in blue and amyloid (ThT-stained) in green; in the bottom right images of panels E & F, all three channels are merged. Scale bars 10 $\mu$m.

To determine if the aggregation process or specific aggregate species are involved in membrane disintegration we simultaneously followed the kinetics of αS aggregation and the leakage of dye from LUVs. When αS was incubated with calcein-filled POPC/POPG (1:1) LUVs and imaged over time, the vesicles were observed to lose their content (Fig. 2A), while in the absence of protein the vesicles remained intact (S2). The calcein release and αS aggregation could both be described with a sigmoidal curve but the rates of the two processes differed. The onset of leakage

from the POPC/POPG (1:1) LUVs seemed however to coincide with start of the increase in ThT fluorescence.

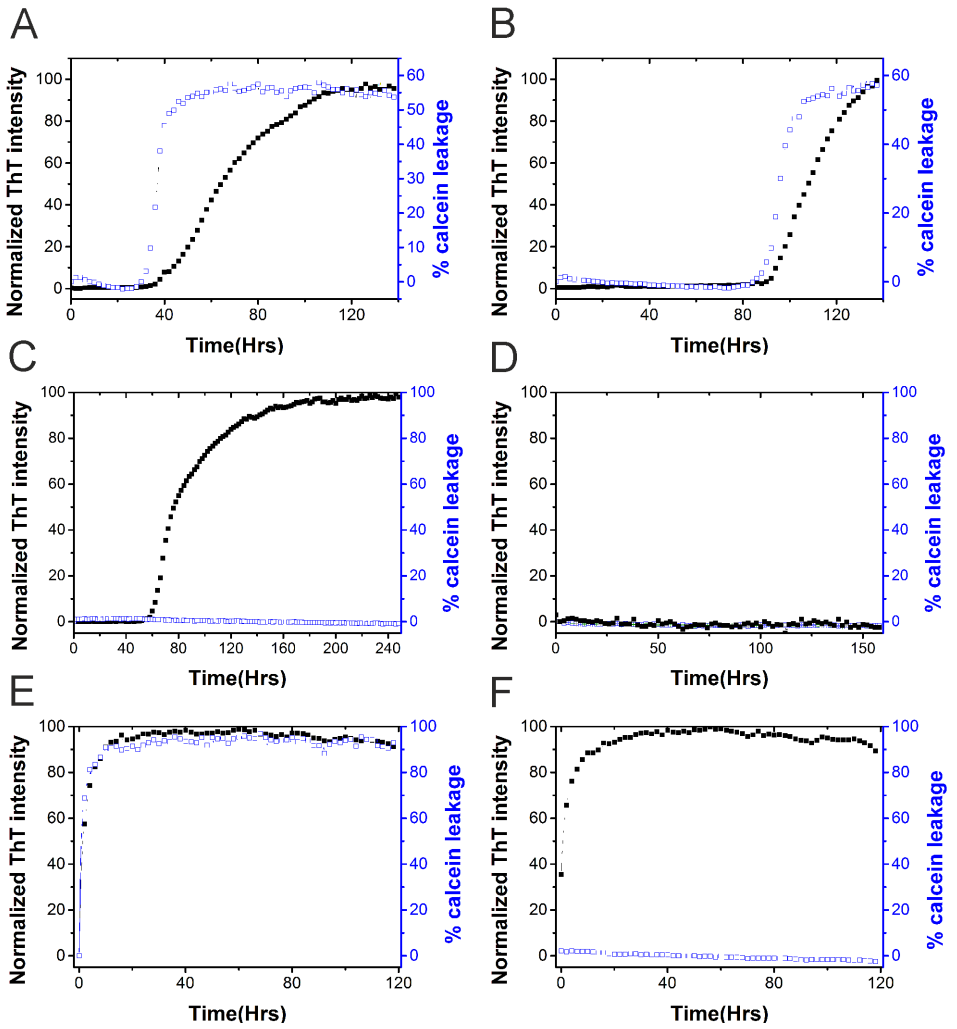


**Figure 2:** Effect of αS fibrillization on membrane leakage monitored using ThT fluorescence (black) and calcein leakage (blue). (**A**) POPC/POPG(1:1) LUVs were incubated with 1:1 molar ratio αS:lipid. (**B**) PC/PE/PI (55:15:30) LUVs were incubated with 100µm of αS monomers (**C**) POPC LUVs were incubated with 1:1 molar ratio αS. (**D**) POPC/POPG(1:1) LUVs were incubated with 20µm stable αS oligomers. (**E**) The aggregation was seeded by 10% preformed sonicated fibrils in presence of 90µm monomeric αS and POPC/POPG (1:1) LUVs. (**F**) The aggregation was seeded by 10% preformed sonicated fibrils in presence of 90µm αS monomers and POPC LUVs. The aggregation was performed at 37±1$^0$C without any agitation.

To be able to assess if the onset of fibril growth and calcein leakage indeed occur concurrently we plotted the normalized calcein leakage as a function of the normalized ThT fluorescence (Fig. 3). Figure 3 shows that ThT fluorescence and calcein leakage both start from the origin of the plot, but that leakage is complete before all αS in the solution has fibrillized. The simultaneous increase in ThT fluorescence and calcein leakage from LUVs suggests that fibril growth at the membrane could be responsible for membrane disruption. However, the aggregation lag times in the presence of POPC/POPG (1:1) LUVS were high compared to the lag times observed in the absence of vesicles, indicating that the lipid bilayer did not enhance fibril nucleation at the protein to lipid ratio used. Instead fibrils or oligomers that appear in solution may be responsible for the observed effect.

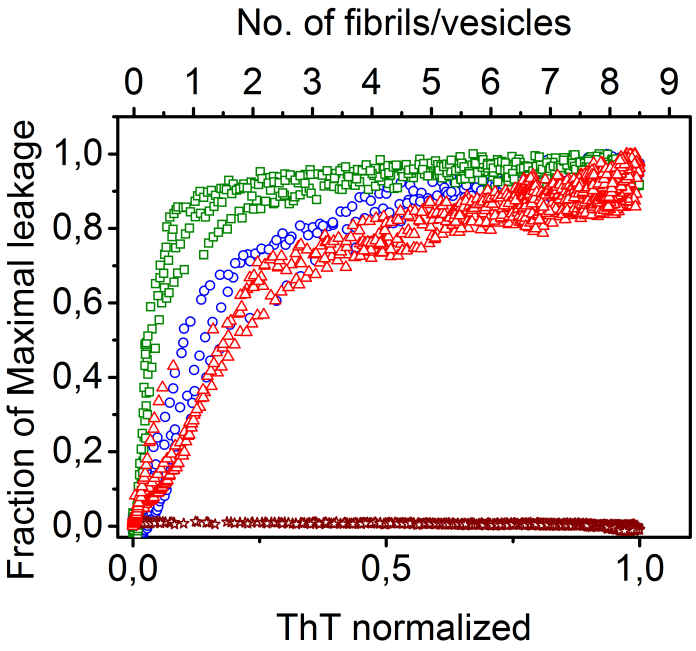


**Figure 3.** Membrane leakage as a function of the normalized ThT fluorescence (bottom) and the calculated average number of fibrils per vesicle present in solution (top). The average number of fibrils per vesicle was estimated from the median fibril length assuming that all αS monomers are in fibrils at the maximal ThT signal and that ThT intensity is proportional to the fibril

content. Leakage is presented in triplicates for vesicles of the following membrane compositions POPC/POPG(□) POPC/POPE/POPI (□), POPC/POPE/CL(Δ), POPC (★).

Oligomers are intermediate, possibly toxic species, formed during fibrillization of proteins such as αS. The toxicity of oligomers has been putatively attributed to membrane binding and permeabilization. To verify if *in vitro* produced soluble oligomers [17] can also be responsible for the membrane disintegration observed in GUVs and LUVs we followed the oligomer-induced leakage of calcein filled POPC/POPG LUVs. We incubated 20$\mu$M (equivalent monomer concentration) of isolated oligomers with calcein filled LUVs, and followed the membrane leakage kinetics for 120 hours. We did not observe any signs of membrane damage in the presence of these oligomers (Fig. 2 D). We therefore conclude that this stable oligomer species cannot be responsible for the observed membrane disintegration. Preformed fibrils are known to enhance aggregation, resulting in decreased lag times. When aggregation is seeded by fibrils, monomers add to the seeds, circumventing the formation of any oligomeric species. To test whether the observed αS-induced membrane damage is caused by αS fibrils or fibril growth rather than by oligomeric species, the aggregation of a 100 $\mu$M monomeric αS solution was seeded by preformed sonicated fibrils. Figure 2E shows that in the presence of 10% αS seeds the lag time associated with calcein leakage from POPC/POPG vesicles completely disappears. Moreover, it has previously been shown that purified mature fibrils do not damage POPC/POPG LUVs [17]. These results suggest that the elongation of fibrils is an important cause of membrane damage.

To see if, in the aggregation experiment presented in Figure 3, enough fibrils are present in solution to damage all vesicles we estimated the number of fibrils per vesicle from the ThT fluorescence. Although we are aware that the ThT signal can saturate while the fibril content is

still increasing we simplify our calculation by assuming that at the ThT plateau all protein is aggregated[29]. We employ a linear relation between the ThT signal and the concentration of aggregated protein. This simplification may result in an overestimation of the number of fibrils in solution. To obtain the number of fibrils from the amount of aggregated protein, we used the median fibril length measured by atomic force microscopy at comparable buffer conditions [24]. The vesicles were obtained by extraction though a 100 nm polycarbonate filter. The number of vesicles in solution was therefore obtained assuming a vesicle diameter of 100 nm and an average head group area of 0.70 $nm^2$. In Figure 3 the observed vesicle leakage is presented as a function of the calculated number of fibrils per vesicle. The data shows a good correlation between the number of fibrils per vesicle and the observed leakage, supporting the idea that growth of fibrils is responsible for the observed damage. For POPG:POPC vesicles, leakage is complete when each vesicle is associated with a fibril of median length.

Fibril growth is however not enough to cause damage. When membrane leakage and protein aggregation were followed in the presence of calcein-filled POPC vesicles the ThT signal intensity was observed to increase, indicating the formation of amyloid fibrils, but no calcein leakage was observed (Fig. 2C). In this experiment the calcein-filled vesicles remained intact for at least 240 hrs. Similar results were obtained when aggregation was seeded in the presence of POPC vesicles (Fig. 2F). The inability of growing fibrils to impair the integrity of POPC membranes may be related to the low affinity of αS for these bilayers. Membranes of negatively charged lipids have a high affinity for αS compared to zwitterionic POPC bilayers [26, 30]. Although growth does not necessarily start at the membrane surface, the ability of αS to bind the lipid bilayer seems to play an important role in the membrane disruption mechanism.

For lipid bilayers containing more than the 20% negatively lipids required for αS binding[26], such as POPC:POPE:PI (1:1:1) and CL:POPE:POPC (3:4:5) vesicles, aggregation does indeed cause membrane leakage (Fig. 2B, 3). However compared to POPG:POPC vesicles more fibrils per vesicle are required to obtain a comparable number of leaky vesicles. This difference reflects the lower binding affinity of αS for membranes with lower surface charge density. In the literature it has been shown that protein aggregation can result in lipid extraction[31]. The colocalization of ThT signal and lipid fluorescence in Figure 1D suggest that amyloid fibrils bind or contain lipids. The mechanism by which membrane adsorbed fibrils damage the membrane therefore probably involves lipid extraction by surface bound monomers that undergo a conformational transition from α-helix to β-sheet. We have observed that fibrils can bind lipids. When αS amyloid fibrils are incubated with the relatively soluble Rhodamine-labeled phospholipid DOPE we see an incorporation of lipids into the fibrils (Fig. 4A). The fluorophore Rhodamine alone did not show this high affinity for αS amyloid fibrils (Fig. 4B). The very low solubility of phospholipids would prevent lipid extraction by fibrils in solution at relevant time scales. The low solubility of the phospholipid can however not prevent lipid extraction when membrane bound αS is incorporated into fibrils.

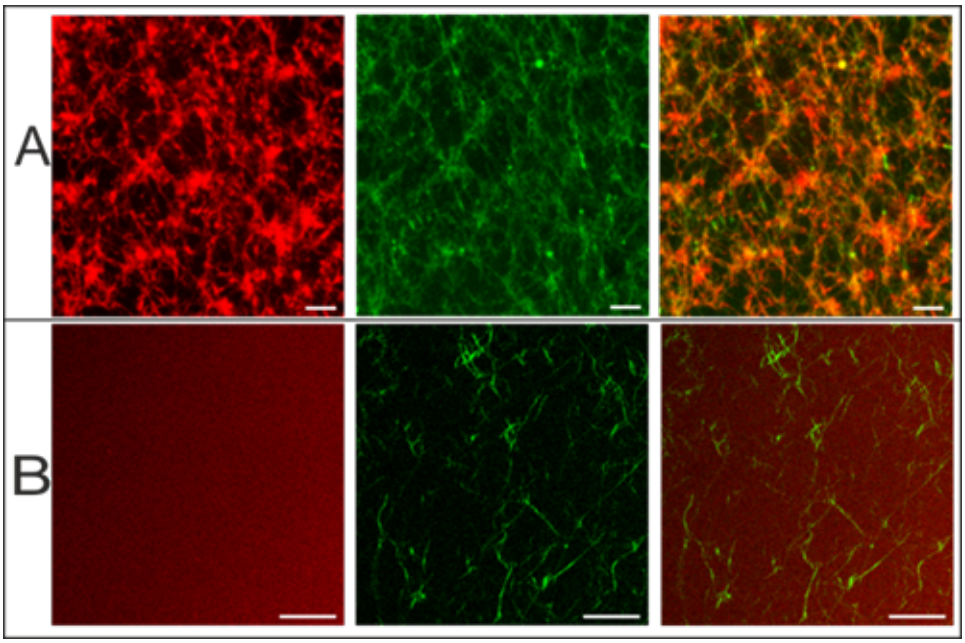


**Figure 4** Rh-DOPE lipids co-localized with α-synuclein fibrils. (A). Fibrils were incubated with ThT(green) and Rh-DOPE(red). Fibrils are visualized using ThT fluorescence and Rh-DOPE lipids co-localize with the α-synuclein fibrils. (B) Controls show ThT fluorescence from fibrils, but no signal of rhodamine B binding to fibrils. Scale bar 10 $\mu$m

## 4. DISCUSSION

Membrane damage has been implicated as the mechanism of toxicity of protein aggregation in several diseases. There is an ongoing discussion on the nature of the aggregate species involved in membrane disruption. In Parkinson's disease no clear correlation has been observed between the amount of αS inclusions, or Lewy bodies, and the stage of the disease [32]. The absence of a clear link between fibrillar aggregates and cell death has resulted in a shift of research efforts towards pre-fibrillar oligomeric species. The mechanism of αS oligomer-induced membrane disruption has drawn a lot of attention [33]. However exogenous αS fibrils can seed αS aggregation inside cells [34] and spread the pathology from cell to cell by endocytosis [35]. Fibrils may therefore play an important role in membrane disruption. Both GUVs and LUVs

composed of a 50/50 mixture of the negatively charged lipid POPG and the zwitterionic lipid POPC lost their integrity when incubated with high concentrations of αS (Fig. 1, 2, 3). Although aggregating αS damaged membranes with a charged lipid content as low as 30% (Fig. 3), damage could not be induced by either aggregating αS monomer or elongating seeds in LUVs composed of the neutral phospholipid POPC (Fig. 3, 2C, 2F). Electrostatic interactions are important for the binding of αS to membranes. The amphipathic α-helices of αS contain positively charged residues at neutral pH. The fact that no membrane damage is observed for POPC vesicles reflects the very low binding affinity of αS to zwitterionic lipids (Fig. 2C, 2F). The relatively low affinity of αS for PC bilayers [36] can apparently not support disruptive membrane deformations at the timescales studied.

Membrane binding is required for αS aggregation to be disruptive. *In vivo*, αS has been observed to colocalize with both the nuclear envelope [37] and mitochondrial membranes [20, 21, 38]. The POPC/POPE/POPI and POPC/POPE/CL (3:4:5) membranes used in the experiments presented in Figure 3 mimic the more complex phospholipid composition of these respective membranes [18]. As observed for POPG/POPC vesicles these more complex cellular membrane mimics with lower surface charge density are also vulnerable to aggregation-induced damage. Although we realize that these 3-component lipid vesicles oversimplify the complexity of cellular membranes, these results may indicate that the membrane damaging mechanism presented here can be physiologically relevant.

In contrast to aggregating αS solutions, isolated *in vitro* produced stable oligomers were not able to induce leakage in POPC/POPG LUVs, which remained intact for at least 160 hours (Fig. 2 D). This is in agreement with experiments in which calcein leakage after 30 minutes of oligomer addition was studied [17]. Incubation of isolated oligomers with both LUVs and GUVs

composed of 100% negatively charged lipids has been reported to result in transient pore formation [33]. When the negatively charged lipid content was decreased to 50%, oligomers were no longer able to induce leakage of calcein from the LUVs. Considering the percentage of negatively charged lipids in naturally occurring membranes it seems unlikely that the stable soluble oligomers that we are able to isolate are responsible for cellular membrane damage. Although the disruption mechanism does not involve these stable isolated oligomers, it cannot be excluded that transient or less stable oligomer species can damage cellular membranes.

Aggregation lag-times have been reported to depend on the protein to lipid ratio [39]. At the protein to lipid ratio used here, the lag-time in the presence of vesicles was increased compared to the control that did not contain vesicles. This result suggests that, compared to the timescales involved in aggregation in solution, the α-helical secondary structure of the membrane-bound protein is stable [39]. Fibrils that form in solution and bind to the membrane are therefore a likely cause of membrane damage (Fig. 3). It has been argued that the growth of mechanically stiff amyloid fibrils can penetrate membranes and in this way cause cell death [40]. However, although POPC, POPC/POPG, POPC/POPE/POPI and POPC/POPE/CL membranes are probably comparably soft, no calcein leakage was observed for POPC vesicles in the presence of αS amyloid fibrils, a result which speaks against this mechanism (Fig. 2C, 2F, 3)

The adsorption of mature IAPP and β2-microglobulin fibrils has been shown to result in deformation of LUVs [12, 13]. In these cases the curvature of the membrane was high compared to the length and persistence length of the amyloid fibril[41, 42]. Strong adsorption of long fibrils is therefore expected to result in deformation. The curvature radius of GUVs is comparable to the mean length and persistence length of the fibril, and although membrane deformations may occur due to adsorption of fibrils to the relatively flat GUV membrane, these

shape adjustments are probably supported by the permeability of the membrane for $H_2O$, membrane undulations, and flexibility. The mechanism of membrane distortion may therefore depend on the curvature of the membrane involved. In our experiments both GUVs and LUVs were disintegrated upon incubation with monomers. Membrane deformations resulting from fibril adsorption are therefore most likely not the only cause of membrane disruption.

The addition of sonicated fibrils to a solution containing both monomers and POPC/POPG LUVs resulted in the disappearance of the lag phase, leading to immediate calcein loss from vesicles. The growth of membrane-adsorbed fibrils is therefore a likely mechanism of membrane disruption. Growth may damage the membrane by extracting lipids from the bilayer. Aggregation of αS on SUVs and supported lipid bilayers has been reported to involve the extraction of lipids from the bilayer and clustering of lipids around growing αS aggregates [31, 43]. The colocalization of fluorescent lipids and amyloid fibrils indicates that lipids have affinity for the fibril (Fig. 4). The extremely low solubility of phospholipids makes the extraction of lipids by preformed fibrils very unlikely. However, the amphiphilic properties of αS may make extraction possible when the fibrils grow on the membrane surface. Lipid extraction and amyloid growth result in mixed amyloid/lipid structures and small vesicles as observed for GUVs in (Fig. 1D, 1E and 1F). These aggregates are reminiscent of Lewy bodies suggesting a similar formation mechanism for these lipid-rich amyloid inclusions inside cells.

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**Notes**

The authors declare no competing financial interests.

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