A structural and kinetic link between membrane association and amyloid fibril formation of α-Synuclein

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24   **ABSTRACT:**

25   The protein α-Synuclein (αS) is linked to Parkinson's disease through its abnormal aggregation, which is

26   thought to involve an interplay between cytosolic and membrane-bound forms of αS. Therefore, better insights into

27   the molecular determinants of membrane association and their implications for protein aggregation may help

28   deciphering the pathogenesis of Parkinson's disease. Following previous studies using micelles and vesicles, we

29   present a comprehensive study of αS interaction with phospholipid bilayer nanodiscs. Using a combination of NMR-

30   spectroscopic and complementary biophysical as well as computational methods we structurally and kinetically

31   characterize αS interaction with defined stable planar membranes in a quantitative and site-resolved way. We probe

32   the role of αS acetylation as well as membrane charge, plasticity and available surface area in modulating αS membrane

33   binding modes and directly link these findings to their consequences for αS amyloid fibril formation.

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35   **KEYWORDS:** α-Synuclein, membrane interaction, solution NMR, dynamic nuclear polarization (DNP) solid-state

36   NMR, nanodiscs, amyloid formation, lipid-modulated protein aggregation.

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38   The protein α-Synuclein (αS) is associated with various synucleinopathies including Parkinson's disease through its

39   abnormal aggregation, fibril formation and formation of Lewy bodies (Jucker and Walker, 2013, Luk, et al., 2012, Spillantini, et al.,

40   1997, Tuttle, et al., 2016). While the exact native function is yet not fully understood, αS is found in synaptic vesicles and supposed

41   to be involved in membrane interactions, e.g. in synaptic vesicle homeostasis (Bellani, et al., 2010, Gitler, et al., 2008) and SNARE-

42   like vesicle-to-vesicle or vesicle-to-membrane fusion (Diao, et al., 2013, Fusco, et al., 2016). Membrane association of αS has been

43   shown to modulate its aggregation propensity (Dikiy and Eliezer, 2012, Zhu, et al., 2003), and αS oligomeric species have been

44   proposed to be the toxic species in Parkinson's disease, especially through membrane pore formation mechanisms (Auluck, et al.,

45   2010, Butterfield and Lashuel, 2010). αS has been shown to be N-terminally acetylated, which is thought to act as an important mode

46   of regulation of protein-membrane association (Dikiy and Eliezer, 2014, Nemani, et al., 2010, Theillet, et al., 2016).

47   Previous data recorded using micelle and vesicle preparations already provided valuable information of the αS-membrane

48   interactions, including binding and lipid specificity (Bodner, et al., 2009, Jo, et al., 2000, Rhoades, et al., 2006), effect of mutations

49   on membrane association (Bodner, et al., 2010, Fusco, et al., 2016), micelle-bound structure (Ulmer, et al., 2005), vesicle-bound

50   structural insights (Drescher, et al., 2008, Fusco, et al., 2014, Jao, et al., 2008) and conformational dynamics (Eliezer, et al., 2001,

51   Fusco, et al., 2014, Fusco, et al., 2016). Two structural models of lipid-bound αS were proposed, i.e. the "extended helix" consisting

52   of one roughly 100-residue long α-helix (Georgieva, et al., 2008) and the "horse-shoe", consisting of two helices with different lipid

53   affinities separated by a kink at residues 42-44 (Drescher, et al., 2008). Furthermore, various effects of lipids for αS aggregation were

54   reported including inhibition of aggregation (Zhu and Fink, 2003), triggering of fibrillation (Galvagnion, et al., 2015, Zhao, et al.,

55   2004) and modification of fibril structure (Comellas, et al., 2012). Membrane binding and its effect on aggregation have been shown

56   to be strongly dependent on chemical properties of the lipids including head group charge content (Drescher, et al., 2008) and fatty

57   acid type (Galvagnion, et al., 2016).

58   The intrinsic features of the phospholipid bilayer nanodisc (ND) system (Bayburt, et al., 2002) offer the potential to provide

59   additional insights that are complimentary to the information obtained on micelle and vesicle preparations. Notably, NDs have been

60   used before to study the effect of calcium ions on the membrane interaction of αS (Zhang, et al., 2014) as well as lipid and monomer

61   specificity of the Alzheimer's associated Aβ peptide (Thomaier, et al., 2016). In general, NDs are very homogenous, stable in a wide

62   buffer range (Viegas, et al., 2016) and allow the preparations of well-defined lipid mixtures with an accurate estimate of the bilayer

63   size (Denisov, et al., 2004), charge (Her, et al., 2016), and lipid molarity (Inagaki, et al., 2012). The increased stability offers e.g. the

64   possibility to consistently determine the interaction with a stable planar bilayer surface. In contrast, it is known for small unilamellar

65   vesicles (SUVs) that the interaction with αS can considerably and rapidly change the lipid environment (e.g. from homogenous SUVs

66   to rather heterogeneous particles (Comellas, et al., 2012, Eichmann, et al., 2016, Fusco, et al., 2016, Ouberai, et al., 2013)).

67   Additionally, the smaller size of the NDs should, in theory, allow the detection of the lipid-bound state using suitable solution NMR

68   techniques (Etzkorn, et al., 2013, Hagn, et al., 2013, Viegas, et al., 2016). In general, the well-defined size and lipid composition of

69 NDs, paired with their accessibility, homogeneity and stability should therefore permit quantitative insights into membrane

70 association as well as its role in aggregation.

71   Here we make use of this potential and report on a comprehensive NMR investigation of the effects of lipid charge, bilayer

72 fluidity and αS acetylation on the structural aspects of αS membrane association. We corroborate these insights with molecular

73 dynamics (MD) simulations as well as a series of complementary biophysical measurements to further characterize membrane

74 plasticity, overall affinities as well as binding and aggregation kinetics. Based on this data we correlate structural insights, such as

75 residue specific affinities and competition for accessible membrane surface area, to their potential role in modulating αS aggregation

76 properties. Our study provides insights into (i) the different lipid binding modes of αS to stable planar bilayers of defined lipid quantity

77 and composition, (ii) the effect of membrane plasticity for αS binding, (iii) the modulation of membrane plasticity through αS, and

78 (iv) the connection between binding modes and their effect on αS aggregation. Additionally, it gives an initial estimate of the number

79 of lipid-associated αS molecules that are required to induce/promote nucleation, and allows to develop a basic structural,

80 thermodynamic and kinetic model of the modulation of αS aggregation through its interaction with different membrane surfaces.

81 Our *in vitro* data help to better understand the molecular determinants of αS-membrane association and point to possible *in vivo*

82 implications in the context of Parkinson's disease.

83

84 **RESULTS**

85 **Effects of phospholipid head-group charge on the αS membrane binding mode.**

86 To obtain residue specific insights into the interaction of αS with lipid bilayer nanodiscs (NDs) of various composition, we recorded

87 a series of solution NMR 2D TROSY-HSQC spectra (see Figure 1 – figure supplement 1 for full list of measured samples). The NMR

88 spectrum of αS in the presence of NDs containing only DMPC lipids perfectly overlays with the spectrum of αS in the absence of

89 NDs (Figure 1a-d). This finding provides additional evidence that αS does not interact with non-charged lipid bilayers (Rhoades, et

90 al., 2006), a point that is still controversial in the literature (Davidson, et al., 1998). Additionally, it also shows that αS does not

91 interact with the membrane scaffold protein (MSP), confirming that the effects described in the following are not biased by

92 (unspecific) αS-MSP interactions.

93   In a similar way as reported previously using liposomes (Drescher, et al., 2008, Iyer, et al., 2016), we further tested the

94 influence of increasing amounts of negatively charged lipid head groups on αS membrane association, keeping a molar ratio of one

95 αS molecule per membrane leaflet (Figure 1a-d). Note that lipid ratios and proper mixing of the different lipid types inside the

96 nanodiscs was also observed by NMR spectroscopy (Figure 1 – figure supplement 2a). Our NMR data show a gradually increasing

97 bilayer interaction of αS with increasing lipid charge content, dividing the protein into rather distinct regions with different binding

98 behaviors (Figure 1e). The first region spans the N-terminal residues 1-38, which are already weakly interacting at 25% content of

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99 negatively charged lipids and strongly interact at 50% (or higher) charge content. The region of residues 38-60 interacts more

100 gradually at 50% charge content. Amino-acids 60-98, corresponding approximately to the aggregation-prone non-amyloid-β

101 component (NAC region), displays some interactions with membranes containing 75% anionic lipids and strongly interacts at 100%

102 anionic lipid content. The 98-120 region is (partly) affected by 100% net charge content only. Finally, the last 20 C-terminal residues

103 never show any membrane interaction. This data is largely in line with an expected predominantly electrostatic model (the first 60

104 residues displaying a net positive charge, the last 40 residues a net negative charge, and the NAC region being mostly hydrophobic),

105 as well as the three regions dynamic model reported before using SUVs (Fusco, et al., 2014).

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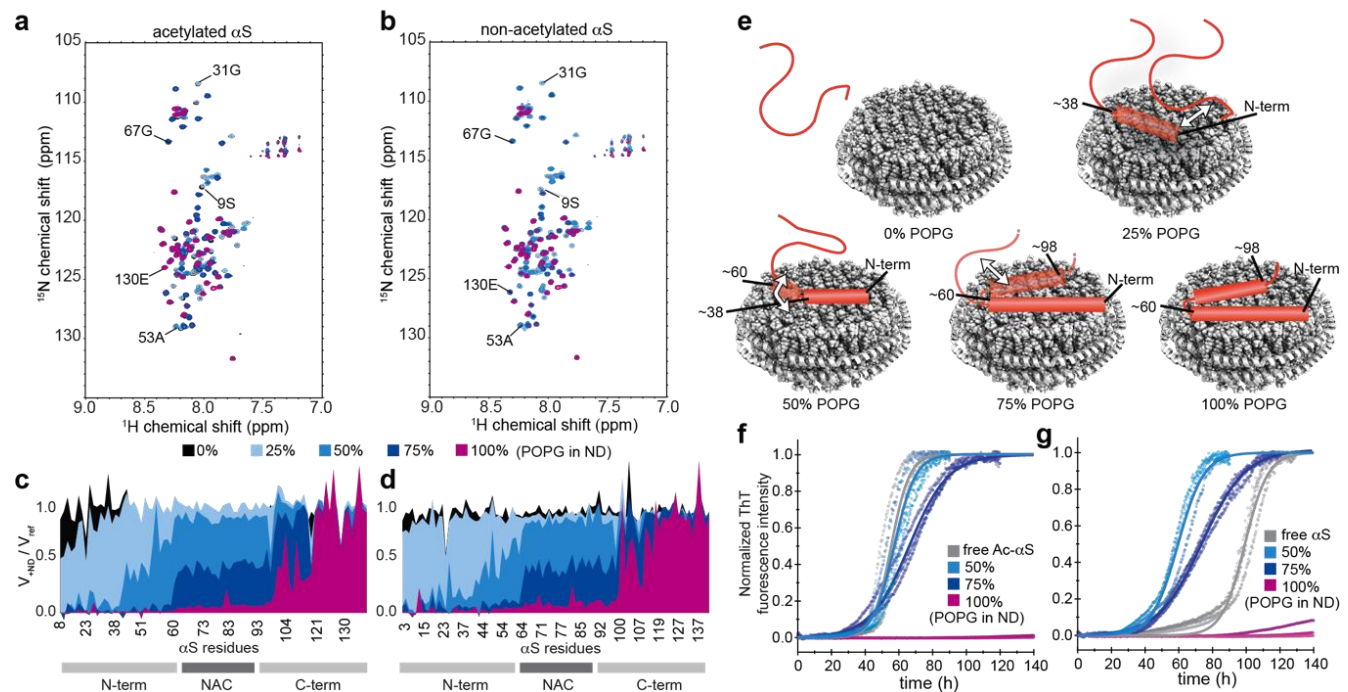
108 **Figure 1. Lipid charge content modulates αS membrane binding modes and different binding modes show different effects on αS**

109 **aggregation properties.** NMR spectra of [^{15}N]-αS (50 µM) in the absence or in the presence of 25 µM NDs of different POPG (negative

110 charges) contents. [^{15}N-^{1}H]-TROSY-HSQC spectra of acetylated (a) and non-acetylated (b) αS. Spectra in the absence (grey) or in the

111 presence of NDs containing 0% POPG (black), 25% POPG (light blue), 50% POPG (blue), 75% POPG (dark blue) and 100% POPG (purple)

112 are displayed. Selected residue assignments corresponding to differently affected parts of αS are indicated. Corresponding NMR attenuation

113 profiles, i.e. the ratio of peak volumes in the presence and absence of NDs, are plotted against αS primary sequence for acetylated (c) and

114 non-acetylated (d) αS. (e) Molecular model visualizing the gradual binding of different parts of αS to NDs with increasing charge content.

115 (f,g) αS aggregation assays (normalized ThT fluorescence) in the absence and presence of NDs with indicated POPG charge content for

116 acetylated (f) and non-acetylated (g) αS (data of triplicate measurements until reaching saturation and their respective fits are shown, color

117 code as in a-d).

118

119   Using Thioflavin T (ThT) fluorescence as a reporter for fibril formation, we also measured aggregation kinetics of αS in the

120   absence and presence of the different ND compositions (Figure 1f-g). These experiments were performed under conditions where αS

121   amyloid fibrils form spontaneously, mainly by interface-driven nucleation and subsequent amplification through fragmentation

122   (non-repellent plates, glass balls, shaking, see below for complementary kinetic assays) and therefore mainly report on the

123   potential interference of nanodiscs with the lipid-independent aggregation pathway of αS (Campioni, et al., 2014, Gaspar, et al., 2017,

124   Rabe, et al., 2013, Vacha, et al., 2014). Interestingly, despite the fact that the NMR data show interaction, the presence of NDs up to

125   an anionic lipid content of 50% does not appear to affect aggregation kinetics (note that the free non-acetylated αS reference (Figure

126   1g, gray) shows a different behavior, this point will be discussed below). When increasing the negative charge content to 75% the

127   aggregation half-time slightly increases (Figure 1f,g dark blue) and a strong aggregation-inhibiting effect is detected in the presence

128   of NDs with 100% anionic lipids (Figure 1f,g purple).

129   While the NMR data visualize the modes of αS binding to membranes of different charge contents, the ThT kinetic data

130   allow to directly link these molecular determinants to their effect on αS aggregation. In this respect, one of the most striking

131   connections is that αS interaction with NDs comprising up to 50% negatively charged lipids does not involve the NAC region and

132   that under the same conditions no detectable effect on the aggregation behavior of (acetylated) αS is found in ThT assays. When

133   further increasing the charge density above 50% negatively charged lipids, NMR data show first a partial (75% POPG, Figure 1a-d,

134   dark blue) and then a full (100% POPG, Figure 1a-d, purple) signal attenuation of the NAC region. This correlates well with a slight

135   inhibitory effect of the 75% charged NDs on αS aggregation (Figure 1f,g dark blue) and a very strong inhibitory effect of 100%

136   charged NDs (Figure 1f,g, purple). Our data strongly suggest that for the tested conditions (high anionic lipid content and high lipid-

137   to-αS ratios) membrane association of the NAC region seems to be the dominant factor for protecting αS from aggregation.

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139   **Nanodisc-bound state of αS is predominantly α-helical.**

140   It is worth noting that the NMR results described above mainly refer to the decrease in peak intensity as a reporter for interactions,

141   which is in line with the effects seen before using SUVs (Bodner, et al., 2009). While it is clear that SUVs have particle sizes

142   (associated with slow tumbling rates) well above the detection limit of conventional solution NMR techniques, the smaller size of the

143   ND system should, in principle, allow detection of NMR signals, as has been reported before for several ND-bound or ND-integrated

144   proteins (Viegas, et al., 2016). Nonetheless, neither the usage of Transverse Relaxation Optimized Spectroscopy (TROSY)

145   (Pervushin, et al., 1997) with increased signal accumulation (i.e. 10-fold longer as for spectra shown in Figure 1a-b) nor the

146   measurement at increased temperatures (35°C) and the usage of an NMR-optimized smaller membrane scaffold protein

147   (MSP1D1ΔH5) (Hagn, et al., 2013) forming NDs of smaller size and higher tumbling rates, resulted in appearance of a new set of

148   peaks indicative for the bound sate (and the presence of slow exchange processes) or a collective shift of peaks indicative of fast on-

149   off exchange processes (e.g. Figure 3 – figure supplement 1). In theory, three effects may explain this observation and obstruct

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150   detection of the ND-bound residues of αS: (i) the bound-to-free exchange rate is in the order of the NMR time scale (so-called

151   intermediate exchange), (ii) the presence of a non-negligible part of αS protruding out of the ND (namely at least residues 98-140),

152   slowing down molecular tumbling and increasing relaxation leading to line broadening beyond the detection limit, and/or (iii)

153   membrane-bound αS shows a significant amount of plasticity leading to inhomogeneous broadening of the NMR lines. While

154   intermediate exchange can be largely ruled out due to the observed binding kinetics (*vide infra*, Figure 4a,b, t_{on} and t_{off} of around 4

155   µs·M and 65 s, respectively) it is at this point difficult to further distinguish between slower tumbling and molecular plasticity (or a

156   combination thereof) that interfere with solution NMR detection of the bound conformation.

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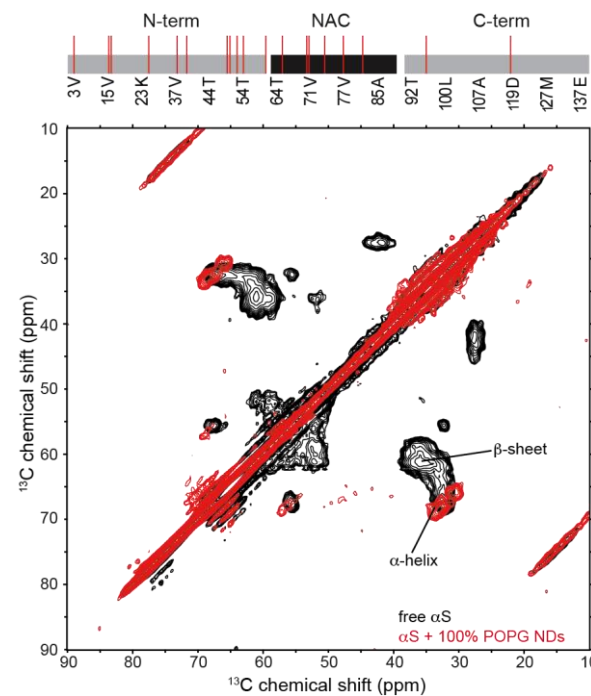
159   **Figure 2. Nanodiscs binding induces α-helical structure in αS.** [^{13}C-^{13}C]-Proton Driven Spin Diffusion MAS-DNP spectra of free αS in

160   frozen solution (black) and when bound to NDs with 100% POPG lipids (red). Selective isotope labelling was used to specifically monitor

161   Valine Cα-Cβ chemical shift distribution. Peaks position indicative of the β-sheet and α-helical secondary structure are labelled. Occurrence

162   of Valine residues in the αS sequence is shown on top (18 out of 19 Valines are present in the expected binding site of the used ND).

163

164   In order to still gain insight into the conformation of αS bound to NDs, we used magic angle spinning (MAS) solid-state

165   NMR which is not subject to size effects. Moreover, we took advantage of the very low temperatures (100 K) used in Dynamic

166   Nuclear Polarization (DNP) to additionally eliminate exchange processes, as well as to increase the sensitivity of the experiment. To

167   avoid problems of signal overlap arising from severe inhomogeneous line broadening often seen in this range of temperatures (Siemer,

168   et al., 2012), we used a sparse isotope labelling scheme (Hong and Jakes, 1999), leading to the simplification of ^{13}C-^{13}C spectra to

169   secondary structure sensitive Cα-Cβ cross-correlations of valines (and leucine Cβ-Cγ). Notably according to the αS primary sequence

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170 (Figure 2, top) and our solution NMR observations (Fig. 1a-b), 95% of the valine residues (i.e. 18 out of the 19) should be membrane-

171 bound at the used charge content and αS-to-ND ratio. While in the absence of NDs the DNP ^{13}C-^{13}C spectrum (Figure 2, black) shows

172 a continuous distribution of the Valine Cα-Cβ cross peaks reflecting the carbon chemical shifts of the allowed Ramachandran space

173 (expected for an intrinsically disordered protein such as αS), a very strong peak shift to a defined chemical shift range typical for α-

174 helical structure is visible after addition of NDs (Figure 2b, red). The DNP data thus show that αS binds the ND lipid surface in α-

175 helical conformation corroborating previous studies using CD and vesicles, solution NMR and detergents micelles and solid-state

176 NMR and SUVs (Fusco, et al., 2014, Galvagnion, et al., 2015, Ulmer, et al., 2005).

177

178 **Membrane plasticity and αS-membrane interaction: a two-way street?**

179 While Figure 1 shows the effect of gradually increasing the level of POPG in DMPC lipids it should be noted that both lipids do not

180 only differ in their head group charge but also in their hydrocarbon chain length and number of unsaturated bonds, i.e. 16:0-18:1 PG

181 and 14:0 PC for POPG and DMPC, respectively (nomenclature refers to 'number of carbons in the fatty acid': 'number of

182 unsaturations'). Both of these features will affect the fluidity and phase transition temperature T_m of the lipid bilayer. In general,

183 native membranes are heterogeneous mixtures of numerous different lipids and proteins, both of which can strongly influence lipid

184 phase properties. In this respect it was also suggested before that the MSP-lipid interactions may not only be seen as an artificial

185 border of the bilayer, but may partly mimic protein-lipid interaction occurring in native membranes (Alvarez, et al., 2010). While the

186 usage of additional transmembrane proteins is rather restricted due to the limited bilayer area of the NDs, the usage of different

187 heterogeneous lipid mixtures may generate a suitable mimetic for rather heterogeneous physiological membranes.

188   To further investigate the effect of different lipid properties we recorded additional NMR spectra of αS in the presence of

189 NDs containing different lipids and lipid mixtures. The data recorded with 100% POPC NDs do not show interaction (Figure 3a,

190 grey), comparable to 100% DMPC NDs (Figure 1a-d, black) and demonstrates that in the absence of negative net charges on the head

191 groups, properties such as an increased chain length and unsaturation, do not induce a significantly stronger interaction. We also used

192 lipid mixtures where charged and uncharged lipids have the same fatty acid chains (i.e. POPC/POPG and DMPG/DMPC) or mixtures

193 where the position of negative charge and hydrocarbon chain is swapped (i.e. DMPG/POPC vs. POPG/DMPC). In these mixtures,

194 we kept the overall net charge content constant at 50%. Our data show that the heterogeneous mixtures DMPG/POPC and

195 POPG/DMPC behave very similarly (Figure 3a, turquoise and dark blue, respectively) suggesting that the position of charge with

196 respect to chain length and unsaturation is not critical in the tested conditions. Also, when using a more homogenous mix of

197 POPG/POPC, a similar pattern is found (Figure 3a, yellow), suggesting that 'surface roughness' of the membrane, as potentially

198 introduced by the heterogeneous lipid chain lengths, does not significantly change the αS-membrane interaction.

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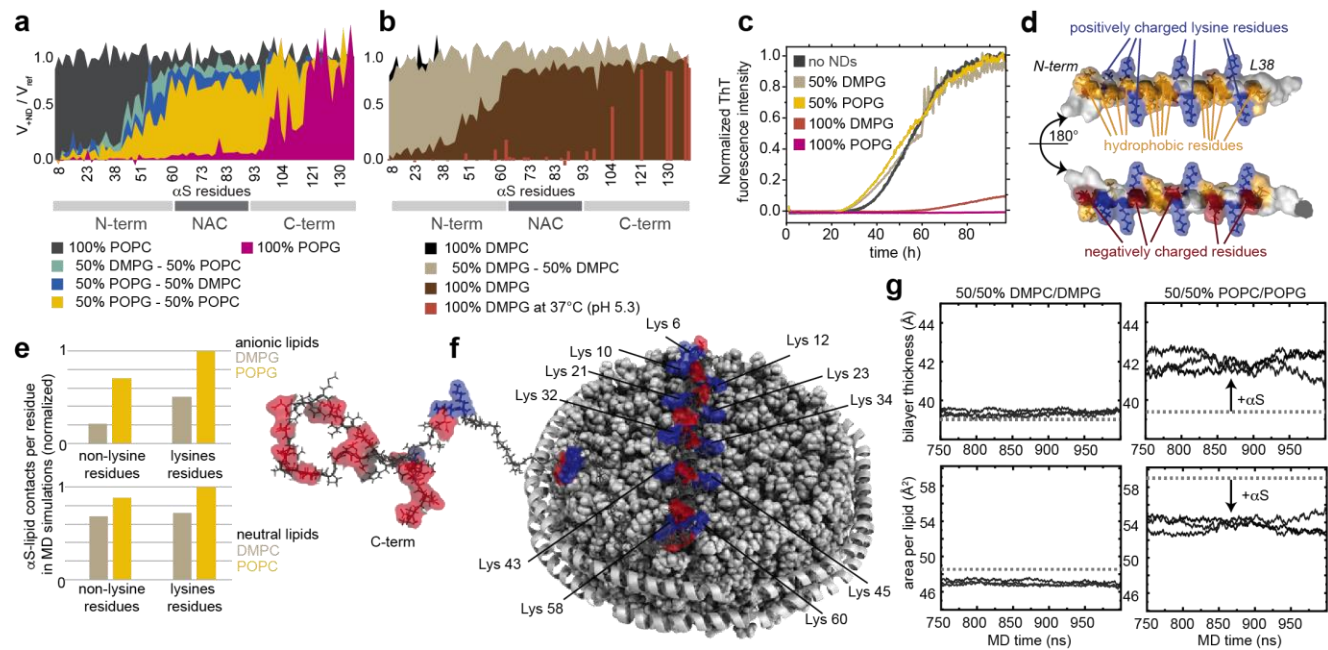
Figure 3. αS-lipid interaction is favored by, and potentially modulates, membrane plasticity. αS NMR attenuation profiles in the presence of NDs with indicated lipid composition (molar ratio 2:1, αS-to-NDs) for bilayers that are in a more fluid (**a**) or gel phase (**b**) at 10°C. Note that all NMR data, except the indicated 100% DMPG measurement (b, red bars), are recorded at 10°C. (**c**) Corresponding ThT aggregation assays for selected conditions (color code as in (a,b), for better visibility only the mean values of triplicate measurements are shown). (**d**) αS structural model of key lipid binding mode (see text for more details). Periodically and symmetrically appearing lysine residues (blue) form a positively charged 'grid'. (**e**) αS-to-lipid contacts (<4 Å) per residue as occurring during the time course of MD simulations. Normalized values for interactions of either lysine or all other residues with anionic lipids (upper diagram) or neutral lipids (lower diagram) for gel/fluid-phase membranes (beige/yellow bars) are shown, respectively. (**f**) Model of αS-ND interaction, lysine (blue) and negatively charged residues (red) are highlighted. (**g**) Effect of αS interaction in MD simulations on the two different membrane systems. Bilayer thickness (upper panels) and area per lipid (lower panels) in the presence (solid lines, three independent simulations) and in the absence of αS (dashed line, one simulation) are shown.

Interestingly, when using a homogeneous mix with fully saturated lipids (DMPC/DMPG), albeit having the same net charge content of 50%, a very different pattern in line with no significant interaction with αS, is visible (Figure 3b, beige). Notably, at the temperature of the NMR experiments (10 °C) the bilayer formed by DM-lipids is in the gel phase (T_m around 28 °C, Figure 1 – figure supplement 2d). This data is in line with previous data on SUVs that identified an important role of the lipid phase for αS-lipid interaction (Galvagnion, et al., 2016). When increasing the charge content to 100%, but remaining in the gel phase (100% DMPG, Figure 3b, brown) the first 60 residues of αS show a clear interaction with the membrane resembling a binding mode that is found for 50% charge content in the fluid phase (e.g. Figure 3a, yellow). While increasing the temperature for the NMR measurements above T_m leads to a previously observed loss of NMR signals due to amide-water exchange processes for most residues relevant for the

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221   interaction (Figure 3 – figure supplement 1c-d), lowering the pH from 7.4 to 5.3 (Figure 3 – figure supplement 1e-f) can counter this

222   effect and allows to confirm that when measuring the interactions to DMPG lipids in the fluid phase, a much larger binding interface

223   is found (Figure 3b, brown-red), comparable to the binding mode found for 100% POPG (Figure 3a, purple). Consequently, when

224   the lipid charge density is high enough, the lipid phase state and related bilayer fluidity in a stable planar bilayer plays an important

225   role in αS association. Taken together, these data on αS-lipid association can be summarized as follows: (i) unsaturations in the

226   hydrocarbon chains, leading to increased membrane fluidity, are not sufficient to induce binding, (ii) the presence of heterogeneity

227   in fatty acid chains and the combination of charge and unsaturation on the same lipid molecule are not critical and (iii) in addition to

228   charge, a lipid phase state that introduces an increased membrane fluidity is important for binding.

229   We also performed ThT aggregation assays with NDs containing selected lipid mixtures as investigated by NMR, again

230   under conditions in which spontaneous aggregation of αS is observed in the absence of lipids. All mixtures that contain 50%

231   negatively charged lipids, independently of tail heterogeneity or charge position show consistently unaffected aggregation behavior

232   (Figure 3c). However, αS aggregation is drastically impeded in the presence of 100% DMPG NDs (Figure 3d, red-brown). Since

233   aggregation assays were measured at 37 °C (i.e. above the DMPG phase transition) this is well in line with the corresponding NMR

234   attenuation profile (Figure 3c, red-brown) suggesting that under this conditions αS is in a lipid binding mode that involves the NAC

235   region and thus inhibits aggregation.

236   From the evaluation of the different membrane interaction modes of αS with NDs observed in this study, it is clear that in

237   particular the initial about 38 residues comprise a central lipid binding motif. Based on previously reported solid-state NMR data

238   (Fusco, et al., 2014) and CD data (Galvagnion, et al., 2015, Jo, et al., 2000) of vesicle preparations as well as the DNP solid-state

239   NMR data of αS in the presence of NDs that we report here, it is also clear that the lipid interacting residues of αS form a helical

240   secondary structure. Analysis of the primary sequence and secondary structure of this interacting region highlights key features of its

241   architecture which is highly suitable for interactions with charged lipid (bi)layers (Figure 3d) (Davidson, et al., 1998). These features

242   include the very exposed and symmetric distribution of positively charged lysine side chains (Figure 3d, blue), the occurrence of

243   hydrophobic residues on one side of the helix (Figure 3d, yellow) and the distribution of negative charges on the opposite side (Figure

244   3d, red). Based on this architecture it is tempting to speculate that the positively charged residues will interact with negatively charged

245   lipid head groups, the hydrophobic residues will be oriented towards the hydrophobic lipid chains and the negatively charged residues

246   will be oriented towards the solvent (and compensate the net charge of the protein). Noteworthy these protein features are again found

247   in the next binding region (residue 39-60). In this picture, it would be likely that the lipids as well as the lysine side chains (partly)

248   rearrange, from their 'unbound' conformation, to ideally accommodate electrostatic interactions in the bound conformation. This

249   rearrangement may be favored by a more fluid lipid phase, which would explain the lower interaction found for NDs with DMPG

250   lipids below the phase transition.

251   To test this hypothesis, we performed molecular dynamics (MD) simulations of αS-membrane interactions. Our simulations

252   focus on the first 61 residues of αS and its interactions with membranes formed by a mixture of either 50% POPG - 50% POPC lipids

253   in the fluid phase or a 50% DMPG - 50% DMPC mix in the gel phase (see methods for more details). Indeed, the MD data confirm

254   that the lysine residues play a key role in the membrane interaction, as e.g. visible by promoting considerably more contacts to anionic

255   lipids than other residues during the time course of the simulations (Figure 3e, upper diagram). Additionally, the MD data also show

256   a generally stronger interaction of αS with the anionic lipids in the fluid membranes (POPG) as compared to the gel-phase membranes

257   (DMPG) (Figure 3e, yellow vs. beige). Noteworthy, these effects are much less pronounced for contacts to the neutral lipids (Figure

258   3e, lower diagram). These findings correlate well with the effects of lipid charge and membrane plasticity seen in the NMR and

259   aggregation assays.

260   Interestingly, the MD data also report on the effect of αS interaction from the lipid point of view. According to this data,

261   the already well ordered DMPC/PG-lipids (gel phase) experience only very small effects due to the presence of αS. On the other

262   hand, the less ordered POPC/PG-lipids (fluid phase) are strongly affected. Here the presence of αS induces a considerably more

263   ordered lipid state as evident by an increased bilayer thickness, reduced surface area per lipid and increased order parameters for the

264   hydrocarbon chains (Figure 3g and Figure 3 - figure supplement 3). Generally, the MD data suggest that αS-membrane interaction is

265   (initially) facilitated by increased membrane plasticity (e.g. more contacts for fluid phase). The increased plasticity may facilitate

266   reorientations of the lipids to support favorable interactions with αS. This view is supported by our MD data showing significantly

267   higher occurrence of short lysine-lipid distances, which would allow lipid-mediated salt bridges, in the simulations with more fluid

268   membranes (Figure 3 - figure supplement 3). These interactions may consequently confine lipids and lead to a reduced membrane

269   plasticity. The latter is in line with recent experimental data showing that αS binding can increase lipid packing (Iyer, et al., 2014) an

270   effect that has also been suggested to play a role in αS function as chaperone for SNARE-mediated vesicle fusion (Burre, et al., 2010).

271

272   **NDs can simultaneously interact with multiple αS proteins facilitating formation of aggregation seeds.**

273   While our NMR data clearly reveal binding modes with different contributions of the αS primary sequence, we were also interested

274   in the overall affinity of the protein for NDs. We therefore measured interaction kinetics using bio-layer interferometry (BLI) with

275   immobilized NDs of different charge content. In line with the NMR data, no αS binding was detected when NDs containing 100%

276   DMPC were immobilized (data not shown). In the case where NDs with 100% anionic lipid content were immobilized, a clear

277   response upon addition of different αS concentrations was observed (Figure 4 a,b) and dissociation constants K_D in the order of 60-

278   100 nM (one αS to one ND) were calculated. In addition, kinetic information could be extracted which shows a tight binding with a

279   fast association and a slow off-rate of approximately 10^{-2} s^{-1}.

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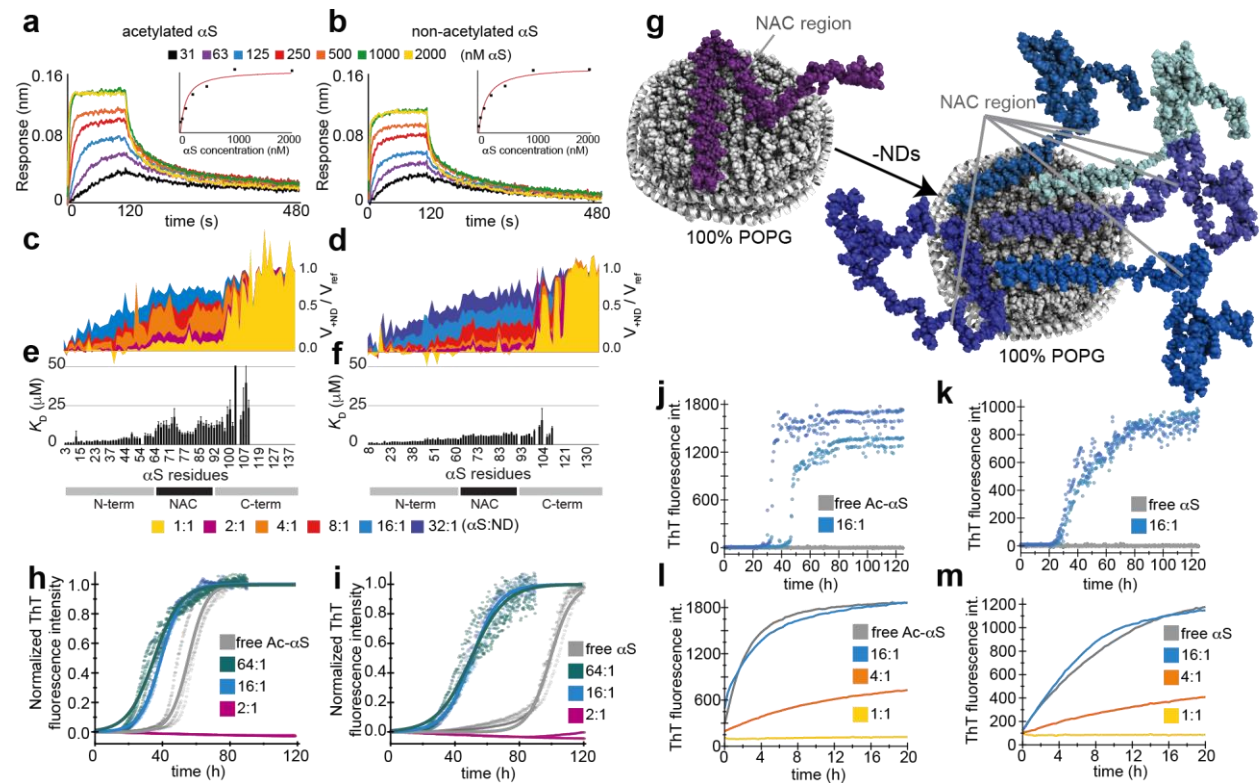
Figure 4. Limited but highly charged lipid surface area can induce an αS binding mode capable of promoting aggregation through induction of primary nucleation. BLI sensorgrams using 100% POPG immobilized NDs and acetylated αS (a) or non-acetylated αS (b) in different concentrations ranging from 31 (black) to 2000 nM (yellow). Corresponding steady-state response plots are shown as inserts. The extracted global affinities (K_D) are about 60 and 100 nM, respectively. NMR attenuation profiles of a titration of 50 µM acetylated (c) or non-acetylated (d) αS with varying concentrations of 100% POPG NDs (αS-to-NDs molar ratios ranging from 32:1 to 1:1, see color code). (e,f) Corresponding residue-specific affinities (K_D) extracted from NMR titration data shown in (c,d), respectively. (g) Model of different αS-lipid binding modes in the presence of excess (upper picture) or limited amount (lower picture) of highly charged NDs (for simplicity only one side of the bilayer is occupied by αS). (h,i) Normalized ThT fluorescence kinetic curves for selected αS-to-ND ratios for acetylated (h) and non-acetylated (i) αS. While high amount of NDs (upper binding mode in g) inhibit aggregation, limited amount of highly charged membrane surface (lower binding mode in g) enhances aggregation. (j,k) Normalized kinetic data in quiescent conditions and absence of preformed seeds at pH 5.3 for acetylated (j) or non-acetylated (k) αS. While under these conditions no aggregation (nucleation) is observed in the absence of NDs (grey) the presence of 16:1 molar ratio of 100% POPG NDs (lower binding mode in g) induces primary nucleation. (l,m) Raw data of quiescent ThT fluorescence aggregation assay in the presence of 2.5% preformed seeds for acetylated (l) or non-acetylated (m) αS for different molar ratios (see color code) of 100% POPG NDs.

In order to obtain residue-specific insights into the membrane affinity of αS, we additionally conducted NMR titration experiments using 100% negatively charged NDs (Figure 4c-f). In general, dissociation constants can be extracted from NMR titrations attenuation profiles by fitting the concentration dependency of the attenuation with a single exponential decay for each

12

300 resolved peak (corresponding to one assigned residue) (Figure 4e-f). Our data reveal differential membrane affinities for different

301 regions of the αS primary sequence. The regions with differential affinities largely overlap with the regions of the different binding

302 modes identified before, i.e. four distinct regions of decreasing affinity range (1-38, 39-60, 61-98, 99-140).

303 Importantly, it appears that one ND with 100% negatively charged lipids can simultaneously interact with up to 16 αS

304 molecules (8 per bilayer side) in the course of the NMR time scale, as seen from the almost complete disappearance of the signals of

305 the very N-terminal residues (Figure 4e-f, light blue). Moreover, while these first residues are interacting with the membrane,

306 independently of the number of αS molecules bound to one ND, the binding of the NAC region is strongly modulated by the number

307 of bound αS molecules. This suggests that, due to the higher lipid affinity of the N-terminal region as compared to the NAC region,

308 the free energy of the system is minimized by favoring N-terminal interactions in cases where the accessible membrane surface is

309 limited. Note that due to the geometry of the used nanodiscs up to five αS molecules can simultaneously bind with a 38-residue long

310 α-helix (first binding mode) to one side of one ND. If 8 molecules are accommodated together on the surface, (on average) a 23-

311 residue long helix per monomer could be formed.

312 To characterize the effect of the accessible membrane surface area (as given by the αS-to-ND ratio) and the resulting stoichiometry

313 on αS aggregation behavior, we measured ThT aggregation kinetics on samples with different αS-to-ND ratios ranging from 2:1 to

314 64:1 by varying the ND concentration at constant αS concentrations (Figure 4h-i). Interestingly, a higher ratio of αS-to-ND leads to

315 a prominent decrease in aggregation lag-times when using 100% POPG NDs (Figure 4h,i, blue and cyan). These data show, in line

316 with previously reported behavior on SUVs (Comellas, et al., 2012, Galvagnion, et al., 2015, Zhao, et al., 2004), that under specific

317 conditions lipid bilayers can accelerate the fibrillation process. Here, these conditions represent a limited membrane surface area with

318 a high charge density. According to our NMR data this will introduce an αS-lipid binding mode that brings several αS molecules with

319 exposed NAC regions in close proximity.

320 Noteworthy, it has been reported in the case of SUVs (Galvagnion, et al., 2015, Hellstrand, et al., 2013) that lipids can be

321 incorporated in fibrils and change their morphology. Nonetheless, in the case of the more stable nanodiscs this is not expected, indeed

322 AFM images of αS fibrils formed in the absence or presence of NDs do not show different morphology (Figure 4 – figure supplement

323 1) and the size of nanodiscs does not change after interaction with αS (as seen from SEC, Figure 1 – figure supplement 2). This

324 indicates that a perturbation of the aggregation pathway by lipid incorporation is very unlikely in our case.

325 We additionally carried out the same BLI measurements, NMR titrations and ThT assays for ND containing only 50%

326 POPG lipids. For these NDs no clear signature of binding could be obtained in the BLI measurements (data not shown), suggesting

327 a weak affinity and/or too fast off rates to allow detection via BLI. This is in line with SEC profiles that also point to a more transient

328 interaction (Figure 1 – figure supplement 2).

329 NMR titrations, however, show clear concentration dependent attenuation profiles that allow the calculation of residue

330 specific affinities (Figure 5a-d). Noteworthy, the affinities for the αS residues in the first binding region (residues 1-38) are

13

331 comparable to the values obtained for 100% charged NDs (Figure 4e-f). In contrast, for the following binding regions much lower

332 affinities are found (at the edge of detection for residues 39-60, and no interaction for residues > 60), including the absence of

333 interactions of the NAC region. In line with an exposed NAC region the ThT data for NDs with 50% negatively charged lipids at low

334 αS-to-ND ratios are consistently showing no effect on aggregation half-times. The data at higher ratios are less reproducible and show

335 a slight tendency to prolonged half-times (Figure 5e-f). At this point, it is not clear whether this feature has mechanistic relevance or

336 is just an artefact caused by the limited reproducibility of this condition.

337

338 **The molecular, thermodynamic and kinetic determinants of membrane-modulated αS aggregation.**

339 It has recently been shown in a systematic study that under appropriate conditions, which minimize the intrinsic nucleation rate

340 (quiescent conditions and protein repellent plate surfaces), lipid bilayers in the form of small unilamellar vesicles can largely

341 accelerate the nucleation of αS amyloid fibrils (Galvagnion, et al., 2015). In order to determine whether NDs can have a similarly

342 accelerating effect, we performed ThT aggregation experiments under similar conditions, i.e. where no αS aggregation should be

343 detected in the absence of lipids (see methods for more details). These experiments were performed at mildly acidic pH (5.3), as it

344 was recently shown that under these conditions, αS amyloid fibrils can amplify autocatalytically through surface-catalyzed secondary

345 nucleation (Buell, et al., 2014, Gaspar, et al., 2017). This should in principle enable even very low primary nucleation rates to be

346 detected through autocatalytic amplification. Membrane binding behavior of αS at these buffer conditions was also checked by NMR

347 for 100% POPG NDs and comprises the same region (residues 1-96 at low αS-to-ND ratios) as seen for neutral pH (Figure 3- figure

348 supplement 1c,d).

349 As expected, the ThT assay under the quiescent conditions does not show any aggregation in the absence of NDs (Figure

350 4j,k grey). However, the samples with 100% POPG NDs (in a αS-to-ND ratio of 16:1) do display aggregation, indicating that this

351 type of membrane is indeed enhancing primary nucleation to a degree sufficient that the subsequent secondary nucleation leads to

352 detectable quantities of amyloid fibrils (Figure 4j,k blue). Interestingly, since our data also allow an estimation of the total number of

353 αS monomers that are brought in close proximity due to their interaction with the same ND, this result may also provide a first

354 approximation of the number of αS monomers needed for the formation of a nucleus. Our data suggest that this 'minimal critical

355 nucleation number' has an upper limit of around 8 αS molecules.

356 In order to further disentangle the effect of NDs on the various individual steps in the αS aggregation pathway, we next

357 designed strongly seeded aggregation assays under quiescent conditions at neutral pH, where fibril elongation is the only process that

358 occurs at a significant rate. Seeding experiments carried out in the presence of 100% charged (POPG) NDs show a clear reduction in

359 elongation rate with increasing ND concentration (Figure 4l,m). While at high molar excess of αS (16:1, αS-to-ND), according to our

360 NMR data, nearly all αS monomers should interact through their most N-terminal regions with the NDs, no significant effect on the

361 elongation rate was observed (Figure 4l,m blue). At molar ratios of 4:1 (Figure 4l,m orange) a decrease in elongation is observed,

14

362   however at this ratio our NMR data clearly indicate that all αS monomers are interacting with the NDs. Nevertheless, in both cases

363   (molar ratios of 16:1 and 4:1) larger fractions of the monomer population should still have accessible NAC regions, which may

364   explain their ability to participate in the fibril elongation process. However, the limited interaction surface may also influence the

365   dynamic nature of membrane association leading to a certain population of free monomeric αS at any given time.

366

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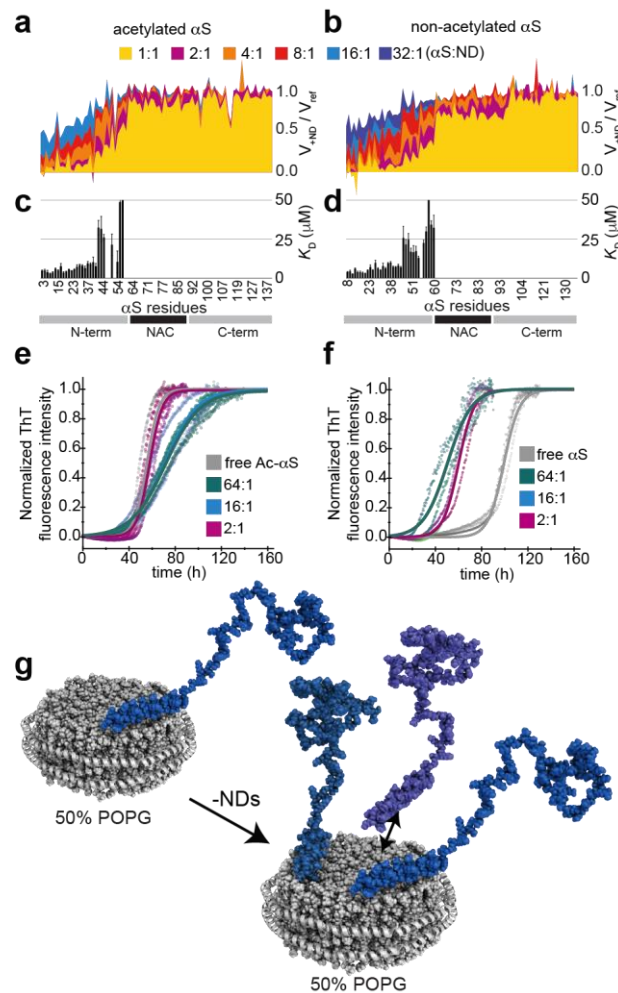
368   **Figure 5. Moderately charged membrane surfaces interact more transiently with αS and do not induce primary nucleation.** NMR

369   attenuation profiles of a titration of 50 µM acetylated (a) or non-acetylated (b) αS with varying concentrations of 50% POPG NDs (αS-to-

370   NDs molar ratios ranging from 32:1 to 1:1, see color code). (c,d) Corresponding residue-specific affinities (K_D) extracted from NMR titration

371   data shown in (a,b), respectively. (e,f) Normalized ThT fluorescence kinetic curves for selected αS-to-ND ratios for acetylated (e) and non-

372   acetylated (f) αS. (g) Model of αS-lipid binding modes in the presence of excess (upper picture) or limited amount (lower picture) of

373   moderately charged NDs (for simplicity only one side of the bilayer is occupied by αS).

374

375   Unlike for 100% POPG NDs and in line with the previously discussed moderate effects of 50% POPG NDs on the overall

376   aggregation process (Figure 1f,g blue, Figure 3d yellow and beige, Figure 5e,f), we did not observe accelerated αS nucleation in the

15

377   presence of NDs with 50% POPG nor a clear perturbation of fibril elongation in seeded experiments (Figure 5 – figure supplement

378   1c-i). This data is difficult to explain given that elongation is, in all cases of amyloid formation, responsible for the generation of the

379   bulk of fibril mass. Hence, its inhibition should slow down the overall aggregation kinetic, also under non-seeded conditions.

380   Noteworthy, we observe that ThT signal intensity can be strongly affected by the presence of NDs and that the absolute ThT intensity

381   does not correlate with absolute fibril mass when comparing data recorded in the absence or presence of NDs (as seen from SDS-

382   PAGE, Figure 5 – figure supplement 1e). Although our NMR data show that these NDs do not interact with the αS, the corresponding

383   ThT signal (of the identical samples) show very different intensities (Figure 5 – figure supplement 1f). It is therefore likely that the

384   ThT (unspecifically) interacts with NDs, leading to an overall decrease in ThT intensities. We therefore normalized most ThT assays.

385   Noteworthy, this effect is less pronounced for ND with 100% POPG (as e.g. visible in the data in Figure 4m,l), which we attribute to

386   the high coverage of the lipid surface by αS molecules that may reduce the unspecific ThT-ND interactions. In line with our other

387   data recorded using 50% POPG NDs, albeit significantly reduced ThT sensitivity, only very moderate effects of the NDs on the fibril

388   elongation process are visible after normalizing ThT intensities in the seeded aggregation assays.

389

390   **N-terminal acetylation has moderate effect on membrane association and leads to different behavior in aggregation assays.**

391   As visible in Figures 1 and 3-5, in addition to using the N-terminally acetylated form of αS, which represents the native post-

392   translational modification of αS and is known to be relevant for membrane association (Nemani, et al., 2010, Theillet, et al., 2016),

393   we also recorded most experiments with the non-acetylated form of αS. In line with previous findings (Maltsev, et al., 2012), N-

394   terminal acetylation leads to clear chemical shift perturbations in the NMR spectra for the first 10 residues of αS (Figure 1a,b).

395   Overall, most of the above discussed features of αS membrane interaction are rather similar in acetylated and non-acetylated

396   αS, however there are a number of distinct differences. For instance, the peaks which are already shifted in free αS due to the

397   acetylation are also the ones that are affected most by the presence of nanodiscs with low amount of charges (close to physiological

398   concentration). Our data (Figure 1a-d) show a rather small but significant increase in the membrane association of the first 15 residues

399   due to the N-terminal acetylation, which is in line with previous observation using SUVs (Bartels, et al., 2014, Dikiy and Eliezer,

400   2014). αS acetylation is known to increase N-terminal helix propensity (Kang, et al., 2013, Maltsev, et al., 2012), which may facilitate

401   formation of the initial binding mode and be of significance for naturally occurring processes.

402   A similar effect is also seen for the global binding as determined by BLI, which shows a (slightly) higher membrane affinity

403   of the acetylated (Figure 4a, K_D of 60 nM) as for the non-acetylated αS construct (Figure 4b, K_D of 100 nM). When looking deeper

404   into the NMR titration data, it appears that another effect takes place, namely a slightly increased membrane affinity of the NAC

405   region for the non-acetylated αS NAC region (Figure 4e *vs.* f and Figure 5c *vs.* d). At current stage, it is not easy to explain why a

406   modification at the N-terminus will affect the lipid interaction of a protein region that is sequentially separated by roughly 60 residues.
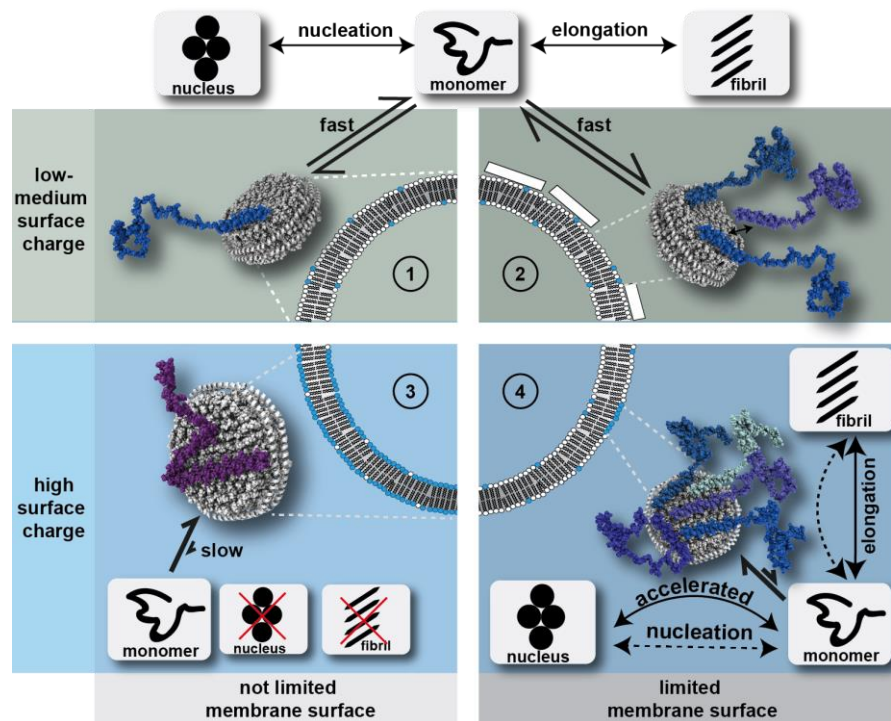
407 Such a behavior could however either be related to intermolecular interactions and/or long range intramolecular interactions (in a

408 'horseshoe'-conformation) that may or may not be artificially introduced by the limited surface area of the NDs.

409   Strikingly, the reference kinetic curve of non-acetylated αS reproducibly shows under the applied conditions a strongly

410 delayed aggregation as compared to the acetylated reference. In the setup used, primary nucleation processes are likely to happen at

411 the air-water or plate-water interface (Campioni, et al., 2014, Vacha, et al., 2014), thus a lower hydrophobic propensity of non-

412 acetylated αS could explain this effect. While this may be the dominant process in the absence of lipids, it may not be the case

413 anymore in the presence of NDs (Galvagnion, et al., 2015), either because NDs shield these interfaces or because nucleation happens

414 primarily at the membrane surface. The much lower differences due to acetylation state in the presence of NDs fit this explanation,

415 as well as additional tests we ran using different types of plates (data not shown). Higher order processes, namely different

416 fragmentation behaviors, can however not be excluded.

417   It appears that the biggest effect of acetylation is related to assay parameters that are normally not the matter of interest,

418 which nevertheless may be important for future studies (Iyer, et al., 2016). Still the results from systematic measurement of the effects

419 of N-terminal acetylation via different methods point to subtle changes in membrane interaction in respect to NAC region specific

420 affinities at high lipid charge densities as well as to N-terminal binding at a lipid charge density comparable with the overall

421 composition found for membranes in e.g. synaptic vesicles (Fusco, et al., 2014). Hence, both effects may be of particular importance

422 under physiological conditions.

423

424

425 **DISCUSSION**

426

427 Overall our data demonstrate that the nanodisc system allows to study the interaction of αS with stable, planar membranes in a

428 quantitative and site-resolved way. It also provides insights into the correlation between the identified membrane binding modes as

429 well as binding kinetics and their consequences for αS amyloid fibril formation, both in respect to the nucleation and elongation

430 process. In summary, our data show that (i) residue specific αS-membrane affinities are rather similar for the N-terminal αS region

431 for 100% and 50% negatively charged NDs, (ii) for 100% anionic lipids the αS can adopt a substantially expanded binding mode as

432 compared to 50% anionic lipids content, leading to considerably higher global affinities, (iii) the exchange rate between free αS in

433 solution and membrane-bound αS is rather slow in the 100% charged case and likely to be rapid in the 50% case, (iv) region-specific

434 membrane affinities (especially the NAC region) are correlated with aggregation properties, (v) with sufficient excess of lipids and

435 sufficient charge density, NDs can inhibit primary nucleation as well as fibril elongation by sequestering monomers out of solution,

436 (vi) competition of αS monomers for highly charged lipid surface generates a membrane-bound αS conformation that can induce

437 primary nucleation, and (vii) the number of αS monomers that are brought together on one ND and which can promote amyloid fibril

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438 nucleation is in the order of 8 αS molecules. Figure 6 summarizes these molecular, thermodynamic and kinetic determinants of

439 membrane-modulated αS aggregation.

440

441

442 **Figure 6. Model of the influence of different NDs on the αS aggregation pathways and its potential implication in the context of**

443 **cell/vesicle membranes.** Four scenarios are depicted according to their membrane surface charge and accessibility, respectively. In cases

444 where membranes/NDs with only low charge densities are present (scenario 1 and 2) αS interacts with its N-terminal residues and most likely

445 forms a fast exchanging equilibrium between soluble and membrane associated αS monomers. This equilibrium does not seem to strongly

446 interfere with the slow process of αS nucleation, it may however (slightly) decrease the pool of free monomers available for fibril formation.

447 Notably these conditions are more likely to better resemble the average charge densities found in physiological membranes. However,

448 specific, abnormal and/or stochastic processes may also lead to highly charged membrane surfaces with limited (scenario 4) or not limited

449 surface access (scenario 3). αS will strongly interact with the latter in a binding mode that will largely inhibit both, αS nucleation and fibril

450 elongation. In cases where several αS monomers compete for a limited highly charged membrane surface area (scenario 4), the amyloid fibril

451 nucleation process can be accelerated, most likely due to an αS binding mode that brings exposed NAC regions of several αS monomers in

452 close proximity. Under these conditions the fibril elongation rate is largely unperturbed, probably due to sufficient monomers with limited

453 membrane association and/or due to induction of higher order processes.

454

455   While our *in vitro* data show strongest effects at lipid charge densities well above the average lipid compositions of native

456 membranes, the membrane composition may vary locally in physiological membranes. Due to the normally found high lipid diffusion

457 rates, clusters of higher negative charges may form spontaneously and/or be induced by an initially transient αS interaction. For the

18

458   latter, the N-terminal acetylation may play an important role, since it increases membrane interaction at average native lipid charge

459   densities.

460   Our data suggest that clusters of around 60-80 negatively charged lipids suffice to form a strong interaction (this may

461   however not be the lower limit). Sporadically formed lipid charged clusters could also induce a competition of several αS monomers

462   for the accessible surface area. Our data show that due to the different residue-specific membrane affinities this will generate a binding

463   mode that, once the rather low αS critical oligomerization number is reached, can act as an aggregation seed. Such a scenario could

464   promote the initial step of primary nucleation in the pathogenesis of Parkinson's disease and is in line with recent *in vivo* findings

465   suggesting that shielding αS from membrane interactions can inhibit initial steps of amyloid fibril formation including the formation

466   of cell toxic species (Perni, et al., 2017).

467

468

469   **MATERIALS AND METHODS**

470

471   **αS and N-terminally acetylated αS expression and purification**

472   Codon-optimized αS in the pT7-7 vector was expressed in *E. coli* BL21 DE3. For acetylated αS, the N-terminal acetylation enzyme NatB from

473   *Schizosaccharomyces pombe* was coexpressed in a second vector, pNatB (Johnson, et al., 2010). PNatB (pACYCduet-naa20-naa25) was a gift from

474   Dan Mulvihill (Addgene plasmid # 53613). Expression was conducted in 50 mM phosphate-buffered 2YT-medium (pH 7.2) with 0.4% glycerol and

475   2 mM $MgCl_2$, protein production was induced at OD 1-1.2 with 1 mM IPTG and run for 4 h at 37 °C. For ^{15}N-labelled protein, αS or acetylated αS

476   was expressed in M9 medium with 0.2% $^{15}NH_4Cl$.

477   Sparsely labelled αS for DNP experiments was non-acetylated, expression was done in a similar way, in M9 medium using 0.4% [2-^{13}C]-glucose

478   and 0.2% $^{15}NH_4Cl$. Isotope labelling of Phe, Gln, Glu, Pro, Asn, Asp, Met, Thr, Lys, and Ile was suppressed by supplementing sufficient quantities

479   (150 µg/ml of each) of these unlabeled amino acids in the expression media as reported previously(Hong and Jakes, 1999).

480   Purification of αS or acetylated αS was carried out as previously described(Hoyer, et al., 2002), some changes to the original protocol have been

481   made. Except for sparse labelled αS for which previous lysis in 20 mM Tris-HCl pH 8.0, 1 mM EDTA was done, cell lysis and release of αS or

482   acetylated αS was performed by directly boiling the frozen cell pellet at 95 °C in a threefold volume of 20 mM sodium phosphate buffer, pH 7.4, for

483   30 min. Thermostable αS or acetylated αS remained in the supernatant after 30 min of centrifugation at 15000·g and 4 °C and was subsequently

484   subjected to an ammonium sulfate precipitation by slowly adding saturated ammonium sulfate solution to 50% saturation at 4 °C. Precipitated protein

485   was pelleted at 15000·g and 4 °C, dissolved in 50 mL of 50 mM Tris-HCl pH 8, sterile-filtered and loaded onto an ion exchange chromatography

486   column (HiTrap Q FF, GE Healthcare), where αS or acetylated αS eluted at around 300 mM NaCl in 50 mM Tris-HCl pH 8. Elution fractions

487   containing αS or acetylated αS were subjected to another ammonium sulfate precipitation and finally purified by a SEC run (Superdex 75 10/300,

488   GE Healthcare) in 20 mM sodium phosphate pH 7.4, 50 mM NaCl.

489   N-terminal acetylation of acetyl-αS was checked by HPLC, mass spectrometry, and NMR, proved to be about 95% when coexpressed with NatB.

490

19

Membrane scaffold proteins expression and purification

As reported in before(Bayburt, et al., 1998) *E. coli* BL21 (DE3) were transformed with MSP1D1 or MSP1D1ΔH5 plasmid DNA in vector pET28a. Cells were grown in LB medium, induced by 1 mM IPTG at an optical density of 0.7 and incubated 5-6 hours at 37°C, then pelleted down. Cells were resuspended in 50 mM Tris-HCl pH 8, 500 mM NaCl (buffer B) supplemented with 6 M GdnHCl and EDTA-free Complete protease inhibitors (Macherey-Nagel) lysed by sonication (Bandelin Sonopuls MS72 probe), centrifuged at 17000·g for 1 hour (Beckman J2-21 rotor JA-20.1) and incubated 1 hour with previously equilibrated 2.5 ml Ni-NTA agarose resin/3L culture (Macherey-Nagel). Column was washed with 4 CV buffer B, 4 CV buffer B supplemented with 1% Triton X-100, 4 CV buffer B + 60 mM Na-cholate, 4 CV buffer B, 4 CV buffer B + 20 mM imidazole. Four fractions of 1 CV were eluted with 250 mM imidazole. The whole process was kept at 4°C in a cold room. The elution fractions were pooled and dialysed against 100-fold 200 mM Tris-HCl pH 7.5, 100 mM NaCl. N-terminal His-tag was cleaved using TEV protease incubated overnight at 4 °C. ΔHis-MSP was separated from MSP by IMAC and concentrated to the desired molarity using a Vivaspin centrifugal device of 10 kDa MWCO.

Nanodiscs assembly

Nanodiscs were assembled according to established protocols(Bayburt, et al., 2002). In short, lipids chloroform stocks were dried under nitrogen flow to obtain a lipid film and stored under vacuum overnight. ΔHis-MSP1D1 or MSP1D1ΔH5 and the appropriate amount of lipids (Avanti Polar Lipids) solubilized in 60 mM Na-cholate were mixed together in 20 mM Tris-HCl pH 7.5, 100 mM NaCl, 5 mM EDTA. The scaffold-to-lipids molar ratio was calculated from geometrical considerations. 20% w/v of previously washed Biobeads SM-2 (Biorad) were added and the mixture incubated at room temperature overnight. The Biobeads were removed by centrifugation and once again 20% w/v were added for an additional 4-5 hours. Finally, they were purified by SEC on a HiLoad 16/600 Superdex 200 pg column (GE Healthcare) equilibrated with 20 mM sodium phosphate pH 7.4, 50 mM NaCl using a Äkta pure device at a flow rate of 1 ml/min. The quality of NDs preparation was check by the SEC chromatogram as well as by DLS (PSS Nicomp). NDs were concentrated to the desired molarity using a Vivaspin centrifugal device of 10 kDa MWCO.

Bio-layer interferometry (BLI)

NDs were immobilized on the sensor surface of amine reactive biosensors (AG2R) (fortéBIO, PALL Life Science) after EDC/NHS activation to a final level between 1.2 and 1.8 nm depending on the NDs type using an Octet RED96 instrument (fortéBIO, PALL Life Science). All biosensors were quenched with 1 M ethanolamine for 3 min. All experiments were carried out in multi cycle kinetics at 25 °C. Association of αS in running buffer (20 mM sodium phosphate pH 7.4, 50 mM NaCl) on NDs and reference biosensors was recorded for 120 s, followed by a dissociation phase of 360 s. Sensorgrams were double referenced using the reference biosensors and a buffer cycle. Steady-state analysis was realized by fitting the αS concentration dependency of the highest response against with a simple 1:1 binding model. After normalization, all on and off curves were fitted against simple exponential build-up or decays and led to similar on- and off-rates.

Solution NMR spectroscopy

Solution NMR experiments were performed on a Bruker Avance III HD spectrometer operating at 600 MHz ^{1}H Larmor frequency, equipped with a triple resonance TCI (^{1}H, ^{13}C, ^{15}N) cryoprobe and shielded z-gradients. If not stated otherwise, all experiments data were recorded at 10 °C with an αS concentration of 50 μM in 20 mM sodium phosphate pH 7.4, 50 mM NaCl, 10% (v/v) ^{2}H$_2$O and ND concentration was set to 25 μM (one αS per membrane leaflet). All [^{1}H-^{15}N]-TROSY-HSQC NMR spectra were acquired with 32 scans and 256 indirect increments, processed with TOPSPIN

526   3.2 (Bruker) and analyzed with CCPN(Vranken, et al., 2005). Peaks were automatically integrated and the ratio of volumes in the presence and

527   absence of NDs plotted against the primary sequence. Outliers as results of peak overlap and/or ambiguities were removed.

528

529   **ThT fluorescence aggregation assays**

530   50 µM of αS or acetylated αS was mixed with either 25 µM (2:1), 3.125 µM (16:1) or 0.781 µM (64:1) nanodiscs with different lipid mixtures.

531   Assays were conducted in 20 mM sodium phosphate buffer pH 7.4 or 20 mM acetate buffer pH 5.3 with 50 mM NaCl, 0.02% NaN_3 and 10 µM

532   Thioflavin T. Unless otherwise stated, triplicates of 120 µl were pipetted into 96-well half area well plates with non-binding surface (Corning

533   No. 3881, black, clear bottom) containing a glass ball for mixing and incubated at 37 °C for up to 7 days. Orbital shaking at 217 rpm was used for

534   15 s every 20 min. Thioflavin T fluorescence was excited at 445 nm and measured at 485 nm every 20 min with 15 s of shaking prior to the

535   measurement in a plate reader (Tecan Spark 10M or Tecan infinite M1000PRO).

536   For the seeded experiments, fibril seeds of αS or acetylated αS were prepared as follows: 300 µl of 100 µM αS or acetylated αS was fibrillated at

537   37 °C and 800 rpm for 3 days in a 2 ml tube containing a glass ball in a Thermomixer (Eppendorf). The fibril solution was diluted to 50 µM and

538   sonicated with a tip sonicator (Bandelin Sonopuls HD3200, BANDELIN electronic) at 10% power (20 W) for 60 s, with 1 s pulses on and 4 s off in

539   between. Seed solution was diluted 20-fold for the aggregation assays (2.5 µM, 5%).

540   Kinetic curves were corrected by subtracting the curve of buffer (containing NDs) in the presence of ThT and normalized to highest fluorescence

541   intensity (in line with comparable fibril mass seen in SDS-PAGE after the aggregation assay). The corresponding triplicates are shown as transparent

542   circles in order to visualize the reproducibility of each experiment. In the case of quiescent nucleation and seeded assays no normalization was applied

543   and data were recorded without the presence of glass balls and without plate shaking.

544

545   **Sodium Dodecylsulfate – Polyacrylamide Gel Electrophoresis (SDS-PAGE)**

546   In order to compare the amounts of soluble and fibrillated αS or acetylated αS in the aggregation samples, 100 µl of each triplicate sample were

547   taken out of the well plate, combined in 1.5 ml tubes and spun down at 20000·g and 20 °C for 30 min. Supernatants (~290 µl) was removed and

548   pellets were resuspended in 280 µl buffer and SDS-sample buffer (4-fold) was added. Samples were boiled for 15 min at 98 °C and subsequently

549   10 µl were loaded onto a 15% SDS-gel together with standards of αS or acetylated αS and nanodiscs.

550

551   **Dynamic Nuclear Polarization (DNP) NMR spectroscopy**

552   Magic-angle spinning solid-state DNP experiments were performed on a Bruker Avance III HD spectrometer operating at 600 MHz, equipped

553   with a 395.18 GHz second-harmonic gyrotron and a 3.2 mm ^{1}H,^{13}C,^{15}N triple resonance low-temperature MAS probe. Data were collected at 100 K,

554   9 kHz MAS speed and 9 W continuous-wave microwave power. The samples were prepared from sparsely labelled non-acetylated αS (250 µg) in

555   the presence or in the absence of 2:1 molar ratio of 100% POPG nanodiscs and filled into 3.2 mm sapphire rotors. Final sample conditions were 15

556   mM sodium phosphate pH 7.4, 25 mM sodium chloride, 30% ^{2}H$_2$O, 60% glycerol-d$_6$ and 2.5 mM AMUPOL(Sauvee, et al., 2013). Two-dimensional

557   [^{13}C-^{13}C]-Proton-Driven Spin Diffusion (PDSD) experiments with 1 s mixing time were performed. ^{1}H decoupling using SPINAL64 with a

558   decoupling field of 104 kHz was employed during evolution and detection periods. Both experiments were conducted using 300 t_1 increments with

559   16 and 48 scans each for αS in the absence and in the presence of nanodiscs, respectively. A recycle delay of 5 s was used in both experiments. Both

560   spectra were processed using Topspin 3.2 (Bruker) using identical parameters with squared and shifted sine bell function (qsine 2.5) for apodization.

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Molecular Dynamics simulations

As starting conformation for the MD simulations, the NMR structure of micelle-bound αS (PDB 1XQ8) was used, considering only the first 61 residues in order to concentrate on the membrane binding region of αS. The Amber99sb-ILDN force field (Lindorff-Larsen, et al., 2010) was used for αS, which was simulated in its non-acetylated form (i.e., with NH3+ at the N-terminus) and with a C-terminal N-methyl amide capping group to account for the fact that αS would continue beyond residue 61. All lysine side chains were modeled as positively charged, glutamate and aspartate as negatively charged, while glutamine and histidine residues were considered to be neutral corresponding to a pH of 7.4. The protein was placed either 0.5 nm or 1.5 nm above the membrane surface. A starting orientation with the negatively charged side chains pointing away from the membrane and the lysine side chains being oriented towards the membrane surface were chosen (Fig. 3d). For modeling the lipid bilayer, membrane patches consisting of POPC/POPG (1:1) or DMPC/DMPG (1:1) involving 512 lipids (256 lipids per leaflet) were built using CHARMM-GUI (Lee, et al., 2016) and modeled with Slipids force field parameters (Jambeck and Lyubartsev, 2012, Jambeck and Lyubartsev, 2013). Before αS was added, both lipid bilayers were solvated and simulated for 500 ns (POPC/POPG) or 1000 ns (DMPC/DMPG) to obtain relaxed membranes. Here, the same simulation procedure was employed as described below. αS was placed above the membrane, the protein-membrane complex solvated using the TIP3 water model, Na+ and Cl- added to neutralize the system and to mimic the Na+ concentration used in the experiments. The ion parameters of Smith and Dang (Smith and Dang, 1994) were used. The system was then subjected to steepest descent energy minimization, followed by MD equilibration in the NVT ensemble for 1 ns at 10 °C using the V-rescale thermostat (Bussi, et al., 2007) with a time constant of 0.5 ps and separate temperature coupling for the protein, membrane and water/ions. Afterwards, 1 ns of NPT equilibration was performed using the Nose-Hoover thermostat (Hoover, 1985, Nosé, 1984) and Parrinello-Rahman barostat (Parrinello and Rahman, 1981) with semiisotropic pressure scaling, a reference pressure of 1 bar, a time constant of 10.0 ps and an isothermal compressibility of $4.5 \times 10\text{-}5$ bar-1. During both equilibration steps, restraints were applied to the positions of the P-atoms of the lipids and terminal C-atoms of their tails with a force constant of 1000 kJ mol-1 nm-2. All bond lengths were constrained using the Lincs algorithm (Hess, et al., 1997). The Coulombic interactions were calculated using the Particle mesh Ewald (PME) method (Darden, et al., 1993, Essmann, et al., 1995) with a cut-off of 1.0 nm for the short-range interactions and a Fourier spacing of 0.12 nm. The cut-off for the van der Waals interactions was set at 1.4 nm. Periodic boundary conditions were employed in all directions. For the MD production runs the same settings as for the NPT equilibration were used, except that all position restraints were removed. All MD simulations were performed at 10 °C with a time step of 2 fs for integration using the GROMACS 4.6 molecular dynamics package (Hess, et al., 2008). For the analysis, which was performed using Gromacs and Membrainy tools (Carr and MacPhee, 2015), only the last 250 ns of each production run was used. An overview of the production runs, can be found in Figure 3 – figure supplement 2.

Differential Scanning Calorimetry (DSC)

Samples of approximately 5 µM NDs of different types (and if stated 10 µM αS) were degassed for at least 20 min at 30 °C and measured in a Microcal VP-DSC instrument (Malvern Instruments). The thermograms were acquired up-scan from 5°C to 45°C at a scanning rate of 0.5°C/min and corrected by subtracting the thermogram of buffer.

Atomic Force Microscopy (AFM)

AFM images were taken in air, using a Nanowizard III atomic force microscope (JPK, Berlin). Samples were taken at the end of aggregation experiments and diluted in pure water to ca. 1 µM protein concentration. 10 µl were added onto freshly cleaved mica and left to dry. Then, they were

597   gently rinsed with water to remove excess salt. Imaging was performed using tapping mode with NSC 36 cantilevers (MikroMasch), with resonant

598   frequencies between 70 and 150 kHz.

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COMPETING INTERESTS

610   The authors declare that no competing interests exist.

AUTHOR CONTRIBUTION

612   H.H., B.S., D.W., W.H., A.K.B. and M.E. designed the experiments. T.V., M.M.W., H.S., B.U. and C.P. performed the experiments. T.V.

613   and M.M.W. analyzed the data. T.V. and M.E. wrote the manuscript. All authors commented on the manuscript.

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793   **FIGURE SUPPLEMENT**

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αS type	pH	MSP type	Lipids in ND	αS-to-ND ratio
acetylated	5.3	-	-	-
	7.4	-	-	-
		D1	100% DMPC	2:1
			25% POPG – 75% DMPC	
			50% POPG – 50% DMPC	1:1 2:1 4:1 8:1 16:1
			75% POPG – 25% DMPC	2:1
		Δ5		
	5.3	D1		2:1
	7.4	D1	100% POPG	1:2 1:1 2:1 4:1 8:1 16:1
			100% POPC	2:1
			50% POPG – 50% POPC	
			50% DMPG – 50% POPC	
			50% DMPG – 50% DMPC	
			100% DMPG	
	5.3	D1	100% DMPG, 37°C	
non-acetylated	7.4	D1	100% DMPC	2:1
			25% POPG – 75% DMPC	
			50% POPG – 50% DMPC	1:1 2:1 4:1 8:1 16:1 32:1
			75% POPG – 25% DMPC	2:1
			100% POPG	1:1 2:1 4:1 8:1 16:1 32:1

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796   **Figure 1 – figure supplement 1. Summary of NMR samples used in the study.**

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799 **Figure 1 – figure supplement 2. Formation of lipid mixtures in NDs, lipid phase transitions and stability of αS association.** (a) NMR

800 ¹H-1D spectra of αS in the presence of NDs containing different POPG-to-DMPC ratios corresponding to the samples shown in Fig. 1a-b.

801 Chemical shifts ranges for signals from either DMPC (left, choline methyl groups) or POPG (right, ¹H next to the unsaturation) are displayed.

802 The volumes of the peaks reflect within an error of approximately 10% the aimed lipid compositions. In addition to volume changes also

803 chemical shifts perturbations for both lipid specific peaks are visible and follow a rather linear dependence on the composition. The NMR

804 data therefore report on both the presence and mixing of both lipid types in single nanodiscs. (**b**) Size exclusion chromatography (SEC)

805 profiles (Superdex 200 13/300 gl, GE Healthcare) of αS alone (grey), 50% POPG NDs alone (black) and both after mixing (same amounts

806 as in the isolated case) and incubation for 24h at room temperature (blue). The clear separation of αS from NDs in the mixture points to a

807 fast-kinetic exchange between free and bound αS. The conserved total absorbance additionally confirms the stability of the NDs in the

808 presence of αS. (**c**) Same as in (b) but using 100% POPG NDs. The SEC profile of the mixture of αS with 100% POPG NDs shows a clear

809 reduction of free αS and a size increase of the ND peak, pointing to a strong interaction between the two components. (**d**) Differential

810 scanning calorimetry (DSC) thermograms of nanodiscs prepared with either 100% DMPC (black), 100% POPG (purple) or a 50%-50%

811 mixture thereof (blue), showing phase transitions temperatures around 28°C, below 5°C and 13°C, respectively. Temperatures at which

812 different measurements were performed are highlighted. (**e**) DSC profile of 100% DMPG NDs in the absence (brown) or presence of αS

813 (orange). While the presence of αS leads to a lower phase transition temperatures of the lipid bilayer, in line with what was reported using

814 SUVs, the transition temperature differences is much smaller for NDs (ΔT_m=2.5 °C) as compared to SUVs (ΔT_m=11 °C) in line with a largely
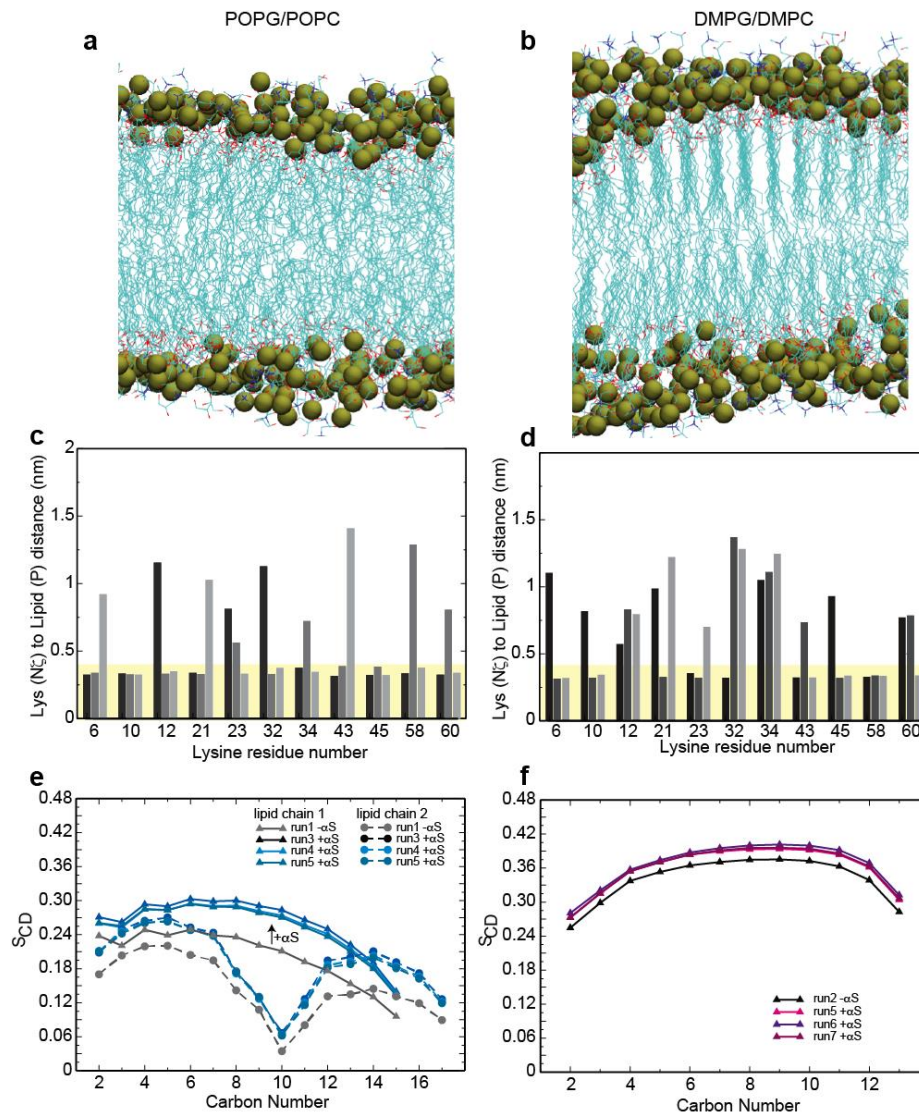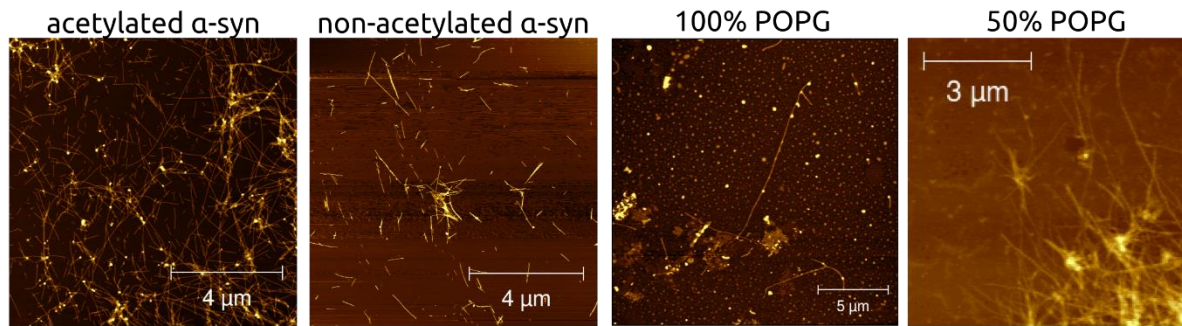
815 increase stability of the NDs.

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Figure 3 – figure supplement 1. αS-ND interactions using different MSP constructs, lower pH and higher temperature. NMR spectra (**a**) and the respective attenuation profiles (**b**) of [^{15}N]-acetylated-αS (50 µM) in the absence or in the presence of 25 µM NDs with 75% POPG content, assembled using the regular MSP1D1 construct (blue) or with the smaller MSP1Δ5 (red). Spectra (**c**) and attenuation profiles (**d**) corresponding to the binding of αS to NDs containing 100% POPG lipids at pH 7.4 (purple) and pH 5.3 (pale orange). Transferable assignments (bars) show that no significant difference in binding mode is visible upon pH variation. Spectra (**e**) and attenuation profiles (**f**) corresponding to the binding of αS to NDs containing 100% DMPG lipids in their gel phase (10°C, brown) or their fluid phase (37°C, red). Since normally measurement at 37 °C leads to considerable peak loss of N-terminal αS residues due to water exchange processes, the spectrum was recorded at pH 5.3 (counteracting water exchange). Note that the pH shift alone has no significant effect on binding (see (**c**) and (**d**)). Reference spectra of respective free αS are shown in dark grey.

Membrane models	Systems	αS initial distance to the membrane surface (nm)	Time (ns)
POPC/POPG*	RUN1	-	500
DMPC/DMPG*	RUN2	-	1000
POPC/POPG	RUN3	0.5	1000
	RUN4	0.5	1000
	RUN5	1.5	1000
DMPC/DMPG	RUN6	0.5	1000
	RUN7	1.5	1000
	RUN8	1.5	1000

Figure 3 – figure supplement 2. Summary of MD simulations used for the study.

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Figure 3 – figure supplement 3. Lipid properties and αS-membrane interactions as seen by MD simulations. (**a,b**) Molecular arrangement of lipid bilayer in the absence of αS for POPG/POPC lipids in fluid phase (**a**) and DMPG/DMPC lipids in gel phase (**b**) (end of run1/run2, Table S2). (**c,d**) Distances for indicated lysine residues from Lys-Nξ to the phosphorus atom of the next anionic lipid as found in different runs of MD simulations for POPG/POPC (c, individual bars for run3-5, Table S2) and DMPG/DMPC (d, individual bars for run5-7, Table S2) membranes. Distances that could promote lipid-mediated salt bridges are highlighted in yellow. (**e,f**) Calculated order parameters for indicated carbon atoms of the lipid fatty acids with and without αS for POPG/POPC (**e**) and DMPG/DMPC (**f**) membranes.

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Figure 4 – figure supplement 1. αS fibrils grown in the absence and presence of NDs have similar morphologies. AFM images of αS amyloid fibrils formed using acetylated αS and non-acetylated αS as well as acetylated αS in the presence of NDs with indicated lipid composition (from left to right).
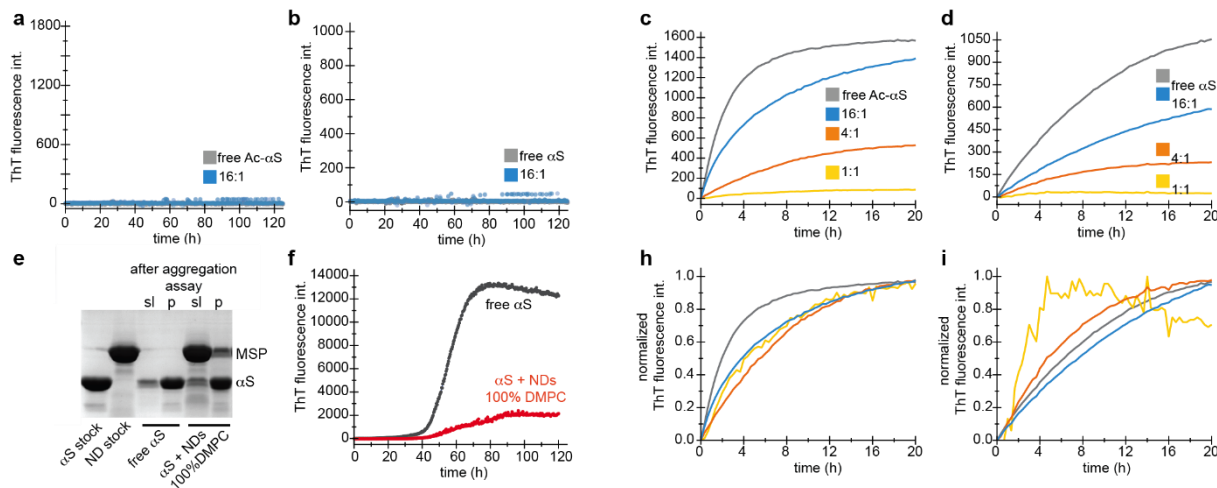
Figure 5 – figure supplement 1. Amyloid fibril nucleation and elongation in the presence of 50% POPG ND and rational for normalization of ThT data. (**a,b**) Quiescent nucleation assay of acetylated αS (**a**) and non-acetylated (**b**) αS in the absence (grey) and presence of 50% POPG NDs (conditions analog to data shown in Fig. 4j,k, for 100% POPG NDs). (**c,d,**) Seeded aggregation assays under conditions analog to data shown in Fig. 4m,l, but using 50% POPG NDs. SDS-PAGE results (**e**) with ThT intensities (**f**) of identical samples. After the aggregation assay the αS-band for soluble (sl) and insoluble (p) protein show very similar intensities in the absence and presence of 100% DMPC NDs. Therefore, ThT intensities in the seeded aggregation assays were normalized (**h,i**).