

Lipid Packing Defects are Necessary and Sufficient for Membrane Binding of α -Synuclein

Corresponding Author: Professor Wade Zeno

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The paper presents a systematic study of how α -synuclein (wt and a variant) bind to lipid membranes with different properties. The research questions are interesting and relevant for the broader field. The study is ambitious in its combination of different of model systems and complementary experimental and simulation methods. I really like this approach. I am therefore sorry that I cannot recommend publication because of some fundamental concerns regarding the choice of model system. To me, it appears that the authors have neglected/overseen complications in the design of model membrane systems in such a way that the conclusions drawn are not convincingly supported by the data.

The impression given when reading the paper is that one makes the assumption that changing lipid chain composition only influence lipid packing in a fluid bilayer. The model system are then designed to separately alter membrane charge and membrane interfacial hydrophobicity (in the paper referred to as defects, which is a bit unlucky terminology, see point 4 below). In doing this assumption, one have neglected that changing the lipid chain also influence other important properties, including lipid solubility in water (more soluble lipids can be depleted from the membrane, highly relevant for DLPS), lipid reactivity (highly relevant for polyunsaturated lipids are easily oxidised), and lipid phase (lipids with bulky chains, like the DPhPC, form nonlamellar phases). These aspects are all likely influencing protein binding. It is therefore very difficult to disentangle effects of membrane charge and membrane interfacial hydrophobicity. These complications are appear to be neglected in the paper.

1. Membrane charged is not well controlled when using shorter-chain charged lipids like DLPS.

DLPS has a solubility in water of ca 0,1 microM, which is not negligible. This is in clear contrast to DOPS that has a water solubility that is ca 3 orders of magnitudes lower. Solubility of DLPS is also clearly higher than for the zwitterionic DLPC. When vesicles composed of DLPC/DLPS vesicles are present in dilute solution, DLPS will be depleted from the membrane to the solution to a much larger extent than the less soluble DLPC. As a consequence, the membrane will have lower charge than one would expect on basis of the lipid ratio in the sample. For the DOPC/DOPS vesicle, the problem is not the same as both lipids negligible solubility in water. As a consequence, the charge density at the DLPC/DLPS vesicles will be lower compared to the DOPC/DOPS vesicles. As there are no measures of surface charge (or surface potential) presented, one do not know what is the charge of the different vesicles compared. Note that the magnitude of this effect will depend on the total lipid concentration, not only the proportions between the lipids. For dilute samples (or samples rinsed in buffer as in the flu microscopy studies), the effect is expected to be more pronounced than for more concentrated samples.

2. Not all lipid mixtures form planar bilayer structures

DPhPC and DPhPS have bulky chains, which promotes the formation of nonlamellar (inverted cubic or hexagonal) phases. Previous studies have shown on quite rich phase behaviour of DPhPC and DPhPC/DPhPG (the later another anionic lipid with similar headgroup size as PS) at conditions relevant for the present study
<https://doi.org/10.1016/j.bbamem.2017.06.003>.

This aspect is not considered in the present paper. Are the vesicles truly vesicles or something else (dispersed cubic or hexagonal phase)? This could impact both stability of the dispersed lipid particles, their interfacial hydrophobicity and the exposed lipid heagroup area.

Also DLPC and DLPS have been shown to form other types of structures than vesicles when dispersed in excess aq solution, including bicelles/discs.

3. Polyunsaturated lipids are prone to oxidation.

It is broadly recognised that polyunsaturated lipids are difficult to handle as they are easily oxidized unless one takes special precautions like working under N₂, adding antioxidants, etc. As far as I can judge from the description, such precautions were not taken here. In case of lipid oxidation, one needs to consider the effects of oxidised lipids and other reaction products (which is an interesting question in itself), which is lacking in the current analysis and discussion of data.

Taken together (point 1-3): In order to evaluate the findings, one needs additional information on the model system

- What is the surface potential (eg zeta potential measurements) in the different model systems at the dilution conditions used
- What are the dispersed lipid particles? Are they vesicles, dispersed hexagonal or cubic phases, lipid discs, or combinations of those?
- If phase and structure is not unilamellar vesicles, what are then the accessible headgroup areas and interfacial hydrophobicities?
- To what extent is there lipid oxidation in samples with polyunsaturated lipids?
- What is the stability of the dispersed particles over time frames comparable to the experiments?

4. Terminology of transient defects is misleading.

The study (incl title) has a strong focus on what one calls (transient) membrane defects. Although this is not unique for this paper, it is an unlucky choice of terminology as it draws the attention away from the relevant property that is the hydrophobicity. Hydrophobic interfaces are an inherent property of the fluid bilayers, not a transient irregularity. It is well established that - as for most lipids, the headgroups are smaller than the projected area of the cylinder representing the lipid chains - there is substantial exposure of the hydrophobic inner layer of the membrane at the interface. This is also in line with the simulation data presented in the paper. Increased hydrophobic exposure can be achieved by having more bulky chains, smaller headgroups, or increased curvature (the latter relevant for some of the comments on curvature in the paper - presently described as a separate mechanism). The terminology is here problematic as one gets the impression that the important property is packing irregularities, rather than the natural explanation that this is driven by hydrophobic interactions.

Some additional comments.

5. There is a huge spread in number of protein per vesicles for most of the systems studied. Is there an explanation for this? Are vesicles present in excess or not? (alt, is protein concentration above or below the saturation condition)? Is it possible to judge if protein binding to these vesicles is cooperative?

6. The cartoon in Figure 2 depicts α -synuclein as a broken α -helix that is mainly located beneath the headgroup layer. Are there data to support this picture? In the text it is stated that α -synuclein is inserted in the membrane, which gives the impression that it goes into the hydrophobic parts. That does not seem to align with the general understanding in the field. As for the broken helix, there seem to be different views on this, where the broken helix has been established for micelles and ultrasmall vesicles, while the extended helix has been proposed for larger vesicles and planar membrane. It would be valuable to comment on that the structure is not fully established and motivate why one used the broken version here.

7. As I understand, the analysis of size from the microscopy data is based on the fluorescence intensity per particle, not on the particle size. If the particle is partly dissolved (as outlined above for the DLPC/DLPS particles, the decreases in size will not be captured by these measurements.

Reviewer #2

(Remarks to the Author)

The authors investigated the influence of the tail and head structures of phospholipids on the membrane binding of α -synuclein, contributing significantly to our understanding of phospholipid-membrane interactions. I appreciate that this work emphasizes lipid packing defects, which I believe will inspire further research in this field. However, my concern lies in the lack of clarity regarding the physical imagery and molecular mechanisms underlying these findings. A more thorough examination of molecular dynamics simulation results could provide valuable insights. I recommend a minor revision before the final publication of this manuscript.

Electrostatic and hydrophobic interactions play distinct roles and mechanisms in the membrane binding of α -synuclein, making it challenging to determine which is more significant. These interactions operate through different mechanisms, potentially resulting in varied membrane binding states. While electrostatic interactions may facilitate the initial approach of α -synuclein to the membrane, hydrophobic interactions likely drive its deeper integration into the membrane, which is often accompanied by the formation of α -helical secondary structures. The authors may find relevant information in Reference 57 and a preprint (https://www.researchgate.net/publication/389636061_Membrane_Binding_of_a-Synuclein_Influenced_by_the_Shortening_of_Phospholipid_Acyl_Chains) for further molecular insights and may consider including additional discussion in their revision.

For instance, the authors should clarify the differing impacts of 25% charged phospholipids on the DL and DO systems. Specifically, it would be important to explain why the NTA variant significantly enhances the membrane binding propensity compared to wild-type alpha-synuclein. The molecular insights into these results are crucial yet remain unclear.

Reviewer #3

(Remarks to the Author)

This study investigates the membrane binding behavior of N-terminally acetylated α -synuclein (NTA- α Syn), the physiologically prevalent form of the protein implicated in Parkinson's disease. Using fluorescence microscopy, circular dichroism spectroscopy, and molecular dynamics simulations, the authors show that lipid packing defects (transient gaps exposing hydrophobic tails) are the primary driver of NTA- α Syn binding, challenging the long-standing emphasis on anionic membrane charge. The paper sounds interesting, but several issues need to be addressed before it can be published.

1. In Figure 1D, Figure 5C, Figure S2A&C, and Figure S3, the authors state that "The black dashed lines indicate the average values of the distributions". However, the black dashed lines on the graphs seem to be exactly equal to 100 nm, and although the authors state in the method that all vesicle sizes were selected to have an average diameter of 100 nm, I think that even so, vesicle sizes will always rise and fall, and it is difficult to achieve an average diameter of exactly equal to 100 nm in an experiment.

2. In the paper, the authors use molecular dynamics (MD) simulations to determine the packing defect of phospholipid membranes of different compositions. However, the MD models used in the paper were all planar with no curvature, whereas the experiments used 100 nm diameter vesicles with a large curvature. The effect of different phospholipid compositions on packing defects at different curvatures may be different. The linear relationship shown in Fig. 4D & Fig. 5D in the paper may be different in the real situation. For example, the authors' simulations do not indicate how different the packing defects formed by the components of DLPC, DOPC, DPhPC, DOPC/DOPS, and DPhPC/DPhPS are in 100 nm diameter vesicles, and whether this difference is sufficient to account for the variability in protein binding.

3. The authors stated that "these results indicate the first reported instance of α Syn exhibiting a preference for neutral membranes over anionic membranes with significant charge". I don't agree with this. In fact, a previous study (DOI: 10.1073/pnas.2310174120) showed that neutral lysophosphatidylcholine can mediate alpha-synuclein-induced synaptic vesicle clustering, indicating that alpha-synuclein can bind strongly to neutral membranes containing lysophospholipids (DOI: 10.1093/nsr/nwae182). More recently, it has shown that N-acetylation of α -synuclein enhances synaptic vesicle clustering mediated by α -synuclein and lysophosphatidylcholine (DOI: 10.7554/eLife.97228.3). The corresponding literature should be included in the discussion.

4. The authors stated that "Our work challenges the long-standing paradigm that anionic lipids are essential for α Syn binding by demonstrating that NTA diminishes α Syn's reliance on anionic membrane charge, leading to membrane packing defects as the primary driver of membrane binding." I don't agree. It is well established that the interaction of alpha-synuclein with the membrane is regulated by both electrostatic interactions and packing defects. The data in this paper only show that alpha-synuclein can bind to phospholipid membranes that do not have negatively charged phospholipids if the membrane has a sufficient packing defect. I agree with this statement. However, the data cannot support membrane packing defects as the primary driver of membrane binding. Previous studies (for examples, DOIs: 10.1016/j.bpj.2024.09.019; 10.1021/acs.jpcb.0c08620; 10.1039/d0cp03546g; etc) have provided significant insight into how the electrostatic interactions and the packing defects influence the membrane binding of alpha-synuclein, and shown that both of the electrostatic interactions and the packing defects play critical roles in the protein binding. The corresponding literature also needed to be included in the discussion.

5. In addition, I suggest that the authors include different concentrations of solutions such as KCl or CaCl₂ in the experiments where the protein binds to DPhPC, DPhPC/DPhPS, etc. to see if the ions have an effect on the binding and thus determine if electrostatic effects play a role in the process.

6. Also, although it is only a published abstract from another group (DOI: 10.1016/j.bpj.2024.11.1091), the title of that abstract is exactly the same as this paper, and the authors might consider discussing it in the discussion.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have responded to all my previous comments and addressed the concerns raised through rigorous new experiments. I have no further comments and I suggest to publish the paper

Reviewer #2

(Remarks to the Author)

The authors have addressed my concerns, and now, the manuscript is recommended for publication.

Reviewer #3

(Remarks to the Author)

The authors have addressed my previous comments and made the necessary revisions. I recommend publishing the current version.

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Reviewer Comments and Author Responses for COMMSBIO-25-0733A

Lipid Packing Defects are Necessary and Sufficient for Membrane Binding of α -Synuclein

David H. Johnson, Orianna H. Kou, John M. White, Stephanie Y. Ramirez, Antonis Margaritakis, Peter J. Chung, Vance W. Jaeger, Wade F. Zeno*

Reviewer 1:

The paper presents a systematic study of how α -synuclein (wt and a variant) bind to lipid membranes with different properties. The research questions are interesting and relevant for the broader field. The study is ambitious in its combination of different of model systems and complementary experimental and simulation methods. I really like this approach. I am therefore sorry that I cannot recommend publication because of some fundamental concerns regarding the choice of model system. To me, it appears that the authors have neglected/overseen complications in the design of model membrane systems in such a way that the conclusions drawn are not convincingly supported by the data.

The impression given when reading the paper is that one makes the assumption that changing lipid chain composition only influence lipid packing in a fluid bilayer. The model system are then designed to separately alter membrane charge and membrane interfacial hydrophobicity (in the paper referred to as defects, which is a bit unlucky terminology, see point 4 below). In doing this assumption, one have neglected that changing the lipid chain also influence other important properties, including lipid solubility in water (more soluble lipids can be depleted from the membrane, highly relevant for DLPS), lipid reactivity (highly relevant for polyunsaturated lipids are are easily oxidised), and lipid phase (lipids with bulky chains, like the DPhPC, form nonlamellar phases). These aspects are all likely influencing protein binding. It is therefore very difficult to disentangle effects of membrane charge and membrane interfacial hydrophobicity. These complications are appear to be neglected in the paper.

Author Response: We appreciate the reviewer taking the time to critically review our work to provide thoughtful and constructive feedback and critiques. We have carefully addressed every concern, adding new data and expanding the discussion. We believe that the revised manuscript now provides a more rigorous and compelling foundation for the conclusions drawn and hope it aligns with the reviewer's expectations.

1. *Membrane charged is not well controlled when using shorter-chain charged lipids like DLPS.*

DLPS has a solubility in water of ca 0,1 microM, which is not negligible. This is in clear contrast to DOPS that has a water solubility that is ca 3 orders of magnitudes lower. Solubility of DLPS is also clearly higher than for the zwitterionic DLPC. When vesicles composed of DLPC/DLPS vesicles are present in dilute solution,

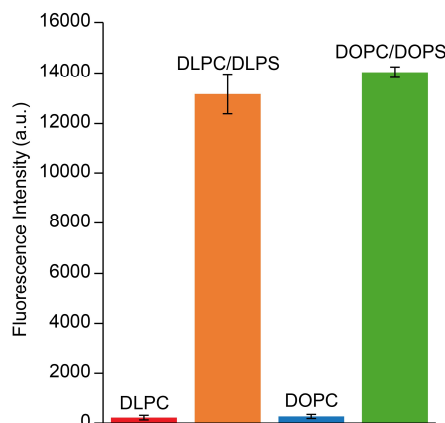
DLPS will be depleted from the membrane to the solution to a much larger extent than the less soluble DLPC. As a consequence, the membrane will have lower charge than one would expect on basis of the lipid ratio in the sample. For the DOPC/DOPS vesicle, the problem is not the same as both lipids negligible solubility in water. As a consequence, the charge density at the DLPC/DLPS vesicles will be lower compared to the DOPC/DOPS vesicles. As there are no measures of surface charge (or surface potential) presented, one do not know what is the charge of the different vesicles compared. Note that the magnitude of this effect will depend on the total lipid concentration, not only the proportions between the lipids. For dilute samples (or samples rinsed in buffer as in the flu microscopy studies), the effect is expected to be more pronounced than for more concentrated samples.

Author Response and Action Taken: We thank the reviewer for their comments clarifying potential issues with vesicle surface charge. We understand that the low lipid concentrations used in the tethered vesicle assay (i.e., $\sim 1 \mu\text{M}$ and lower) could cause disproportionate PS leaching from DL membranes in comparison DO membranes. We'd like point out that we also performed binding experiments in elevated lipid concentration ranges (i.e., 1 mM) with our CD Spectroscopy experiments, where PS leaching should not be an issue. CD experiments at these elevated concentrations indicated that PS-addition negligible impacted αSyn binding to DLPC membranes, which is in line with our results from the tethered vesicle assay in the dilute lipid regime.

Nonetheless, we believe that it was still important to address the concern of DLPS leaching in the dilute lipid concentration regime. Therefore, we developed an assay to test the surface charge of tethered vesicles and included the results as a new Supplementary Figure. In this assay, we monitored the adsorption of fluorescent, positively-charged poly-L-lysine (PLL) to the surface of tethered vesicles. This experimental approach is much more suitable than other approaches (e.g., zeta potential measurements) as it allows for monitoring vesicle surface charge under dilute lipid concentrations where potential DLPS depletion would be most prominent. Our results indicate that the addition of PS significantly enhanced adsorption of PLL to both DLPC and DOPC membranes. Furthermore, we found that adsorption to membranes containing both DLPC/DLPS and DOPC/DOPS were in quantitative agreement, thus strongly suggesting that the two types of vesicles have highly comparable surface charge in the dilute lipid regime. These results therefore confirm that DLPS depletion was negligible when using DLPC/DLPS vesicles in the tethered vesicle assay. We have included the added figure and caption below:

- **Figure S10. Vesicle surface charge assay.** The average fluorescence intensity of ATTO 488-labeled PLL adsorbed to DLPC, 3:1 DLPC:DLPS,

DOPC, and 3:1 DOPC:DOPS SUVs with diameters ranging from 90-110 nm.



In the methods under “SUV Preparation,” the following reference to Figure S10 was added:

- “...surface charge was confirmed with a PLL adsorption assay (Figure S10).”

In the supplementary information, we added the following text to describe the methods and discuss the results of the assay:

- **Poly-L-Lysine (PLL) Adsorption Assay Methods and Discussion**

Because DLPS has non-negligible water solubility with an estimated CMC of $\sim 1 \mu\text{M}$, we performed a tethered vesicle assay with poly-L-lysine (PLL) to verify DLPS was not leaching from the DLPC/DLPS vesicles and influencing the vesicle surface charge (**Figure S10**). PLL was labeled with ATTO 488 by incubating PLL with ATTO 488 NHS-Ester in a 1:1 molar ratio in 20mM HEPES, 150mM NaCl (pH 7.4) at room temperature for 30 minutes. Excess dye was removed by running the solution through a 7k MWCO Zeba Spin Column. The vesicles were prepared and imaged as described in the **SUV Preparation** and **Slide Passivation and Tethered Vesicle Assay**. 500 nM of PLL-ATTO488 was incubated with DOPC, 3:1 DOPC/DOPS, DLPC, and 3:1 DLPC/DLPS, and the fluorescence intensity of the PLL-ATTO488 adsorbed to 100 nm average vesicles was extracted. There was negligible adsorption to the membranes containing only PC lipids and strong adsorption to membranes containing PS lipids. Furthermore, the level of adsorption was comparable between membranes containing DLPS and DOPS, indicating that DLPS leaching was negligible in the tethered vesicle assay used.

2. Not all lipid mixtures form planar bilayer structures

DPhPC and DPhPS have bulky chains, which promotes the formation of nonlamellar (inverted cubic or hexagonal) phases. Previous studies have shown on quite rich phase behaviour of DPhPC and DPhPC/DPhPG (the later another anionic lipid with similar headgroup size as PS) at conditions relevant for the present study <https://doi.org/10.1016/j.bbamem.2017.06.003>.

This aspect is not considered in the present paper. Are the vesicles truly vesicles or something else (dispersed cubic or hexagonal phase)? This could impact both stability of the dispersed lipid particles, their interfacial hydrophobicity and the exposed lipid heagroup area.

Also DLPC and DLPS have been shown to form other types of structures than vesicles when dispersed in excess aq solution, including bicelles/discs.

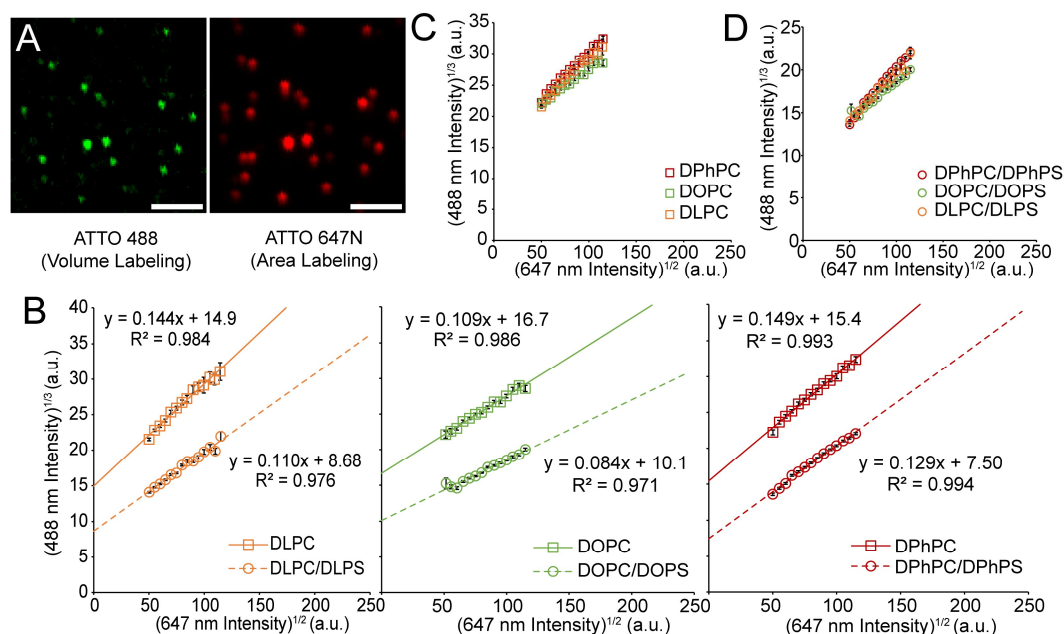
Author Response and Action Taken: The reviewer raises a valid concern regarding the structures of our lipids in solution. We initially assumed that the DPhPC used in our work formed unilamellar vesicles, as several previous works have documented this phase behavior with DPhPC at room temperature, including the work referenced by the reviewer (Kara, et. al., *BBA – Biomembranes*, 2017; Ruiz et. al., *Phys. Chem. Chem. Phys.*, 2023). Non-lamellar phases for DPhPC are typically observed at high temperatures in dehydrated conditions (Hsieh, et. al., *Biophysical J.*, 1997; Kara et. al., *BBA – Biomembranes*, 2017). Regarding DPhPC/DPhPS membranes, the work referenced by the reviewer (Kara et. al., *BBA – Biomembranes*, 2017) indicates that this lipid composition exhibits unique phase behavior at extremely high lipid concentrations (i.e., ~1 M or ~730 mg/mL lipid). These concentrations are 3 orders of magnitude higher than the solutions used in our most concentrated CD Spectroscopy experiment (6 mM lipid). Therefore, the regime at which this non-unilamellar phase behavior is observed is not relevant to our experimental conditions.

Regarding DLPC and DLPS, we acknowledge that DLPC/PS have been used as components in bicelle mixtures (Czerski and Sanders, *Analytical Biochem.*, 2000), but bicelles are multi-component mixtures of multiple chain-length lipids and detergents or detergent lipids (Dürr, et. al., *Prog. in Nucl. Magn. Reson. Spectrosc.*, 2013). Discs also require a detergent or belting molecule that solubilizes the lipids (Bayburt et. al., *Nano Letters*, 2002; Denisov et al, *JACS*, 2004). They are not formed from single-tail-length lipid mixtures. Additionally, bicelles are typically formed at high lipid concentrations (i.e., >10% wt/vol or >10mg lipids/100uL of water) (Angelis and Opella, *Nat. Protocols*, 2007; Czerski and Sanders, *Analytical Biochem.*, 2000), which are ~ 1.5 orders of magnitude higher than our highest lipid concentration used in CD Spectroscopy (6mM DLPC = 0.4% wt/vol). Additionally,

previous work utilizing DLPS has demonstrated that it forms unilamellar vesicles at conditions similar to ours (Hoover et al., *Journal. Phys. Chem. B*, 2021).

Although we strongly believed that our initial assumption of having unilamellar vesicles was valid for DLPC/PS and DPhPC/PS lipid compositions, we went ahead and developed a fluorescence assay to confirm their structures. We constructed an assay in which vesicles were formed in buffer containing a water-soluble dye (i.e., ATTO488), thus encapsulating it within the lumen of vesicular structures and effectively “volume labeling” them. Simultaneously, we incorporated a lipid-anchored fluorophore (i.e., ATTO647N) in the lipid formulations, such that the membranes become fluorescently labeled and vesicles became “surface labeled”. By tethering these lipid products, we were able to perform 2-color fluorescence imaging and observe colocalized ATTO488 and ATTO647N signals. By simultaneously monitoring these dyes, we were able to quantitatively analyze the scaling behavior of the fluorescence intensities. Under the assumption that unilamellar vesicles are present, the following fluorescence intensity scaling behavior is true: $ATTO488 \sim diameter^3$ and $ATTO647N \sim diameter^2$. Therefore, fluorescence intensity plots of $(ATTO488)^{1/3}$ vs $(ATTO647N)^{1/2}$ must yield straight lines. Deviations from this trend would indicate non-unilamellar structures. Not only did all of our compositions exhibit strong linear behavior, but the lines were in quantitative agreement across all compositions (i.e., DL, DO, and DPh), indicating that the DL and DPh structures must be identical to the DO structures. These results were added to the manuscript as Supplementary Figure S9. We have included the figure and its discussion below:

- **Figure S9. Volume labeling assay.** (A) Fluorescence micrographs of SUVs encapsulating ATTO 488 dye for volume labeling and containing ATTO 647N in the bilayer for area labeling. Scale bars represent 2 μ m. (B) Scaling relationship between SUV volume and surface area for PC membranes and 3:1 PC:PS membranes. The intensity values correspond to SUVs with an average diameter of 100 nm. (C) Overlay of the cube root of 488 nm intensity versus the square root of 647 nm intensity for DPhPC, DOPC, and DLPC. (D) Overlay of the cube root of 488 nm intensity versus the square root of 647 nm intensity for DPhPC/DPhPS, DOPC/DOPS, and DLPC/DLPS.



In the methods, under SUV Preparation, we added the following to describe the purpose of the experiment:

- “Unilamellar structures were verified with a volume labeling assay (Figure S9), and their surface charge was confirmed with a PLL adsorption assay (Figure S10).”

In the supplementary information, the following description of the method and primary results was added:

- **Volume Labeling Assay Methods and Discussion**

To ensure the imaged structures were unilamellar vesicles, a volume labeling assay was used (**Figure S9**). SUVs containing lipid-anchored ATTO-647N were formed in the presence of 100 μ M ATTO-488 dye, encapsulating it within the vesicle lumen. The vesicles were then tethered onto the glass surface as described in **Slide Passivation and Tethered Vesicle Assay**. All excess ATTO-488 dye in solution was rinsed away from outside the vesicles in the final buffer rinsing step. All vesicle compositions were then imaged under the same two-channel confocal settings with ATTO-488 representing the vesicle lumen (i.e. volume label) and ATTO-647N representing the vesicle bilayer (i.e., surface label). The following fluorescence intensity scaling behavior must be true for hollow spheres (i.e., unilamellar vesicles): $(\text{lumen intensity or ATTO488}) \sim \text{diameter}^3$ and $(\text{membrane intensity or ATTO647N}) \sim \text{diameter}^2$. Therefore, when the cube root of ATTO488 intensity is plotted vs the square root of ATTO647N

intensity, the plot must exhibit linearity. Linear correlation was indeed observed between the two quantities, indicating the presence of hollow spheres or unilamellar vesicles.

3. *Polyunsaturated lipids are prone to oxidation.*

It is broadly recognised that polyunsaturated lipids are difficult to handle as they are easily oxidatized unless one take special precautions like working under N₂, adding antioxidants, etc. As far as I can judge from the description, such precautions were not taken here. In case of lipid oxidation, one needs to consider the effects of oxidised lipids and other reaction products (which is an interesting question in itself), which is lacking in the current analysis and discussion of data.

Taken together (point 1-3): In order to evaluate the findings, one need additional information on the model system

- What is the surface potential (eg zeta potential measurements) in the different model systems at the dilution conditions used*
- What are the dispersed lipid particles? Are they vesicles, dispersed hexagonal or cubic phases, lipid discs, or combinations of those?*
- If phase and structure is not unilamellar vesicles, what are then the accessible heagroup areas and interfacial hydrophobicities?*
- To what extent is there lipid oxidation in samples with polyunsaturated lipids?*

Author Response and Action Taken: In this query, we will only focus on addressing PUFA oxidation as the other concerns (i.e., surface charge and lipid phase) were addressed in queries 1-2.

Thank you for your feedback. We actually did take steps to mitigate oxidation, but inadvertently omitted the discussion from our initial methods section. We have therefore updated the manuscript to include the appropriate information in the methods section under “SUV Preparation”:

- When handling PUFA-containing mixtures, special precautions were taken to minimize oxidation. All PUFA aliquots were handled in a N₂ glove box, and all buffers were purged with N₂ before rehydrating the lipid films. Additionally, freeze-thaw cycles were omitted to limit exposure to any residual oxygen, and lipid extrusion was carried out within a N₂ glove box. Samples were then imaged within 30 minutes of air exposure.

4. *What is the stability of the dispersed particles over time frames comparable to the experiments?*

Author Response and Action Taken: Regarding particle stability, because our particles are vesicles, vesicles are commonly tested at time scales on the order of 1 week. In our case, we performed all binding assays with vesicles that were made either the same day or day before. A comment reflecting this was added to the methods section under SUV Preparation as follows:

- “[PUFA-containing] Samples were then imaged within 30 minutes of air exposure. All other vesicle preparations were imaged within 48 hours of preparation and stored in a 4 °C refrigerator to minimize degradation of quality.”

5. *Terminology of transient defects is misleading.*

The study (incl title) has a strong focus on what one call (transient) membrane defects. Although this is not unique for this paper, it is unlucky choice of terminology as it draw the attention away from the relevant property that i the hydrophobicity. Hydrophobic interfaces is an inherent property of the fluid bilayers, not a transient irregularity. It is well established that - as for most lipids, the headgroups are smaller than the projected area of the cylinder representing the lipid chains - there is substantial exposure of the hydrophobic inner layer of the membrane at the interface. This is also in line with the simulation data presented in the paper. Increased hydrophobic exposure can be achieved by having more bulky chains, smaller headgroups, or increased curvature (the latter relevant for some of the comments on curvature in the paper - presently described as a separate mechanism). The terminology is here problematic as one gets the impression that the important property is packing irregularities, rather than the natural explanation that this is driven by hydrophobic interactions

Author Response and Action Taken: We understand that the reviewer is highlighting the need to emphasize interfacial hydrophobicity within packing defects instead of referring to packing defects solely as packing irregularities. We agree that packing irregularities do not inherently drive binding and that it is instead hydrophobic interactions with the protein driving binding. In the context of our work, “lipid packing defects” serve as a proxy for this hydrophobic exposure. We quantify the size and frequency of these defect sites with our simulations, where the membrane coverage by defects represents the area of hydrophobic exposure on the surface of the bilayer. We call the defects “transient” to reflect the fluid nature of the bilayer; these defects are not frozen in time and have an associated lifetime due to the fluidity of the bilayer.

While we believe our use of “transient packing defects” is not mutually exclusive from interfacial hydrophobicity, we agree that it is important to explicitly highlight this interfacial hydrophobicity that results from mismatch in size between lipid heads and tails. We have therefore added additional text to the manuscript that reiterates the importance of hydrophobicity when introducing and describing packing defects.

In the abstract, we added the following highlighted text to focus the concept of packing defects on hydrophobicity:

- “...revealing that membrane packing defects (i.e., interfacial hydrophobicity) alone can drive membrane binding.”

We also added additional clarifying text when introducing packing defects in the Introduction:

- “...where the underlying hydrophobic hydrocarbon tails are exposed to solvent. It is important to note that a lipid packing defect, as defined here, is a term to quantify exposed hydrophobic area of lipid bilayers due to non-ideal packing, highlighting the importance of hydrophobic interactions for α Syn binding.

6. *There is a huge spread in number of protein per vesicles for most of the systems studied. Is there an explanation for this? Are vesicles present in excess or not? (alt, is protein concentration above or below the saturation condition)? Is it possible to judge if protein binding to these vesicles cooperative?*

Author Response and Action Taken: The reviewer is correct in noting the somewhat large spread of number of proteins per vesicle. The observed spread of protein per vesicle is due to our selection of a range of vesicle sizes that average to 100nm, in this case 80-120 nm vesicles. For example, if we chose a range of 99-101nm, the spread in protein binding would be much narrower, and the number of vesicles in the population would be much lower. Regarding the saturation regime, the data in Figure 3B indicates that these binding measurements were taken in the sub-saturation regime as the binding curves do not seem to plateau when using the tethered vesicle assay.

Since we were unable to achieve saturation in the tethered vesicle assay, we are unable to properly assess the cooperativity of α Syn binding with this assay. However, we were able to reach saturation using CD spectroscopy and therefore were able to fit the appropriate Langmuir binding curves with Hill cooperativity coefficients in Figure 3E. We included these cooperativity coefficients in Table S4,

showing that α Syn demonstrated cooperative binding on DOPC/PS and DPhPC/PS membranes.

In the discussion section, we highlighted the cooperativity of α Syn binding when discussing the synergy between membrane charge and defects:

- “While increased α Syn binding was strongly correlated with an increase in defect content and neutral DPhPC membranes elicited stronger binding than anionic DOPC/DOPS membranes, it must be noted that anionic charge and defect content exhibited synergy in recruitment of α Syn. Interestingly, our CD Spectroscopy results suggest that the addition of PS enhanced cooperativity of α Syn binding in both DO and DPh membranes (**Table S4**).”

We added Table S4 to the supplement:

- **Table S4. Apparent dissociation constant (K_d) and cooperativity (n) determined by Langmuir-Hill fit to binding curves.** K_d and cooperativity values were determined by fitting data in Figures 3E and 4B to Equation 2 from the main text. The errors listed are the standard error of the mean.

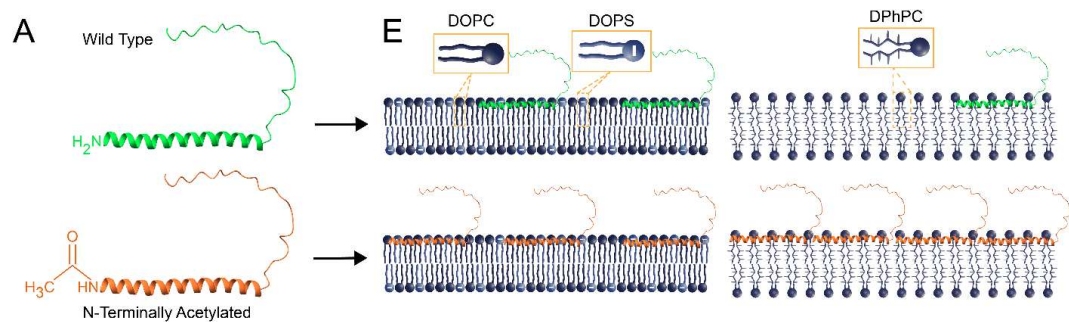
Lipid Composition	K_d	n
DOPC	3376 ± 897	0.97 ± 0.17
DPhPC	330 ± 13	1.69 ± 0.10
DOPC/DOPS	386 ± 13	1.49 ± 0.07
DPhPC/DPhPS	125 ± 8	1.99 ± 0.21

7. *The cartoon in Figure 2 depicts α -synuclein as a broken α -helix that is mainly lie beneath the headgroup layer. Are there data to support this picture? In the text it is stated that α -synuclein is inserted in the membrane, which gives the impression that it goes into the hydrophobic parts. That does not seem to align with the general understanding in the field. As for the broken helix, there seem to be different views on this, where the broken helix has been established for micelles and ultrasmall vesicles, while the extended helix has been proposed for larger vesicles and planar membrane. It would be valuable to comment on that the structure is not fully established and motivate why one used the broken version here.*

Author Response and Action Taken: We thank the reviewer for bringing attention to the importance of distinguishing between different α Syn conformations in the membrane-bound state. We intended for the figure to be a qualitative cartoon representation rather than one to give precise measurements of α Syn penetration depth into the bilayer. We acknowledge that this qualitative description was not clearly articulated in the manuscript. We have therefore added necessary clarification to the figure caption. Regarding α Syn insertion into the hydrophobic parts of the membrane, previous literature has shown that synuclein does interact

with the hydrophobic area in the membrane (Jao et al., *PNAS*, 2008) and that structure does not change with acetylation (Runfola et al., *Scientific Reports*, 2020). The depth of that interaction was skewed deeper in our initial cartoon, so we adjusted it accordingly. The reviewer is also correct in asserting that literature does indicate that there could be more than one equilibrium bound state depending on vesicle size (Jao et al., *PNAS*, 2008; Lokappa and Ulmer, *J Biological Chem.*, 2011), but this was not considered for our binding schematic. To remedy this oversight, the α Syn illustrations in Figure 1 were changed to contain an extended helix as literature indicates that this is the likely conformation on SUVs (Georgiva et al., *JACS*, 2008; Trexler and Rhoades, *Biochem.*, 2009)

Figures 1A and 1E were edited to show the extended helix α Syn conformation:



The following text was added to the caption for figure 1E

- This schematic is a qualitative representation of α Syn-membrane association and is not intended to quantitatively reflect α Syn size and penetration depth relative to the membrane surface.

We added the following highlighted text to the discussion section when mentioning N-terminal acetylation of α Syn and its effect on binding:

- “This state of increased binding activity would, therefore, be highly amenable to defect-rich membranes such as DPhPC, as these membranes contain higher exposure of the underlying lipid tails. It is relevant here to note that we do not evaluate the specific alpha-helical conformation (i.e. broken helix vs extended helix) of α Syn’s N terminus upon membrane binding in this work. This precise structural conformation of the helical N terminus in the membrane-bound state remains context-dependent and lacks strong consensus, as it depends on membrane size and curvature^{60,61}. In **Figure 1E**, α Syn is depicted as an extended helix, which is the likely conformation adopted upon binding to 100 nm vesicles⁶².”

8. *As I understand, the analysis of size from on the microscopy data is based on the fluorescence intensity per particle, not on the particle size. If the particle is partly dissolved (as outline above for the DLPC/DLPS particles, the decreases in size will not be captured by these measurements.*

Author Response and Action Taken: We thank the reviewer for this observation. The reviewer is correct in saying a decrease in size from lipid solubility would not be captured by microscopy. However, our added experiments for queries 1 and 2 address these concerns as 1) The PLL adsorption experiments reveal that DLPS depletion is negligible in our vesicles and 2) the simultaneous area and volume labeling experiments indicate that PC and PC/PS fluorescence intensities remain relatively constant across all tail chemistries. Together, these results indicate that it was highly unlikely for vesicle dissolution to occur in our experiments.

Reviewer #2:

The authors investigated the influence of the tail and head structures of phospholipids on the membrane binding of α -synuclein, contributing significantly to our understanding of phospholipid-membrane interactions. I appreciate that this work emphasizes lipid packing defects, which I believe will inspire further research in this field. However, my concern lies in the lack of clarity regarding the physical imagery and molecular mechanisms underlying these findings. A more thorough examination of molecular dynamics simulation results could provide valuable insights. I recommend a minor revision before the final publication of this manuscript.

Electrostatic and hydrophobic interactions play distinct roles and mechanisms in the membrane binding of α -synuclein, making it challenging to determine which is more significant. These interactions operate through different mechanisms, potentially resulting in varied membrane binding states. While electrostatic interactions may facilitate the initial approach of α -synuclein to the membrane, hydrophobic interactions likely drive its deeper integration into the membrane, which is often accompanied by the formation of α -helical secondary structures. The authors may find relevant information in Reference 57 and a preprint

(https://www.researchgate.net/publication/389636061_Membrane_Binding_of_a-Synuclein_Influenced_by_the_Shortening_of_Phospholipid_Acyl_Chains) for further molecular insights and may consider including additional discussion in their revision.

For instance, the authors should clarify the differing impacts of 25% charged phospholipids on the DL and DO systems. Specifically, it would be important to explain why the NTA variant significantly enhances the membrane binding propensity compared to wild-type α -synuclein. The molecular insights into these results are crucial yet remain unclear.

Author Response and Action Taken: We thank the reviewer for their feedback on the importance of electrostatics and hydrophobicity in aSyn binding. We agree that additional insights can be made by looking at molecular dynamics of aSyn and its interactions with membranes. By incorporating the literature referenced in this comment with other molecular dynamics literature, a much more robust discussion of the intricacies of electrostatic vs hydrophobic interactions was added. We believe this additional discussion gives a more in-depth picture of the interactions that we observe in our findings. We also clarified and added additional insights from literature about the driving force for increased NTA-aSyn binding. The following highlighted text was added to our discussion section:

- “NTA also increases the hydrophobicity of the N-terminus of aSyn, replacing the charged amine with a neutral acetyl group, incentivizing additional hydrophobic interactions⁵⁹. While this reduction in positive charge diminishes electrostatic interactions between the unmodified, N-terminal

methionine and anionic lipid headgroups, recent MD simulations have shown that this loss in electrostatic contribution to binding energy is offset by increased hydrophobic contributions facilitated by the acetylated methionine ⁵⁸. These simulations also demonstrate that acetylation promotes extension of aSyn's helical structure along the length of the N-terminus, enhancing membrane binding. This state of increased binding activity would, therefore, be highly amenable to defect-rich membranes such as DPhPC, as these membranes contain higher exposure of the underlying lipid tails."

Additional text was added to bring additional MD insight to the interplay between α Syn's hydrophobic and electrostatic interactions:

- "This result is particularly interesting in the context of recent MD simulations that have probed the relative contribution of electrostatic and hydrophobic forces for aSyn membrane binding ^{58,63-66}. This literature jointly describes a multi-state binding event in which electrostatic and hydrophobic interactions serve distinct roles in aSyn binding. Electrostatics dominate when the negative headgroups interact with the lysine-rich N-terminus of aSyn, bringing the protein close to the membrane surface. The hydrophobic interactions then dominate when the N-terminus embeds into the membrane upon binding. These hydrophobic interactions become even more relevant in the context of NTA-aSyn where MD simulations show additional helical propensity increases membrane binding affinity ⁵⁸. Throughout this literature, electrostatic interactions were shown to assist in aSyn binding through membrane recognition, but the defects and hydrophobic interactions mediate the physical insertion of the protein beyond surface adsorption ⁶⁶. This necessity of defects for binding and insertion was exemplified with our results utilizing DL membranes, where the addition of 25% charge from PS did not increase the density of packing defects nor the amount of bound aSyn. Even in the presence of substantial anionic charge, which assists with aSyn localization to the membrane, insufficient defects resulted in negligible binding to DL membranes.

Reviewer #3:

This study investigates the membrane binding behavior of N-terminally acetylated α -synuclein (NTA- α Syn), the physiologically prevalent form of the protein implicated in Parkinson's disease. Using fluorescence microscopy, circular dichroism spectroscopy, and molecular dynamics simulations, the authors show that lipid packing defects (transient gaps exposing hydrophobic tails) are the primary driver of NTA- α Syn binding, challenging the long-standing emphasis on anionic membrane charge. The paper sounds interesting, but several issues need to be addressed before it can be published.

- 1. In Figure 1D, Figure 5C, Figure S2A&C, and Figure S3, the authors state that “The black dashed lines indicate the average values of the distributions”. However, the black dashed lines on the graphs seem to be exactly equal to 100 nm, and although the authors state in the method that all vesicle sizes were selected to have an average diameter of 100 nm, I think that even so, vesicle sizes will always rise and fall, and it is difficult to achieve an average diameter of exactly equal to 100 nm in an experiment.*

Author Response and Action Taken: We thank the reviewer for pointing out potential confusion here. We want to clarify that the vesicle distributions presented in the referenced figures were subsets of the vesicle populations with broad diameter ranges. The tethered vesicle assay allows for precise analysis on a single vesicle basis. Using our calibrations between DLS and fluorescence intensity distributions, we were able to select a sub-populations of tethered vesicles that had average diameters of approximately 100 nm when processing our binding data. These subsets contained $\sim 10^3$ vesicles, while the entire populations contained $\sim 10^4$ - 10^5 vesicles. So even though all of the vesicle populations across different samples had different population means and variances, we were able to control for these differences by applying a uniform 80-120 nm cut-off filter in our high throughput analysis. To alleviate this confusion, we added the following text to our manuscript in the methods section:

- All tethered vesicles included in the binding analysis (Fig. 1, Fig. 3-5, Fig. S2-S4, S8-S9) were subsets of their respective vesicle populations and selected in the diameter range 80-120 nm, resulting in average diameters of 100 nm across all compositions studied. This step was necessary to control for curvature-sensing effects in the interpretation of our results.
- 2. In the paper, the authors use molecular dynamics (MD) simulations to determine the packing defect of phospholipid membranes of different compositions. However, the MD models used in the paper were all planar with no curvature, whereas the experiments used 100 nm diameter vesicles with a large curvature. The effect of different phospholipid compositions on packing defects at different curvatures may*

be different. The linear relationship shown in Fig. 4D & Fig. 5D in the paper may be different in the real situation. For example, the authors' simulations do not indicate how different the packing defects formed by the components of DLPC, DOPC, DPhPC, DOPC/DOPS, and DPhPC/DPhPS are in 100 nm diameter vesicles, and whether this difference is sufficient to account for the variability in protein binding.

Author Response and Action Taken: Thank you to the reviewer for highlighting this important discussion point. Based upon previous simulations from Vanni et al. (<https://doi.org/10.1038/ncomms5916>), the curvature of a membrane does indeed affect defect size. See Figures 3 and 4 in Vanni's work. Vanni uses the same Packmem program that we use in our analysis. They observe that the defect size is linearly correlated to inverse curvature with units of nm^{-1} . Thus, as the radius of curvature increases, the defect size constant of a vesicle decreases and approaches the result for a flat plane. Experimentally, we have shown that the radius of curvature of our membranes is about 50 nm or 0.02 nm^{-1} in the units used by Vanni. From Vanni's results, we see that the defect size increases by about 10% due to this degree of curvature and that increased defects are correlated to lower curvature for all membrane lipid compositions. Thus, we argue that our method of using a flat plane as a proxy for a vesicle with a large radius is appropriate to mimic realistic behavior.

For our computer architecture, GROMACS simulations exhibit near linear scaling with the number of atoms in the system. The simulations described in our manuscript typically take 7-10 days to complete. To simulate a full vesicle ($d \sim 100$ nm) in atomistic detail, we would need to increase the size of the box from roughly $10 \times 10 \times 10$ nm to $100 \times 100 \times 100$ nm. Thus, the simulations would be about 1000 times larger and run at about 1/1000th the speed. Such simulations are not feasible with current computational power. We considered several other simulation techniques. Using coarse grained (CG) force fields, simulations can be accelerated by 10-50 times. Optimistically, for a full vesicle, the simulations would take about 20 times as long. Meaning a single simulation would take about 140-200 days to complete. This is possible but also unfeasible for a campaign several simulations covering multiple lipid compositions. Additionally, CG force fields lack atomistic detail, meaning errors can arise to do inaccuracies in intermolecular forces. It is preferable to use atomistic models when possible.

Other methods have been developed to simulate a small patch of a vesicle's surface while inducing membrane curvature by the imposition of external forces. Larsen recently reviewed some common methods for studying curvature (<https://doi.org/10.3390/ijms23158098>). Larsen states, "Investigating curvature sensing and curvature induction in MD simulations is a daunting task, as the researcher must consider several factors, including how to generate and sustain

out-of-equilibrium membrane geometries; how to perturb the membrane as little as possible, despite this non-plane geometry; and how to balance computational costs against having a sufficiently large system, which include the relevant curvature(s) and negligible edge effects.” Inducing curvature in these simulations relies on perturbing the energetics of the system which can also introduce artifacts and edge effects. Ultimately, because Vanni has convincingly demonstrated that flat planes reliably mimic vesicles with 100 nm diameter, we argue that standard MD with atomistic force fields and a flat plane geometry closely approximate the behavior of large vesicles.

To articulate the above discussion in our manuscript and address the reasonable concern brought up by the reviewer, the following text was added to the Supplementary Methods under Simulations to discuss the scaling of defects with curvature:

“While the vesicles used in this work had average diameters of ~100 nm and the simulations were performed on flat bilayers, previous work has verified that defect size increases by only ~10% on 100 nm vesicles when compared to flat bilayers, regardless of lipid composition ¹⁰. Therefore, it can be assumed that the flat bilayers simulated in this work serve as accurate representations of their vesicular counterparts with 100 nm diameters.”

3. *The authors stated that “these results indicate the first reported instance of αSyn exhibiting a preference for neutral membranes over anionic membranes with significant charge”. I don’t agree with this. In fact, a previous study (DOI: 10.1073/pnas.2310174120) showed that neutral lysophosphatidylcholine can mediate alpha-synuclein-induced synaptic vesicle clustering, indicating that alpha-synuclein can bind strongly to neutral membranes containing lysophospholipids (DOI: 10.1093/nsr/nwae182). More recently, it has shown that N-acetylation of α-synuclein enhances synaptic vesicle clustering mediated by α-synuclein and lysophosphatidylcholine (DOI: 10.7554/eLife.97228.3). The corresponding literature should be included in the discussion.*

Author Response and Action Taken: We thank the reviewer for bringing this relevant literature to our attention. While these papers show that αSyn can have significant affinity for neutral membranes, they do not directly compare N-terminally acetylated αSyn binding between anionic and neutral membranes under conditions that control for membrane curvature. For instance, (Zhao et. al., *National Science Review*, 2024 and Wang et. al., *eLife*, 2024) compare highly curved micelles to extruded vesicles while (Zhao et. al., *National Science Review*, 2024) uses unacetylated αSyn in their studies. Our result that neutral membranes can recruit higher levels of N-terminally acetylated αSyn than anionic membranes (while controlling for curvature) is the main novelty and key finding of our manuscript. Nonetheless, the literature that the reviewer has cited is very relevant to our

manuscript and is certainly worth discussing. We have therefore added discussion of this literature to our manuscript.

To the introduction:

- “These packing defects have been shown to enhance membrane binding of wild type α Syn (WT- α Syn) ^{26,27}, especially when phospholipids containing methyl-branched tails ¹⁷ or single tails ^{12,32} were used..”

To the results section:

- “While there is a growing body of recent literature showing the importance of neutral membrane lipids for α Syn-membrane interactions ^{11,12,32}, to the best of our knowledge, our results indicate the first reported instance of N-terminally acetylated α Syn exhibiting a preference for neutral membranes over highly charged anionic membranes (**Figure 1E**). Importantly, these findings were obtained under conditions that controlled for membrane curvature (**Figure 1D**).”

To the discussion section:

- “However, this current study, along with a growing body of work ^{11,12,32}, shows that α Syn interactions with zwitterionic membranes, when sufficient defects are present, can be comparable if not greater than a charged membrane.”

We have also added this literature to various citation locations where applicable regarding vesicle clustering, N-terminal acetylation, packing defects, and α Syn binding.

4. *The authors stated that "Our work challenges the long-standing paradigm that anionic lipids are essential for α Syn binding by demonstrating that NTA diminishes α Syn's reliance on anionic membrane charge, leading to membrane packing defects as the primary driver of membrane binding." I don't agree. It is well established that the interaction of alpha-synuclein with the membrane is regulated by both electrostatic interactions and packing defects. The data in this paper only show that alpha-synuclein can bind to phospholipid membranes that do not have negatively charged phospholipids if the membrane has a sufficient packing defect. I agree with this statement. However, the data cannot support membrane packing defects as the primary driver of membrane binding. Previous studies (for examples, DOIs: 10.1016/j.bpj.2024.09.019; 10.1021/acs.jpcb.0c08620; 10.1039/d0cp03546g; etc) have provided significant insight into how the electrostatic interactions and the packing defects influence the membrane binding of alpha-synuclein, and shown that both of the electrostatic interactions and the packing defects play critical roles in the protein binding. The corresponding literature also needed to be included in the discussion.*

Author Response and Action Taken: We thank the reviewer for drawing attention to this important discussion. We want to highlight an important distinction in our language. Membrane interactions are inherently different than membrane binding or insertion. A protein may interact with a membrane but not bind to or insert into it. Based on the multitude of studies about α Syn binding (including those referenced above), we acknowledge it must be concluded that electrostatics have a role in α Syn interactions when present in the membrane. We see in many studies, including ours, that the introduction of anionic charge increases α Syn binding. However, our study shows that the presence of charge is *not sufficient* for α Syn membrane binding and insertion on its own. While the indicated studies from the reviewer do show that electrostatics, when present, are important in anchoring α Syn to the membrane to better mediate hydrophobic interactions, our paper shows that this is insufficient to mediate membrane binding and insertion. Our observation *necessitates* that a separate mechanism be present to drive α Syn binding when no charge is present (as we see in DPhPC). We show that this mechanism is mediated by membrane defects, namely hydrophobic interactions, that do not require charge to mediate binding and insertion. It is important, however, to discuss that electrostatics do influence α Syn membrane interactions, which should be highlighted. Therefore, the changes below were made.

We changed the wording of the abstract

- Original – “Our work challenges the long-standing paradigm that anionic lipids are essential for α Syn binding by demonstrating that NTA diminishes α Syn’s reliance on anionic membrane charge, leading to membrane packing defects as the primary driver of membrane binding.”
- Revised – “Our work challenges the long-standing paradigm that anionic lipids are necessary for α Syn binding by demonstrating that NTA diminishes α Syn’s reliance on anionic membrane charge, revealing that membrane packing defects (i.e., interfacial hydrophobicity) alone can drive membrane binding.”

The recommended literature was added to the manuscript in conjunction with the following highlighted text added to the Discussion to address the intricacies and findings of the above studies:

- “The observed preference for membrane defects by NTA- α Syn was preserved across both binding assays used. Fluorescence microscopy and circular dichroism spectroscopy combined with all-atom molecular dynamics simulations demonstrated that the presence of defects dictates the ability of α Syn to insert into membranes, and that negatively charged membranes cannot overcome a lack of membrane defects necessary for α Syn binding. This result is particularly interesting in the context of recent MD simulations that have probed the relative contribution of electrostatic and hydrophobic forces for α Syn membrane binding^{58,63-66}. This literature

jointly describes a multi-state binding event in which electrostatic and hydrophobic interactions serve distinct roles in α Syn binding. Electrostatics dominate when the negative headgroups interact with the lysine-rich N-terminus of α Syn, bringing the protein close to the membrane surface. The hydrophobic interactions then dominate when the N-terminus embeds into the membrane upon binding. These hydrophobic interactions become even more relevant in the context of NTA- α Syn where MD simulations show additional helical propensity increases membrane binding affinity⁵⁸. Throughout this literature, electrostatic interactions were shown to assist in α Syn binding through membrane recognition, but the defects and hydrophobic interactions mediate the physical insertion of the protein beyond surface adsorption⁶⁶. This necessity of defects for binding and insertion was exemplified with our results utilizing DL membranes, where the addition of 25% charge from PS did not increase the density of packing defects nor the amount of bound α Syn. Even in the presence of substantial anionic charge, which assists with α Syn localization to the membrane, insufficient defects resulted in negligible binding to DL membranes. In contrast, we note a substantial increase in defect coverage and binding of α Syn to DO membranes when 25% PS was included. This increase in defect content could be due to repulsion between charged lipid headgroups that open additional defects in the DO system or due to a mismatch in headgroup size between PC and PS⁵⁹. While increased α Syn binding was strongly correlated with an increase in defect content and neutral DPhPC membranes elicited stronger binding than anionic DOPC/DOPS membranes, it must be noted that anionic charge and defect content exhibited synergy in recruitment of α Syn.”

5. *In addition, I suggest that the authors include different concentrations of solutions such as KCl or CaCl₂ in the experiments where the protein binds to DPhPC, DPhPC/DPhPS, etc. to see if the ions have an effect on the binding and thus determine if electrostatic effects play a role in the process.*

Author Response and Action Taken: The reviewer has proposed some insightful experiments. Regarding monovalent salts like KCl, previous literature, including papers referenced by the reviewer (Zhao et al., *National Science Review*, 2024; Jacobs et al., *Scientific Reports*, 2019; Davidson et al., *J. Biological Chem.*, 1998), has shown that increasing salt concentration decreases α Syn binding to charged membranes. We would expect to see the same results in our experiments if we repeated them, thus we don't believe that these specific experiments would add significant additional insight to our current work. We would like to point out that there is some modulation of electrostatics in our current work as we varied the PS% (i.e., 0% or 25%). Regarding divalent salts such as CaCl₂, Ca²⁺ is known to cause lipid clustering and other complex membrane interactions (Melcrova et al., *Scientific Reports*, 2016; Pedersen et al., *BBA – Biomembranes*, 2006) that could confound electrostatic vs hydrophobic interactions as Ca²⁺ would change not just electrostatic properties but also some packing properties of the membrane itself.

This type of behavior would be interesting in a follow-up study, as previous literature has shown that Ca^{2+} influences αSyn interaction with membranes because of a specific αSyn - Ca^{2+} interaction (Lautenschläger et al., *Nat. Commun.*, 2018).

6. *Also, although it is only a published abstract from another group (DOI: 10.1016/j.bpj.2024.11.1091), the title of that abstract is exactly the same as this paper, and the authors might consider discussing it in the discussion.*

Author Response and Action Taken: We appreciate the reviewer bringing this to our attention. Their abstract was published shortly after our initial submissions to BiorXiv and peer-review processes. We have since been in contact with the authors. It turns out the similarity in titles is entirely coincidental. While their study uses different lipid compositions and characterization techniques, they independently arrived at a conclusion similar to ours. We believe this convergence highlights the robustness of the underlying phenomenon and reinforces the significance of defect-driven αSyn binding. We would be enthusiastic to cite their findings, but their work is neither published nor available in pre-print form. Should their work become available in either form during our peer review process, we would be happy to update our manuscript to cite and discuss their work.