jz-2022-02126j.R1

Name: Peer Review Information for "Charge of Phospholipids Determines the Rate of Lysozyme Aggregation but Not the Structure and Toxicity of Amyloid Aggregates"

First Round of Reviewer Comments

Reviewer: 1

Comments to the Author

The authors present the results of their studies on aggregation of lysozyme in the presence various phospholipids. They apply a set of physical chemical approaches including imaging with AFM along with the cell-level studies in which the toxicity of aggregates was tested. The finding might be interesting to the JPCL readership, but numerous weaknesses must be eliminated prior to considering suitability of publication of these data.

Major weaknesses

1. Data analysis. The experimental data have been analyzed, but many questions regarding the validity of the conclusions made using the analyzed data remain.

Fig 1A presents the ThT kinetics graphs. What are error bars on these curves? Fig. 1B presents the histograms with error bars – how were these values obtained? How many ThT kinetics graphs were used in generation of these data?

A set of images is shown in Figure 2. Are these representative images? How do they correspond to the ThT results? Various morphologies of aggregates are mentioned in the text. How were they characterized? Oligomers mentioned – what are criteria for assigning the morphology to this category? Specifically, the authors write: “Figure 2. In the presence of PC and PE, we observed only small spherical aggregates that had 5-7 nm in height” Where are such aggregates? I can see non-fibrillar aggregates with various morphologies including elongated ones. How representative are spherical features with the heights indicated in the text? Similar concern is addressed to the following statement: “We also found that PS and CL promote formation of both small oligomers and prolong fibrils that had ~20 nm in height”.

I did not find any evidence supporting the claim.

Figure 3 – AFM-IR data and analyses. How reproducible are the spectra for each type of samples? What is quantitative difference between the spectra of different samples? How were the spectra obtained? Around a particle with selected morphology or by averaging over a set of particles with different morphologies? Even fibrillar species can have different properties such as thickness, lengths – how do these parameters contribute to the IR spectra?

Page 4, lines 2-6. They contain conclusion statements. I did not find a justification for those as no quantitative analysis was presented. The paragraph below (lines 14-23) is a comparison made with other proteins. Such statement is very speculative as all proteins are very structurally different. The conclusion could be made if the authors made a similar analysis for these proteins using the same experimental approaches.

The cell toxicity results along with other data obtained with cells. First, the authors used only acronyms for the cell assays, which need to be explained. Second, assignments of bars to the NS category are not explained. For example, the bar sizes for ROS data for Lys-PS and Lys PG samples are considerably lower than the control, so why are they in the NS category? How many experiments were performed and how the statistics was obtained? What are the samples used in these experiments? How do they relate to the ThT and AFM images?

2. Conclusions. The conclusions on the effect of lipids on the aggregation kinetics are rather straightforward (provided that statistical analysis is done appropriately), but the correlation of these data with other experiments is not well justified as too many concerns need to be addressed.

Minor comment. What are yellow graphs in Fig. 4?

Reviewer: 2

Comments to the Author

This study investigate the characterization of phospholipid-lysozyme interaction on lysozyme aggregation. The authors analyzed aggregation kinetics by thioflavin T assay, aggregate structures by atomic force microscopy (AFM), secondary structure of aggregates by AFM-infrared spectrometry (AFM-IR), and cell toxicity induced by phospholipid-lysozyme aggregates. This study concluded that aggregation kinetics correlated with the charges of phospholipids, but the aggregate structures and cell toxicity associated with the chemical structures and properties of phospholipids rather than their charges. To help readers better understand the results of this study, I recommend that the following should be addressed.

Major:

1. In addition to the IR spectra in Figure 3, AFM-IR could have revealed correlations between AFM images and IR spectra. For example, whether the mapping of lipid-derived signal intensities such as L1, L2, and L3 correlates with the mapping of aggregate structure and Amide I signal in the AFM image is important to show that the aggregates do indeed contain lipids.

2. This study concludes that the charge of lipids correlates with the kinetics of aggregation. However, the charge of lipids is affected by the pH and ionic strength of the solution. For example, at pH 3.0, the pH of the solution used in this study, the carboxyl group of PS appears to dissociate few protons (https://avantilipids.com/tech-support/physical-properties/ionization-constants cited from CRC Handbook of lipid bilayers, Derek Marsh). Thus, the carboxyl group has no charge and the overall electrical situation appears to be neutral and similar to PC. The charge of each phospholipid under the experimental conditions needs to be explained.

3. Lys:PC and Lys:PE do not form long fibers (Figure 2), but their toxicity is quite different (Figure 4). Do they differ in structure, such as size of globular aggregates? For example, it would be easier to understand if the paper could show the distribution of molecular sizes of aggregates in the AFM image (Figure 2).

Minor:

1. The relationship between color scale and height in the z-direction in Figure 2 needs to be added to the figure caption.

Author's Response to Peer Review Comments:

please find point-by-point response attached

Reviewer 1.

1. Fig 1A presents the ThT kinetics graphs. What are error bars on these curves? Fig. 1B presents the histograms with error bars – how were these values obtained? How many ThT kinetics graphs were used in generation of these data?

Response: ThT measurements were made in triplicates. Error bars indicate variability in ThT intensity in the analyzed samples.

We specified in the experimental section of the manuscript that all ThT measurements were made in triplicates.

2. A set of images is shown in Figure 2. Are these representative images? How do they correspond to the ThT results? Various morphologies of aggregates are mentioned in the text. How were they characterized? Oligomers mentioned – what are criteria for assigning the morphology to this category? Specifically, the authors write: “Figure 2. In the presence of PC and PE, we observed only small spherical aggregates that had 5-7 nm in height” Where are such aggregates? I can see non-fibrillar aggregates with various morphologies including elongated ones. How representative are spherical features with the heights indicated in the text? Similar concern is addressed to the following statement: “We also found that PS and CL promote formation of both small oligomers and prolong fibrils that had ~20 nm in height”.
I did not find any evidence supporting the claim.

Response: AFM images are representative images of the samples. We collected 3-4 AFM images per sample. We observed a good correlation between the ThT values and morphologies. Specifically, small oligomers present in Lys:PC and Lys:PE have the lowest ThT intensities. Long Lys:PG fibrils, as well as Lys:CL aggregates exhibited the strongest ThT signal. Morphologies of Lys and Lys:PS are similar, so as their ThT responses.

We used visual examination to characterize the morphologies of protein aggregates. For spherical particles, we used a term oligomer, whereas for elongated aggregates -fibrils. The word “aggregates” were used for both oligomers and fibrils.

We agree that some elongated aggregates were present in Lys:PC and Lys:PE. Therefore, we modified the description of AFM results accordingly:

“In the presence of PC and PE, we observed predominantly small spherical aggregates that had 5-7 nm in height. We also observed a small amount of short fibril species in these samples.”

“We also found that CL promotes formation of both small oligomers and prolong fibrils that had ~20 nm in height, whereas preeminently fibrils were observed in the presence of PS.”

3. Figure 3 – AFM-IR data and analyses. How reproducible are the spectra for each type of samples? What is quantitative difference between the spectra of different samples? How were the spectra obtained? Around a particle with selected morphology or by averaging over a set of particles with different morphologies? Even fibrillar species can have different properties such as thickness, lengths – how do these parameters contribute to the IR spectra?

Response: we collected at least 50 spectra from aggregates present in all analyzed samples. Therefore, reported averaged spectra are representative. The spectra were obtained by positioning the scanning probe at the aggregate. For Lys:PC and Lys:PE, only oligomers were measured since the observed fibrillar species were only occasionally observed. For all other samples we measured fibrils since these species dominated in these samples. Randomly chosen fibrils were measured, which minimizes bias in such structural characterization.

We also performed spectral fitting to provide quantitative information about the protein secondary structure of the analyzed lysozyme aggregates. These results are summarized in the Figure S3.

4. Page 4, lines 2-6. They contain conclusion statements. I did not find a justification for those as no quantitative analysis was presented. The paragraph below (lines 14-23) is a comparison made with other proteins. Such statement is very speculative as all proteins are very structurally different. The conclusion could be made if the authors made a similar analysis for these proteins using the same experimental approaches.

Response: We are grateful to the reviewer for the provided suggestion. We added a histogram of relative contributions of parallel β-sheet (red), unordered protein secondary structure (blue) and antiparallel β-sheet (green) in amide I of AFM-IR spectra collected from lysozyme fibrils (Lys) grown in the lipid-free environment, as well as lysozyme aggregates grown in the presence of PC (Lys:PC), PE (Lys:PE), PS (Lys:PS), PG (Lys:PG) and CL (Lys:CL) to the manuscript (Figure S3).

We did not make a strong comparison of these results with the experimental findings reported by Dou and co-workers for α-Syn and Matveyenka and co-workers for insulin. Therefore, we used the word “resonate with” rather than “in agreement with”.

5. The cell toxicity results along with other data obtained with cells. First, the authors used only acronyms for the cell assays, which need to be explained. Second, assignments of bars to the NS category are not explained. For example, the bar sizes for ROS data for Lys-PS and Lys PG samples are considerably lower than the control, so why are they in the NS category? How many experiments were performed and how the statistics was obtained? What are the samples used in these experiments? How do they relate to the ThT and AFM images?

Response: We modified this section of the manuscript accordingly:

“Amyloid aggregates exert toxicities activating re-action oxygen species (ROS) production in cells.[41-42]; Mitochondrial dysfunction can be determined using JC-1 assay.[24, 25]”

LDH, ROS and JC-1 measurements were made in triplicates. We used T-test to determine statistical significance of the results. We used samples incubated at 65 °C for 18 hours. The sample samples were used for AFM analysis. These samples correspond to the last measured point on the ThT kinetic curves.

6. Conclusions. The conclusions on the effect of lipids on the aggregation kinetics are rather straightforward (provided that statistical analysis is done appropriately), but the correlation of these data with other experiments is not well justified as too many concerns need to be addressed.

Response: We modified the conclusions section of the manuscript accordingly:

“Specifically, in the presence of PC and PE, we found primarily small oligomers. However, in the presence of negatively charged phospholipids domination of fibril-like aggregates were observed.”

We also removed the following sentence:

“It should be noted that in the lipid-free environment, lysozyme formed morphologically different fibrils compared to those grown in the presence of lipids.”

Minor comment. What are yellow graphs in Fig. 4?

Response: Yellow bars correspond to lipids themselves.

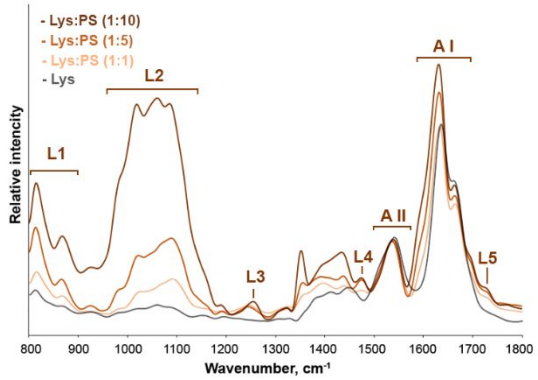
To address this comment, we modified the figure caption accordingly:

“Histograms of LDH (top), ROS (middle) and JC-1 (bottom) toxicity assays of Lys (brown bars), Lys:PC, Lys:PE, Lys:PG, Lys:PS, Lys:CL (orange bars), as we as PC, PE, PG, PS, and CL (yellow bars). Error bars represent standard errors of the mean (SEM) of three replicates.”

Reviewer: 2

1. In addition to the IR spectra in Figure 3, AFM-IR could have revealed correlations between AFM images and IR spectra. For example, whether the mapping of lipid-derived signal intensities such as L1, L2, and L3 correlates with the mapping of aggregate structure and Amide I signal in the AFM image is important to show that the aggregates do indeed contain lipids.

Response: We have previously demonstrated this correlation for α-Syn aggregates (doi.org/10.1021/acs.jpclett.1c00820; doi.org/10.1021/acschemneuro.2c00355). The correlation between the L1-L3 band intensities and presence of lipids could be also observed from the experiments in which Lys:PS aggregates grown in the presence of 1:1, 1:5 and 1:10 protein:lipid ratios were analyzed.



AFM-IR images of Lys and Lys:PS aggregates grown in the presence of 1:1, 1:5 and 1:10 protein:lipid ratios.

2. This study concludes that the charge of lipids correlates with the kinetics of aggregation. However, the charge of lipids is affected by the pH and ionic strength of the solution. For example, at pH 3.0, the pH of the solution used in this study, the carboxyl group of PS appears to dissociate few protons (https://avantilipids.com/tech-support/physical-properties/ionization-constants cited from CRC Handbook of lipid bilayers, Derek Marsh). Thus, the carboxyl group has no charge and the overall electrical situation appears to be neutral and similar to PC. The charge of each phospholipid under the experimental conditions needs to be explained.

Response: We want to thank the reviewer for the excellent comment. Indeed at pH 3, carboxyl group of PS is protonated and this lipid is zwitterionic. To address this, we added the following comment:

“It should be noted that at pH 3.0 used in our experiments, carboxyl group of PS is protonated, therefore, this lipid possesses net positive charge (cationic lipid)”, accordingly to CRC Handbook of lipid bilayers, Derek Marsh

Since PS possesses net positive charge at pH 3, we indicated throughout the manuscript that we dealt with zwitterionic, anionic and cationic lipid.

3. Lys:PC and Lys:PE do not form long fibers (Figure 2), but their toxicity is quite different (Figure 4). Do they differ in structure, such as size of globular aggregates? For example, it would be easier to understand if the paper could show the distribution of molecular sizes of aggregates in the AFM image (Figure 2).

Response: In our previous study, we investigated mechanisms of toxicity exerted by insulin:PC aggregates that similar to Lys:PC and Lys:PE are dominated by small oligomers. We found that both insulin fibrils and insulin:PC aggregates use the same mechanisms by which they exert cell toxicity.

In the current work, we found that structures of Lys:PC and Lys:PE are different, as well as toxicity exerted by these aggregates. Thus, one can expect that structure rather than the aggregation stage (fibrils vs oligomers) determines toxicity of amyloid aggregates.

4. The relationship between color scale and height in the z-direction in Figure 2 needs to be added to the figure caption.

Response: we added the scale bar to describe Z profile in the caption of Figure 2

jz-2022-02126j.R2

Name: Peer Review Information for "Charge of Phospholipids Determines the Rate of Lysozyme Aggregation but Not the Structure and Toxicity of Amyloid Aggregates"

Second Round of Reviewer Comments

Reviewer: 1

Comments to the Author

More work is needed.

The authors responded to some of my comments, but missed a sufficient number of those. I identified unanswered questions of the previous critiques with additional comments.

2. A set of images is shown in Figure 2. Are these representative images? How do they correspond to the ThT results? Various morphologies of aggregates are mentioned in the text. How were they characterized? Oligomers mentioned – what are criteria for assigning the morphology to this category? Specifically, the authors write: “Figure 2. In the presence of PC and PE, we observed only small spherical aggregates that had 5-7 nm in height” Where are such aggregates? I can see non-fibrillar aggregates with various morphologies including elongated ones. How representative are spherical features with the heights indicated in the text? Similar concern is addressed to the following statement: “We also found that PS and CL promote formation of both small oligomers and prolong fibrils that had ~20 nm in height”. I did not find any evidence supporting the claim.

- Various morphologies of aggregates are mentioned in the text. How were they characterized?
- Oligomers mentioned – what are criteria for assigning the morphology to this category?
- Specifically, the authors write: “Figure 2. In the presence of PC and PE, we observed only small spherical aggregates that had 5-7 nm in height” Where are such aggregates? I can see non-fibrillar aggregates with various morphologies including elongated ones. Statistics of the height measurements is missing,
- How representative are spherical features with the heights indicated in the text? Statistics is needed here too to justify the yield of each morphology.
- Similar concern is addressed to the following statement: “We also found that PS and CL promote formation of both small oligomers and prolong fibrils that had ~20 nm in height”. Statistics of the height measurements is needed

3. Figure 3 – AFM-IR data and analyses. How reproducible are the spectra for each type of samples? What is quantitative difference between the spectra of different samples? How were the spectra obtained? Around a particle with selected morphology or by averaging over a set of particles with

different morphologies? Even fibrillar species can have different properties such as thickness, lengths – how do these parameters contribute to the IR spectra?

• How reproducible are the spectra for each type of samples?

• What is quantitative difference between the spectra of different samples?

• How were the spectra obtained? Around a particle with selected morphology or by averaging over a set of particles with different morphologies? Even fibrillar species can have different properties such as thickness, lengths – how do these parameters contribute to the IR spectra?

Reviewer: 2

Comments to the Author

Thanks to the author's excellent response, it is much easier to understand.

On that note, I would appreciate it if this study could clarify the following points before being accepted.

1. My comment #1 is related to the Reviewer 1's comment 3, where the manuscript does not tell the reader where in the AFM image the IR spectrum is measured; the author's response to this comment in Reviewer 1 needs to be included in the Supporting Information. It would be easier for the reader to understand if, in addition to the average spectrum in Figure 3, several revpresentative locations (~3 positions) where the IR spectrum was measured are shown for each image (Figure 2) and the spectrum for each location is shown in the Supporting Information.

2. The IR spectra in the author's response to my comment 1 and Figure 3, and the ThT measurement (Figure 1) should all show the negative control of lipid only (no lysozyme).

Author's Response to Peer Review Comments:

please find point by point response attached.

Reviewer 1.

1. Various morphologies of aggregates are mentioned in the text. How were they characterized?

Response: We used AFM to characterize morphologies of the aggregates. For each sample, an aliquot of the protein suspension was diluted with 1x PBS, pH 3.0 and deposited on the pre-cleaned silicon wafer. Next, the wafer was dried under a flow of dry nitrogen. At least 4-5 images 5x5 μm were collected for each sample. Observed spherical aggregates were classified as "oligomers", whereas elongated aggregates were identified as "fibrils". At least 50 individual oligomers were analyzed to determine height distribution of protein aggregates in each sample.

To address this question, we added the following text to the experimental section of the manuscript in SI.

"For each sample, an aliquot of the protein suspension was diluted with 1x PBS, pH 3.0 and deposited on the pre-cleaned silicon wafer. Next, the wafer was dried under a flow of dry nitrogen. At least 4-5 images 5x5 μm were collected for each sample. Observed spherical aggregates were classified as "oligomers", whereas elongated aggregates were identified as "fibrils". At least 50 individual oligomers were analyzed to determine height distribution of protein aggregates in each sample, Figure S5."

We also added histograms of height distribution of Lys:PC, Lys:PE, Lys, PG, Lys:CL and Lys aggregates to the SI of the manuscript.

2. Oligomers mentioned – what are criteria for assigning the morphology to this category?

Response: as identified all spherical aggregates as "oligomers" and all elongated as "fibrils".

3. Specifically, the authors write: "Figure 2. In the presence of PC and PE, we observed only small spherical aggregates that had 5-7 nm in height" Where are such aggregates? I can see non-fibrillar aggregates with various morphologies including elongated ones. Statistics of the height measurements is missing,

Response: we modified the description of Lys:PC and Lys:PE aggregates:

"In the presence of PC, we observed predominantly small spherical aggregates that had 5-7 nm in height. We also observed a small amount of short fibril species in Lys:PC. However, only small oligomers were observed in Lys:PE."

We also added histograms of height distribution of Lys:PC, Lys:PE, Lys, PG, Lys:CL and Lys aggregates to the SI of the manuscript.

4. How representative are spherical features with the heights indicated in the text? Statistics is needed here too to justify the yield of each morphology.

Response: We added histograms of height distribution of Lys:PC, Lys:PE, Lys, PG, Lys:CL and Lys aggregates to the SI of the manuscript.

5. Similar concern is addressed to the following statement: “We also found that PS and CL promote formation of both small oligomers and prolong fibrils that had ~20 nm in height”. Statistics of the height measurements is needed

Response: We added histograms of height distribution of Lys:PC, Lys:PE, Lys, PG, Lys:CL and Lys aggregates to the SI of the manuscript.

6. How reproducible are the spectra for each type of samples?

Response: We found that spectra reported in our study are reproducible, as we performed AFM-IR analysis of many aggregates from several independently made experiments. It should be noted that some classes of aggregates, such as Lys:PE, Lys:PG, Lys:CL and Lys demonstrated very little if any structural heterogeneity of analysis aggregates. Lys:PC and Lys:PS exhibited greater degree of structural heterogeneity, as can be observed in the acquired spectra. For instance, we observed two distinctly different types of AFM-IR spectra acquired from morphologically identical Lys:PS aggregates. These findings are in a good agreement with the previously reported AFM-IR analysis of insulin aggregates that were grown in the presence of PC and PS. Additional studies are required to fully elucidate observed heterogeneity of Lys:PC and Lys:PS, which are beyond the scope of the current work.

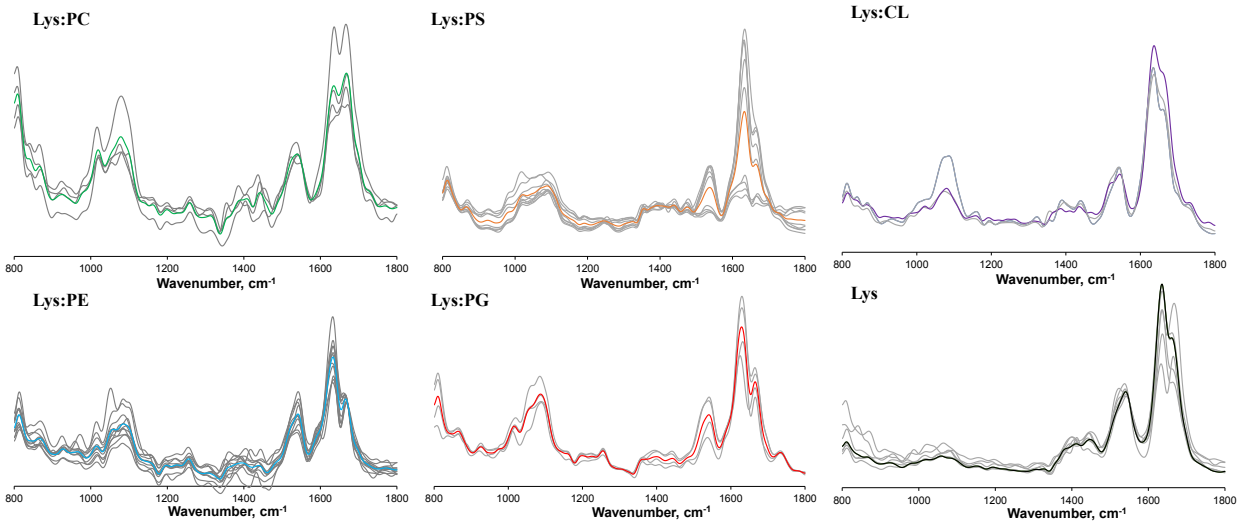


Figure A1. Individual (grey) and averaged (colored) AFM-IR spectra of Lys:PC, Lys:PE, Lys:PS, Lys:PG, Lys:CL and Lys.

To address the reviewer’s question, we added the following paragraph to the manuscript, as well as added the discussed above figure to the SI of the manuscript.

“It should be noted that some classes of aggregates, such as Lys:PE, Lys:PG, Lys:CL and Lys demonstrated very little if any structural heterogeneity of analysis aggregates. Lys:PC and Lys:PS exhibited greater degree of structural heterogeneity, as can be observed in the acquired spectra. For instance, we observed two distinctly different types of AFM-IR spectra acquired from morphologically identical Lys:PS aggregates. These findings are in a good agreement with the previously reported AFM-IR analysis of insulin aggregates that were grown in the presence of PC and PS. Additional studies are

required to fully elucidate observed heterogeneity of Lys:PC and Lys:PS, which are beyond the scope of the current work."

7. What is quantitative difference between the spectra of different samples?

Response: we reported the histogram of relative contributions of parallel β-sheet (red), unordered protein secondary structure (blue) and antiparallel β-sheet (green) in amide I of AFM-IR spectra collected from lysozyme fibrils (Lys) grown in the lipid-free environment, as well as lysozyme aggregates grown in the presence of PC (Lys:PC), PE (Lys:PE), PS (Lys:PS), PG (Lys:PG) and CL (Lys:CL) as Supporting Figure 4.

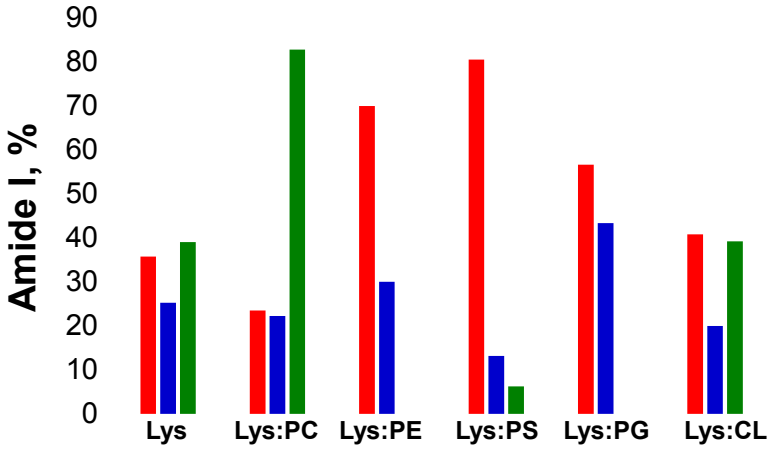


Figure A2: Histogram of relative contributions of parallel β-sheet (red), unordered protein secondary structure (blue) and antiparallel β-sheet (green) in amide I of AFM-IR spectra collected from lysozyme fibrils (Lys) grown in the lipid-free environment, as well as lysozyme aggregates grown in the presence of PC (Lys:PC), PE (Lys:PE), PS (Lys:PS), PG (Lys:PG) and CL (Lys:CL).

As can be seen from histogram, AFM-IR allows for quantification of the protein secondary structures in all analyzed classes of lysozyme aggregates.

8. How were the spectra obtained? Around a particle with selected morphology or by averaging over a set of particles with different morphologies? Even fibrillar species can have different properties such as thickness, lengths – how do these parameters contribute to the IR spectra?

Response: To acquire the spectrum, scanning probe was positioned at the top of the aggregate. We acquired spectra from all morphologically different structures observed in the sample. Variability in the size of the aggregates only altered overall intensity of the AFM-IR spectra. We observed very little if any structural heterogeneity in Lys:PE, Lys:PG, Lys:CL and Lys aggregates, whereas some degree of structural heterogeneity in Lys:PC and Lys:PS. These heteogenaity is reflected by different intensity ratios of 1630 and 1660 cm^{-1}, as well as different intensity of lipid vibrations (1000-1200 cm^{-1}). Changes in amide I (1630-1660 cm^{-1}) are indicative of differences in the secondary structure of Lys:PC and Lys:PS

aggregates, whereas changes in the intensity of lipid vibrations (1000-1200 cm^{-1}) point on the different amount of the corresponding lipid present in these aggregates.

To address this concern, we added the following text to the SI of the manuscript:

"To acquire the spectrum, scanning probe was positioned at the top of the aggregate. We acquired spectra from all morphologically different structures observed in the sample. Variability in the size of the aggregates only altered overall intensity of the AFM-IR spectra. We observed very little if any structural heterogeneity in Lys:PE, Lys:PG, Lys:CL and Lys aggregates, whereas some degree of structural heterogeneity in Lys:PC and Lys:PS. These heteogenaity is reflected by different intensity ratios of 1630 and 1660 cm^{-1}, as well as different intensity of lipid vibrations (1000-1200 cm^{-1}). Changes in amide I (1630-1660 cm^{-1}) are indicative of differences in the secondary structure of Lys:PC and Lys:PS aggregates, whereas changes in the intensity of lipid vibrations (1000-1200 cm^{-1}) point on the different amount of the corresponding lipid present in these aggregates."

We also added the following figure as Figure S5.

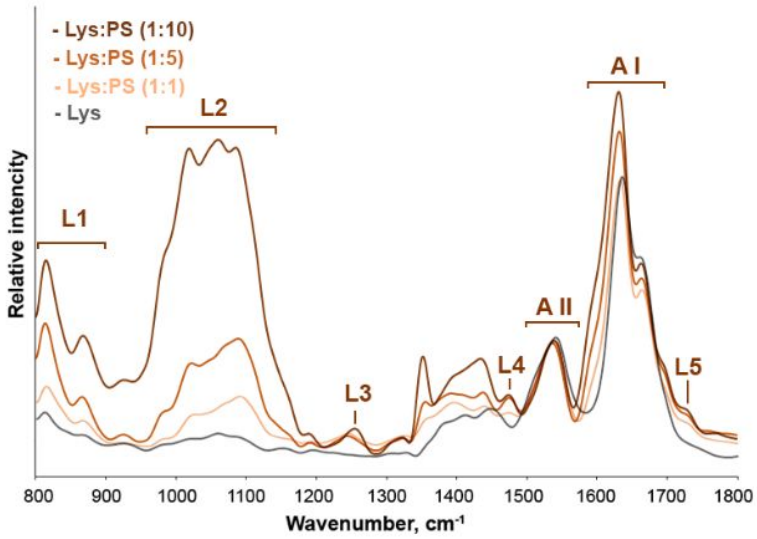


Figure A3. AFM-IR images of Lys and Lys:PS aggregates grown in the presence of 1:1, 1:5 and 1:10 protein:lipid ratios.

Reviewer 2.

1. My comment #1 is related to the Reviewer 1's comment 3, where the manuscript does not tell the reader where in the AFM image the IR spectrum is measured; the author's response to this comment in Reviewer 1 needs to be included in the Supporting Information. It would be easier for the reader to understand if, in addition to the average spectrum in Figure 3, several representative locations (~3 positions) where the IR spectrum was measured are shown for each image (Figure 2) and the spectrum for each location is shown in the Supporting Information.

Response: we added this information to SI section of the manuscript.

3. The IR spectra in the author's response to my comment 1 and Figure 3, and the ThT measurement (Figure 1) should all show the negative control of lipid only (no lysozyme).

We added reference IR spectra of lipids to the SI. Lipids cause to ThT change, therefore, we do not show ThT measurements with lipids themselves.

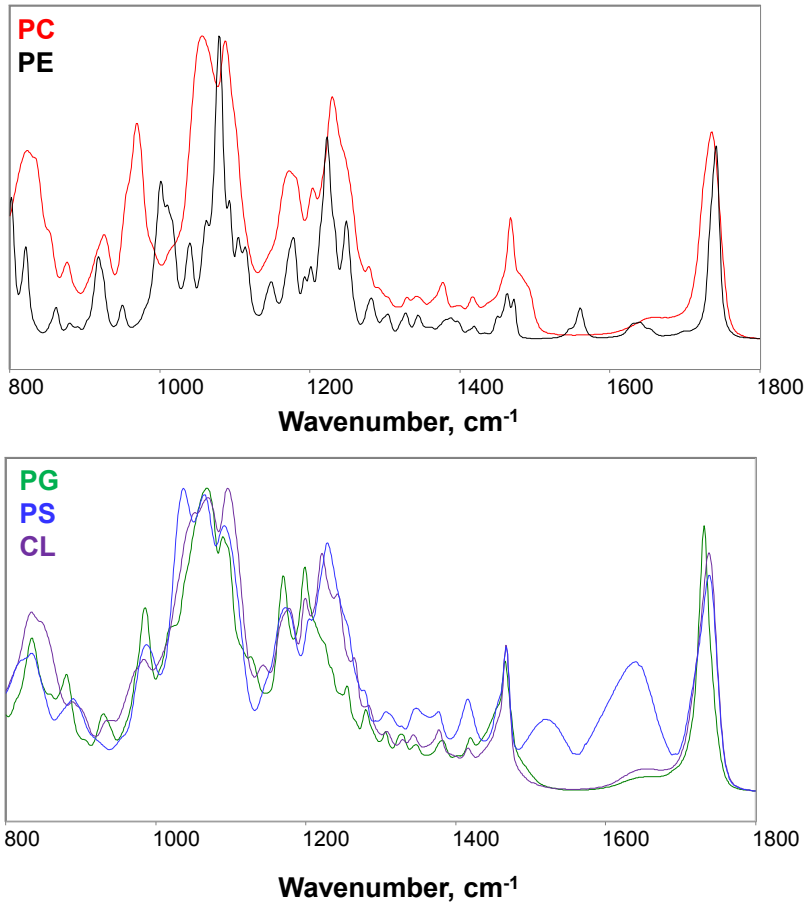


Figure A4. IR spectra of lipids