Charge of Phospholipids Determines the Rate of Lysozyme Aggregation but Not the Structure and Toxicity of Amyloid Aggregates

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Supporting Information

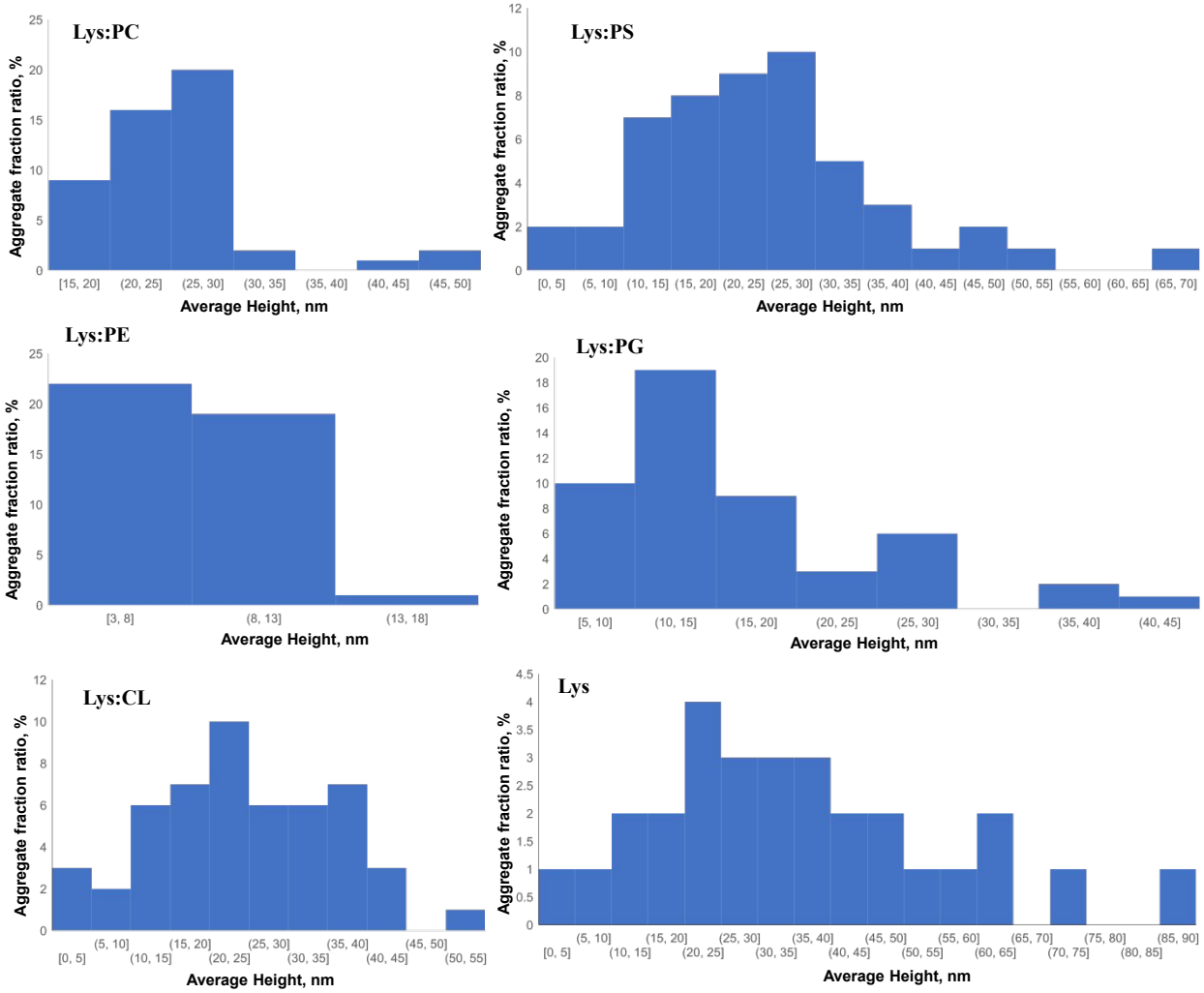


Figure S1. Histograms of height distribution of Lys:PC, Lys:PE, Lys, PG, Lys:CL and Lys aggregates.

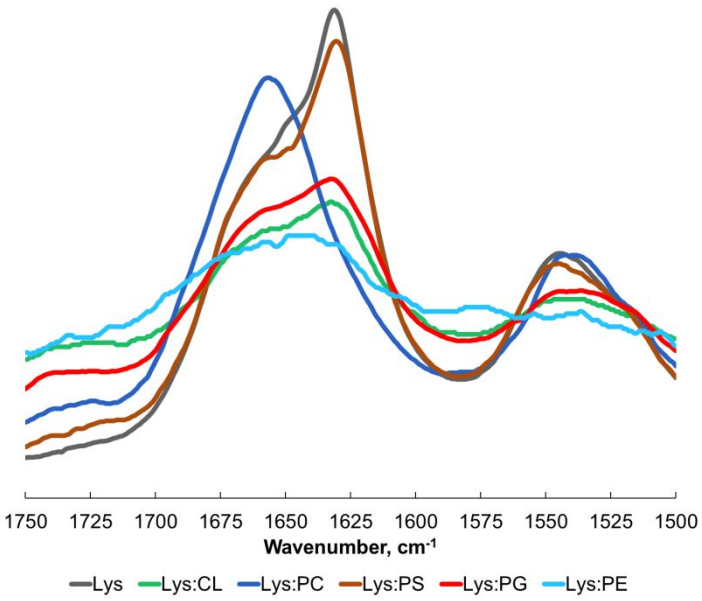


Figure S2. ATR-FTIR spectra of lysozyme aggregates grown in the lipid-free environment and in the presence of phospholipids.

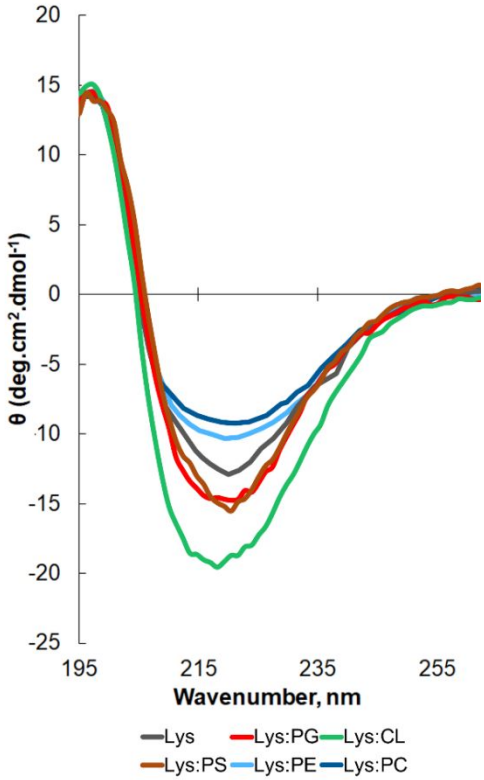


Figure S3. CD spectra of lysozyme aggregates grown in the lipid-free environment and in the presence of phospholipids.

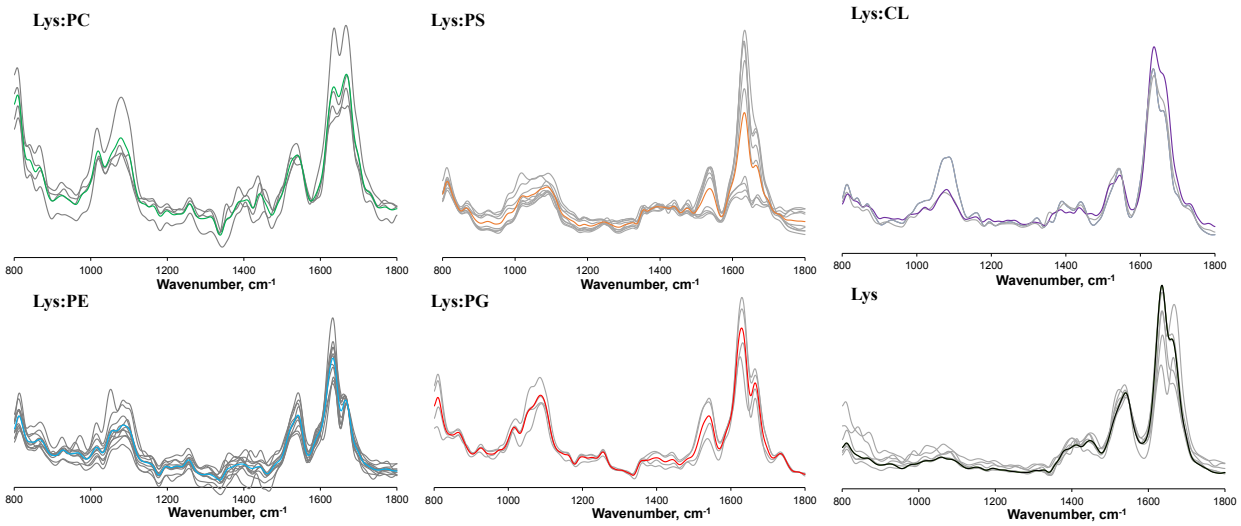


Figure S4. Individual (grey) and averaged (colored) AFM-IR spectra of Lys:PC, Lys:PE, Lys:PS, Lys:PG, Lys:CL and Lys.

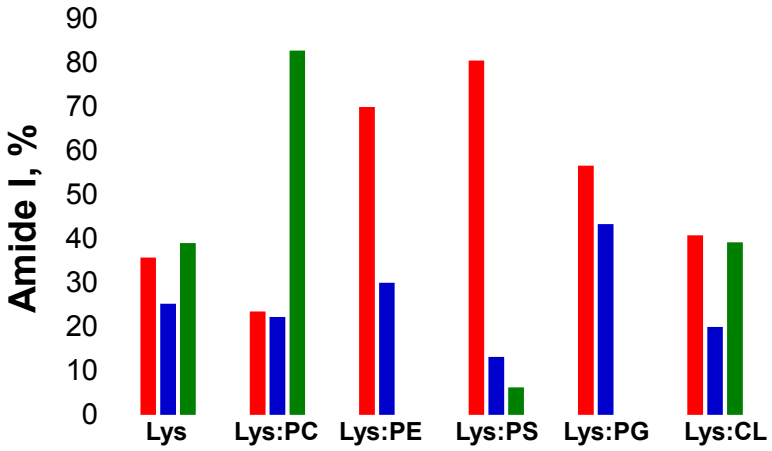
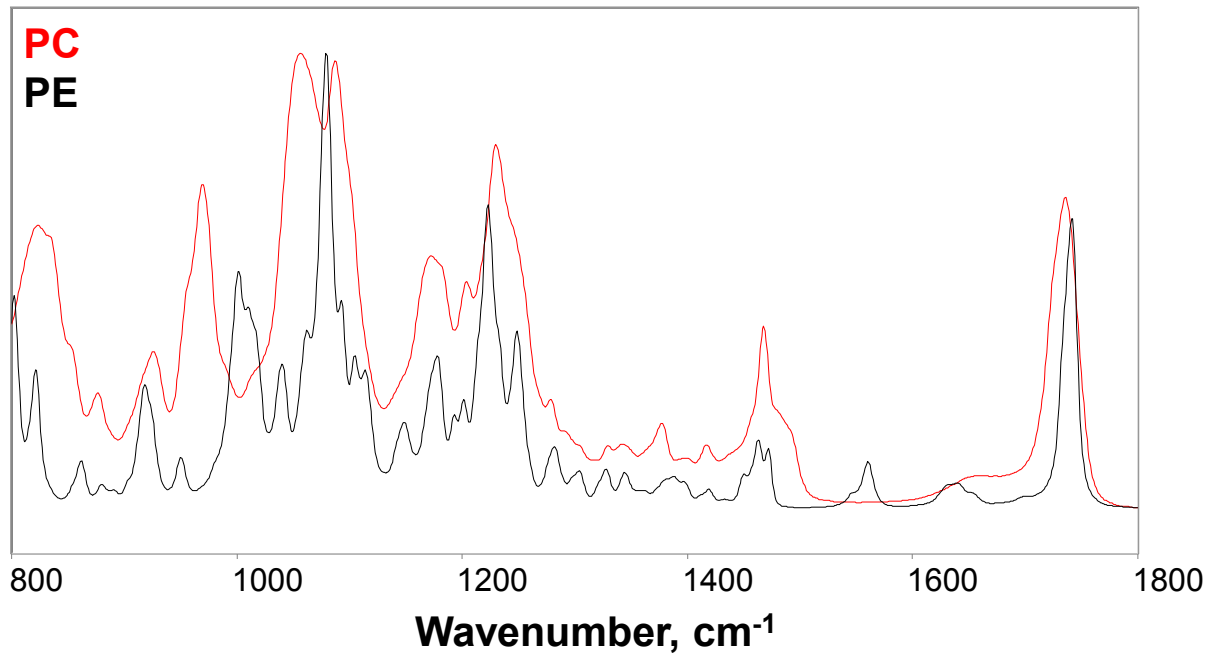


Figure S5. Histogram of relative contributions of parallel β-sheet (red), unordered protein secondary structure (blue) and antiparallel β-sheet (green) in amide I of AFM-IR spectra collected from lysozyme fibrils (Lys) grown in the lipid-free environment, as well as lysozyme aggregates grown in the presence of PC (Lys:PC), PE (Lys:PE), PS (Lys:PS), PG (Lys:PG) and CL (Lys:CL).



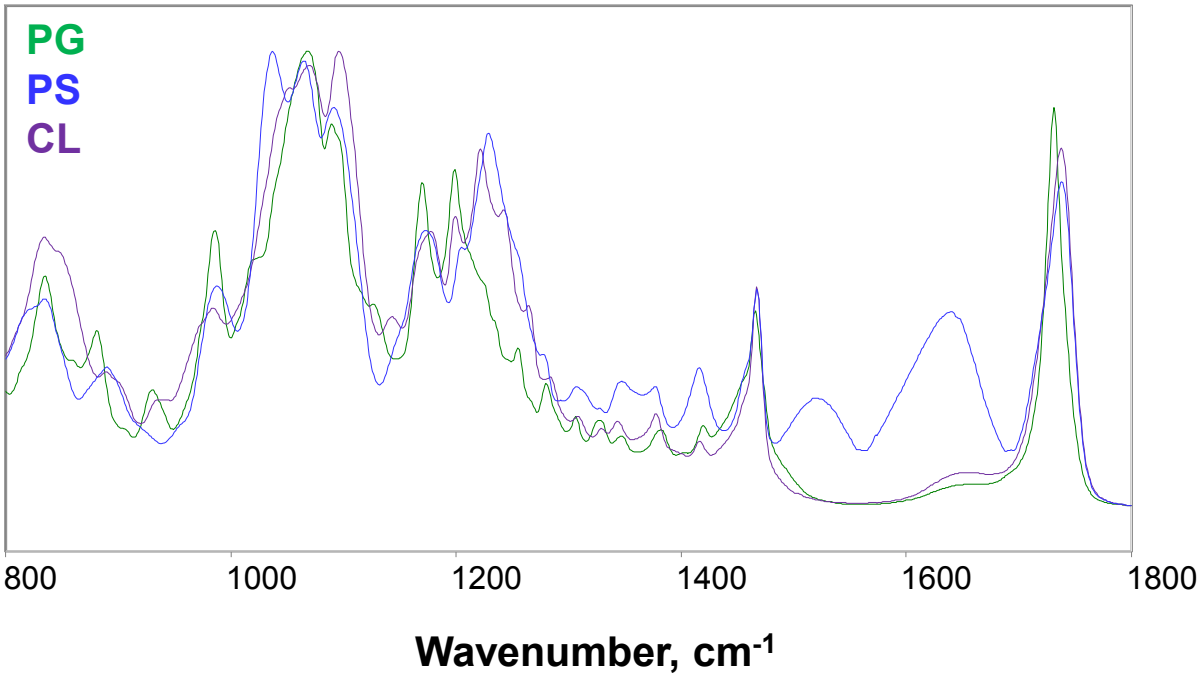


Figure S6. IR spectra of lipids

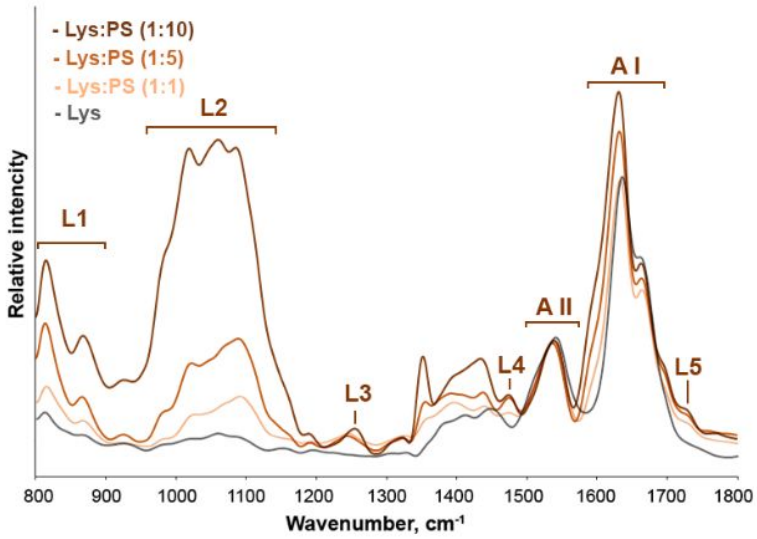


Figure S7. AFM-IR images of Lys and Lys:PS aggregates grown in the presence of 1:1, 1:5 and 1:10 protein:lipid ratios.

Experimental Section:

Materials: Hen egg-white lysozyme was purchased from Sigma-Aldrich (St. Louis, MO, USA) was purchased from Sigma-Aldrich (St. Louis, MO, USA), 1,2-dimyristoyl-sn-glycero-3-phospho-L-serine (DMPS or PS) 1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC or PC), 1',3'-bis[1,2-distearoyl-sn-glycero-3-phospho]-glycerol (18:0 cardiolipin (CL)) were purchased from Avanti (Alabaster, AL, USA).

Liposome preparation: Large unilamellar vesicles (LUVs) of PC, PE, PG, PS and CL were prepared accordingly to the method reported by Galvagnion et al.[33] Briefly, 0.6 mg of each lipid was dissolved in 2.6 ml of phosphate buffered saline (PBS) pH 7.4. Lipid solutions were heated in water bath to ~50 °C for 30 min and then cooled in liquid nitrogen for 3-5 min. This procedure was repeated 10 times. After this, lipid solutions were passed 15 times through an extruder that was equipped with 100 nm membrane (Avanti, Alabaster, AL, USA). The size of LUVs was determined by dynamic light scattering.

Protein aggregation and kinetic measurements: In the lipid-free environment, 400 μM of hen egg-white lysozyme was dissolved in PBS; solution pH was adjusted to pH 3.0 using concentrated HCl. For the Lys:PC, Lys:PE, Lys, PS, Lys:PG and Lys:CL, 400 μM of insulin was mixed with 5x concentration of the corresponding lipid (protein-to-lipid ratio for all samples was 1:5); solution pH was adjusted to pH 3.0 using concentrated HCl. Next, the solutions were placed in the dry block-heater and incubated at 65 °C for 18 hours. For ThT assay, an aliquot of protein samples was mixed with 2 mM of ThT solution and placed in the plate reader (Tecan, Männedorf, Switzerland). Excitation was 450 nm with the emission collected at 488 nm. All measurements were made in triplicates.

AFM imaging: microscopic analysis of protein aggregates was performed on AIST-NT-HORIBA system (Edison, NJ). Silicon AFM probes with force constant of 2.7 N/m and resonance frequency of 50-80 kHz were purchased from Appnano (Mountain View, CA, USA). 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 S1. Analysis and pre-processing of AFM images was performed using AIST-NT software (Edison, NJ, USA).

AFM-IR: AFM-IR analysis of samples was performed on Nano-IR3 system (Bruker, Santa Barbara, CA, USA) equipped with a QCL laser. Contact-mode AFM tips (ContGB-G AFM probe, NanoAndMore, Watsonville, CA, USA) were used to obtain all AFM-IR spectra. 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, Figure S4. This heterogeneity 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, Figure S7.

Attenuated total reflectance Fourier-transform Infrared (ATR-FTIR) spectroscopy: Spectra of lipids were measured using Spectrum 100 FTIR spectrometer (Perkin-Elmer, Waltham, MA, USA).

Cell toxicity assays: Mice midbrain N27 cells were grown in RPMI 1640 Medium (Thermo Fisher Scientific, Waltham, MA, USA) with 10% fetal bovine serum (FBS) (Invitrogen, Waltham, MA, USA) in 96 well-plate (5,000 cells per well) at 37 °C under 5% CO_2. After 24 h, the cells were found to fully adhere to the wells reaching ~70% confluency. N27 cells divide approximately once in 12h. Consequently, after 24 h of cell incubation at 37 °C under 5% CO_2, each well contained approximately 10,000 cells. The cell count and viability were verified by Trypan Blue dye that confirmed the number of cell and their viability in the range of 98-99%. After that, 100 µL of the cell culture was replaced with 100 µL RPMI 1640 Medium with 5% FBS containing protein samples. After 48 hours of incubation, lactate dehydrogenase (LDH) assay was performed on the cell medium using CytoTox 96 non-radioactive cytotoxicity assay (G1781, Promega, Madison, WI, USA). Absorption measurements were made in plate reader (Tecan, Männedorf, Switzerland) at 490 nm. Every well was measured 25 times in different locations. At least two independently prepared protein samples were examined using LDH assay. All measurements were made in triplicates. T-test was used to determine significance level of differences between toxicity of analyzed samples.

In parallel, reactive oxygen species (ROS) assay was performed using the same cell culture. Briefly, ROS reagent (C10422, Invitrogen, Waltham, MA, USA) was added to reach the final concentration of 5 µM and incubated at 37 °C under 5% CO_2 for 30 min. After the supernatant was removed, cells were washed with PBS and resuspended in 200 µL of PBS in the flow cytometry tubes. Sample measurements were made in LSR II Flow Cytomer (BD, San Jose, CA, USA) using red channel (λ=633 nm). Percentages of ROS cells was determined using LSR II software.

For JC-1 staining, 1µL of JC-1 reagent (M34152A, Invitrogen, Waltham, MA, USA) was added to cells and incubated at 37 °C under 5% CO_2 for 30 min. After the supernatant was removed, cells were washed with PBS and resuspended in 200 µL of PBS in the flow cytometry tubes. Sample measurements were made in LSR II Flow Cytomer (BD, San Jose, CA, USA) using red channel (λ=633 nm). Percentages of ROS cells was determined using LSR II software.