Unsaturation in Fatty Acids of Phospholipids Drastically Alters the Structure and Toxicity of Insulin Aggregates Grown in Their Presence

Mikhail Matveyenka[1], Stanislav Rizevsky[1,2] and Dmitry Kurouski[*1,3]

1. Department of Biochemistry and Biophysics, Texas A&M University, College Station, Texas 77843, United States
2. Department of Biotechnology, Binh Duong University, Thu Dau Mot, 820000, Vietnam
3. Department of Biomedical Engineering, Texas A&M University, College Station, Texas, 77843, United States

Corresponding author: Dmitry Kurouski; Email: dkurouski@tamu.edu

Supporting Information.

Experimental Section:

Materials: Bovine insulin was purchased from Sigma-Aldrich (St. Louis, MO, USA), 1,2-Dimyristoyl-sn-glycero-3-phosphocholine (s-PC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (u-PC), 1',3'-bis[1,2-distearoyl-sn-glycero-3-phospho]-glycerol (18:0 cardiolipin (s-CL)) and 1',3'-bis[1,2-dipalmitoleoyl-sn-glycero-3-phospho]-glycerol (18:1 cardiolipin (u-CL)) were purchased from Avanti (Alabaster, AL, USA). S-PC, u-PC, s-CL and s-CL have >99% purity. Purity of bovine insulin is not reported by Sigma-Aldrich. All chemicals used as received.

Liposome preparation: Large unilamellar vesicles (LUVs) of s-PC, u-PC, s-CL and u-CL were prepared accordingly to the method proposed by Galvagnion et al.[1] Briefly, 0.6 mg of the lipid was dissolved in 2.6ml of phosphate buffered saline (PBS) pH 7.4. After lipid was fully dissolved, the corresponding solution was heated in a water bath to ~50 °C for 30 min and then immersed into liquid nitrogen for 3-5 min. This procedure was repeated 10 times. Finally, lipid solutions were processed using an extruder equipped with a 100 nm membrane (Avanti, Alabaster, AL, USA). Dynamic light scattering was used to ensure that the size of the LUVs was within 100±10 nm.

Insulin aggregation: In the lipid-free environment, 400 μM of insulin was dissolved in PBS; solution pH was adjusted to pH 3.0 using concentrated HCl. For **Ins:CL-u**, **Ins:CL-s**, **Ins:PC-u** and **Ins:PC-s**, 400 μM of insulin was mixed with an equivalent concentration of the corresponding lipid; pH of the final solution was adjusted to pH 3.0 using concentrated HCl. Next, samples were placed into 96 well-plate that was kept in the plate reader (Tecan, Männedorf, Switzerland) at 37 °C for 24 hours under 510 rpm agitation.

Kinetic measurements: Rates of insulin aggregation were measured using thioflavin T (ThT) fluorescence assay. For this, samples were mixed with 2 mM of ThT solution and placed in into 96 well-plate that was kept in the plate reader (Tecan, Männedorf, Switzerland) at 37 °C for 24 hours under 510 rpm agitation. Fluorescence measurements were taken every 10 min (excitation 450 nm; emission 488 nm).

AFM imaging: Microscopic analysis of protein aggregates performed on AIST-NT-HORIBA system (Edison, NJ) in tapping mode under ambient air environment using silicon AFM probes (force constant 2.7 N/m; resonance frequency 50-80 kHz) that were purchased from Appnano (Mountain View, CA, USA). Pre-processing of the collected AFM images was made using AIST-NT software (Edison, NJ, USA).

AFM-IR: AFM-IR spectra were collected on Nano-IR3 system (Bruker, Santa Barbara, CA, USA) equipped with a QCL laser using contact-mode AFM scanning probes (ContGB-G AFM probe, NanoAndMore, Watsonville, CA, USA). Collected spectra were pre-processed using Bruker Imaging software.

Circular Dichroism (CD): For all measurements, samples were first diluted to the final protein concentration of 100 μM using PBS and then placed into 1 mm quarz cuvette. CD spectra were acquired on J-1000 CD spectrometer (Jasco, Easton,

MD, USA). Three spectra were collected for each sample within 205-250 nm and averaged using Thermo Grams Suite software (Thermo Fisher Scientific, Waltham, MA, USA).

Attenuated total reflectance Fourier-transform Infrared (ATR-FTIR) spectroscopy: An aliquot (50 µL) of the protein sample was placed onto ATR crystal and dried at room temperature. Spectra were measured using Spectrum 100 FTIR spectrometer (Perkin-Elmer, Waltham, MA, USA). Three spectra were collected from each sample and averaged using Thermo Grams Suite software (Thermo Fisher Scientific, Waltham, MA, USA). To the best of our knowledge, dehydration of amyloid aggregates results in minimal if any changes in secondary structure and supramolecular chirality.[2]

Cell toxicity assays: Mice midbrain N27 cells, a model cell line for Parkinson disease, 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 hours, the cells were found to fully adhere to the wells reaching ~70% confluency. Next, 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 with the sample of the protein aggregates, 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. All experiments were done in triplicates. Every well was measured 25 times in different locations.

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 Accuri C6 Flow Cytomer (BD, San Jose, CA, USA) using red channel (λ=633 nm). Percentages of ROS cells was determined using Acura software.

For both LDH and ROS controls, (PBS) pH 7.4 was added to cells and incubated under the same experimental conditions. T-test was used to analyze the results: *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, relative to untreated cells are shown. NS indicates 'non-significant' differences between the toxicity or ROS response of the samples.

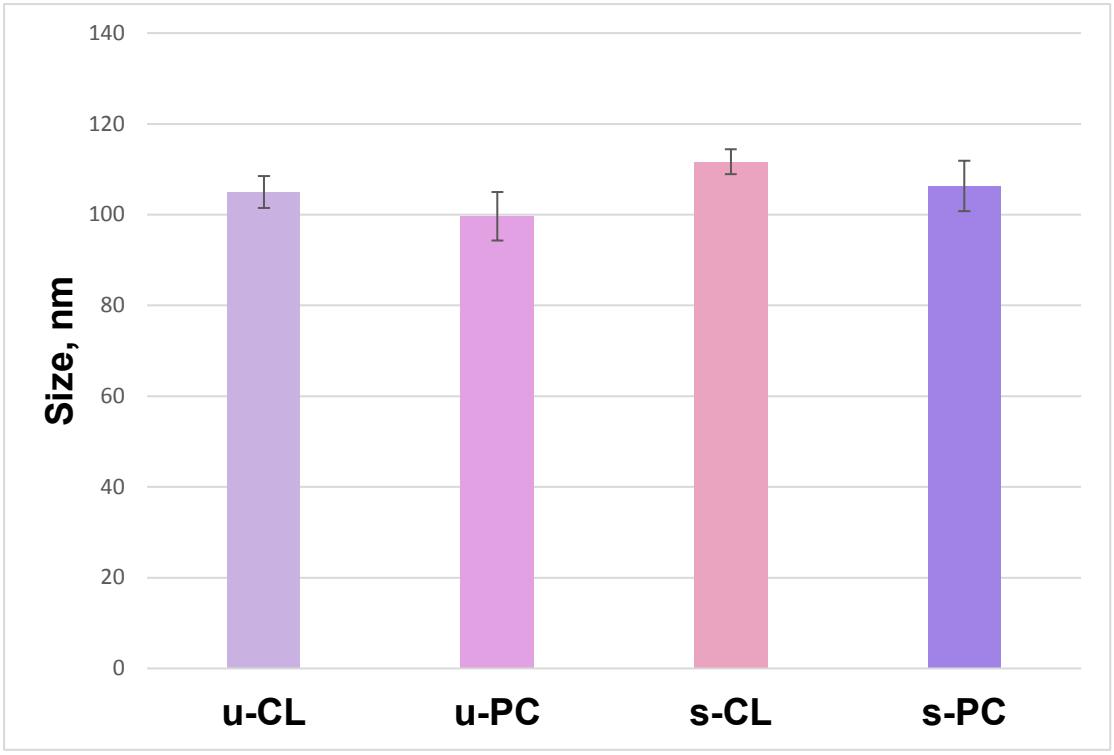

Figure 1. DLS data of LUVs u-CL, u-PC, s-CL and s-CP.

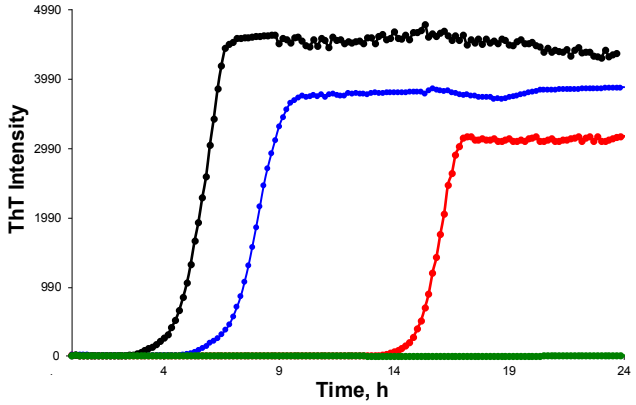

Figure S2. Lipids uniquely alter the rate of insulin aggregation. ThT aggregation kinetics of insulin in the lipid-free environment (red) and in the presence of u-CL (black), s-CL (blue), u-PC (green) and s-PC (purple) at 1:1 molar ratio.

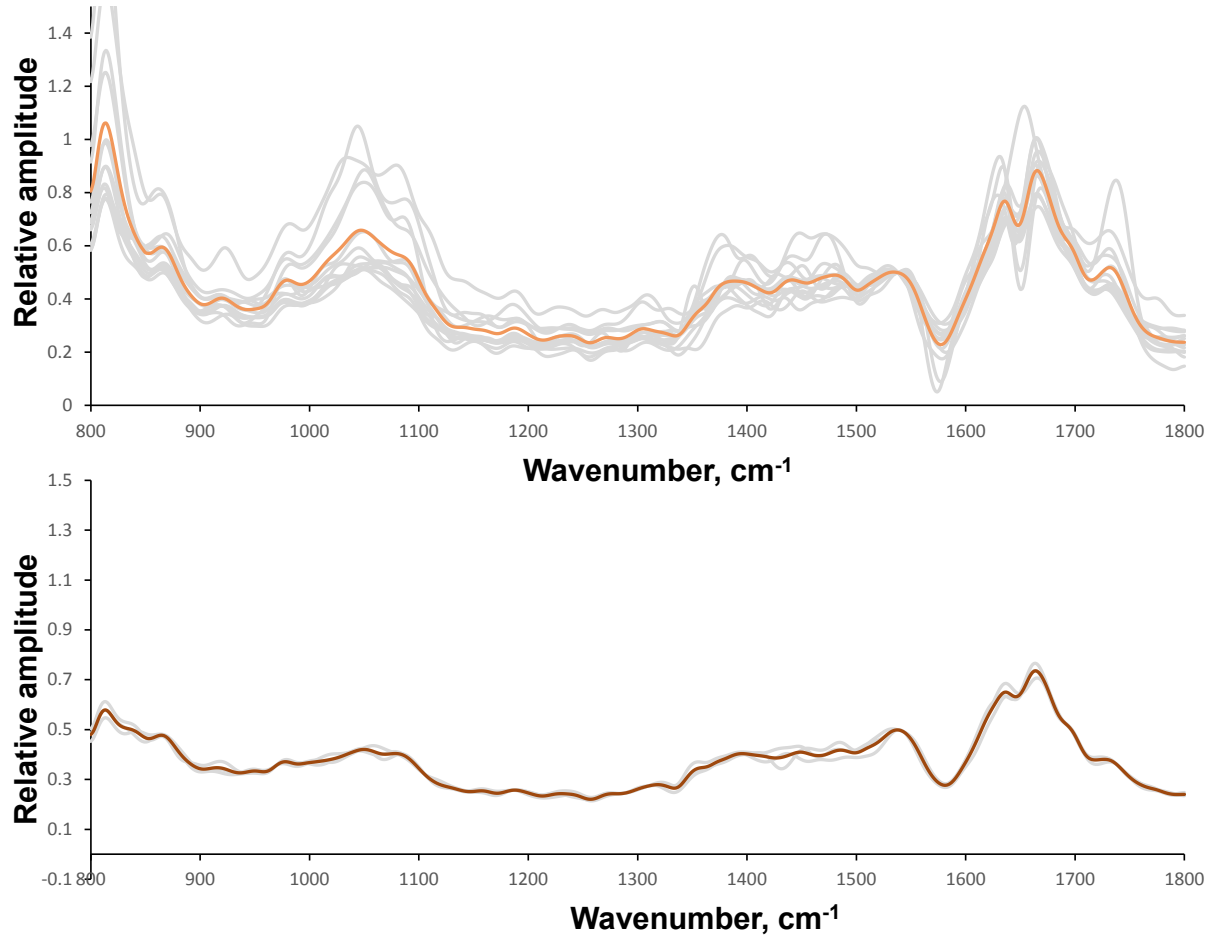

Figure S3. AFM-IR spectra collected from the individual oligomers (gray) and the corresponding averaged spectra (red and maroon) of population A (top) and B (bottom) of Ins:PC-s oligomers.

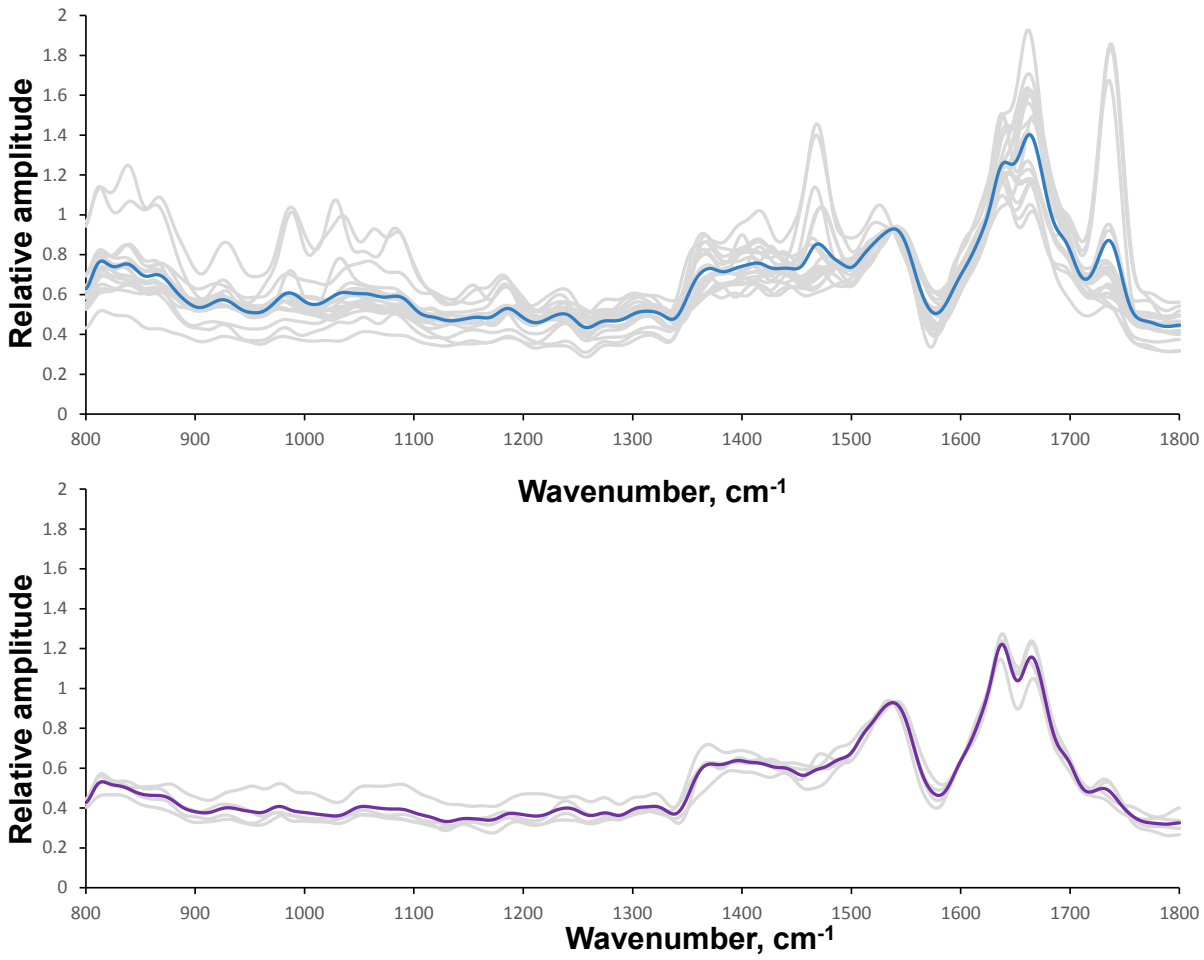

Figure S4. AFM-IR spectra collected from the individual oligomers (gray) and the corresponding averaged spectra (blue and purple) of population A (top) and B (bottom) of Ins:PC-u oligomers.

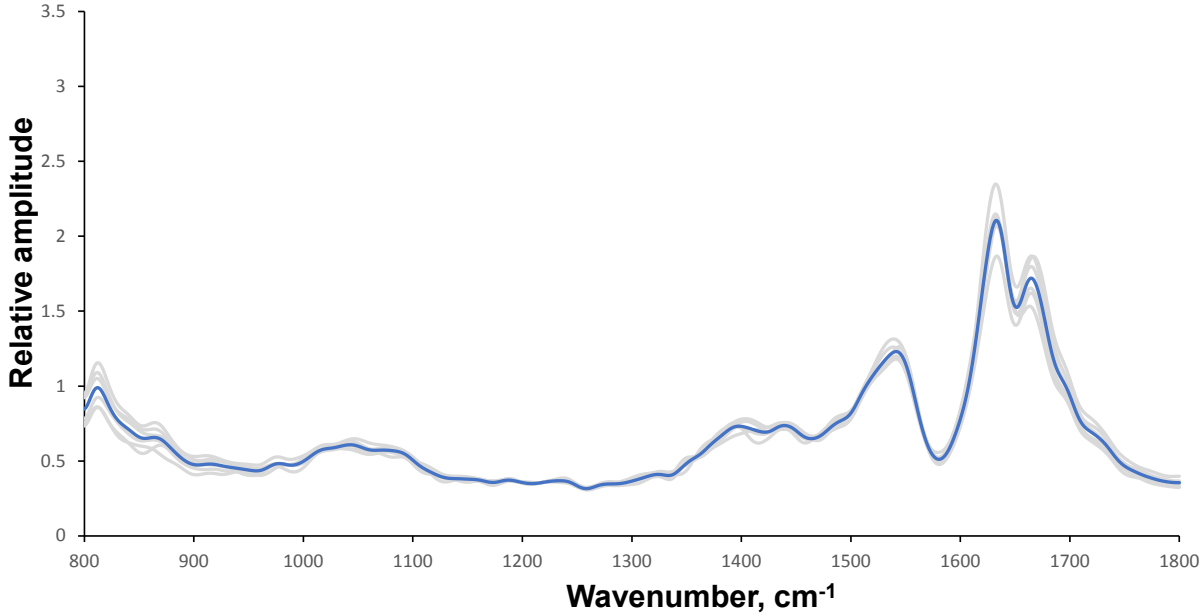

Figure S5. AFM-IR spectra collected from the individual Ins:CL-s aggregates (gray) with the averaged spectrum (blue).

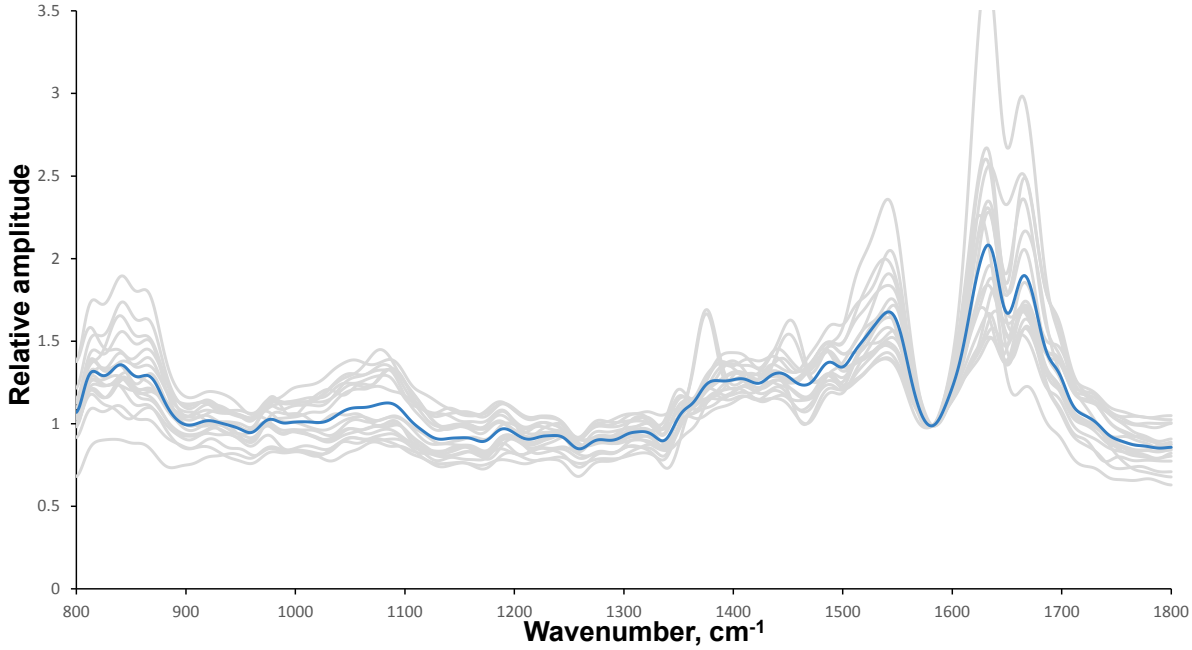

Figure S6. AFM-IR spectra collected from the individual Ins:CL-u aggregates (gray) with the averaged spectrum (blue).

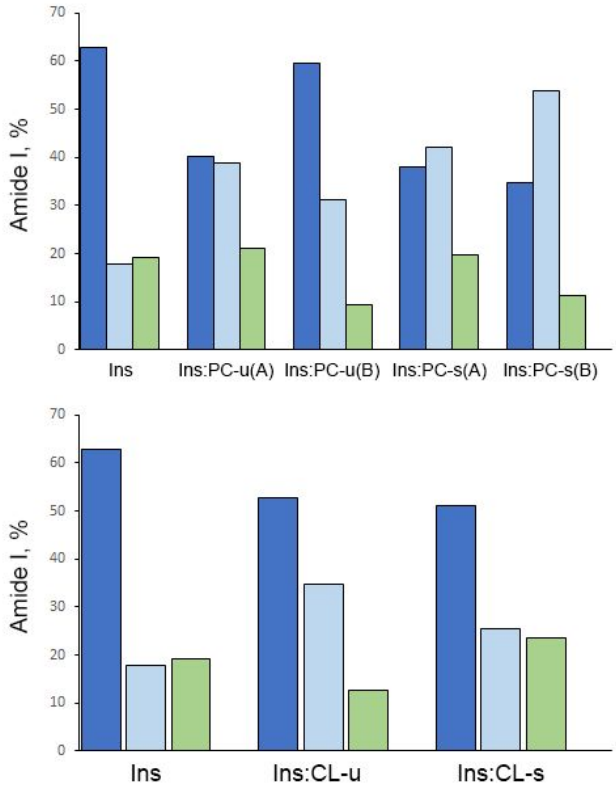

Figure S7. Histograms of relative contributions of parallel β-sheet (blue), unordered protein secondary structure (light blue) and antiparallel β-sheet (green) in amide I of AFM-IR spectra collected from two populations (A and B) of Ins:PC-u and Ins:PC-s (top) and Ins:CL-u and Ins:CL-s (bottom) together with insulin aggregates (Ins) grown in the lipid-free environment.

1. Galvagnion, C.; Buell, A. K.; Meisl, G.; Michaels, T. C. T.; Vendruscolo, M.; Knowles, T. P. J.; Dobson, C. M., Lipid Vesicles Trigger A-Synuclein Aggregation by Stimulating Primary Nucleation. *Nat. Chem. Biol.* **2015**, *11*, 229-234.

2. Kurouski, D.; Lombardi, R. A.; Dukor, R. K.; Lednev, I. K.; Nafie, L. A., Direct Observation and Ph Control of Reversed Supramolecular Chirality in Insulin Fibrils by Vibrational Circular Dichroism. *Chem. Commun. (Camb)* **2010**, *46*, 7154-6.