Electronic Supplementary Information:

Interaction with Prefibrillar Species and Amyloid-Like Fibrils Changes the Stiffness of Lipid Bilayers

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S.1 Materials

Bovine α-lactalbumin (ALA, Batch number JE 010-5-410) was kindly provided by Davisco Foods International (USA). 1,2-Dioleolyl-sn-glycero-3-phosphocholine (DOPC) was purchased from Avanti Polar Lipids (USA). Fluorescent lipids Oregon Green 488 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (DHPE) and Texas Red DHPE were purchased from Thermo Fisher Scientific (Germany). The following dyes were acquired from Sigma Aldrich (Sweden): Atto 647 N, Thioflavin T (ThT), 8-Anilino-1-naphthalenesulfonic acid ammonium salt (ANS). 25% Hydrochlorid acid was purchased from Merch KGaA (Germany). Reactants for acetate and phosphate buffer preparations, as well as sucrose, glucose, and sodium chloride were also purchased from Sigma Aldrich. The G-25 Sephadex column was obtained from GE Healthcare Sciences (UK). The employed 0.22 μm syringe filters were purchased from Merck Millipore (Germany). Uranyl acetate for staining was acquired from Agar Scientific (UK).

S.2 Preparation of native-like ALA samples

ALA is chosen as a protein model system for its ability to easily switch between different folding states and its propensity to form amyloid-like fibrils. Samples of non-incubated (monomeric) ALA were prepared at $4\,\mathrm{mg\,ml^{-1}}$ in the following buffers: a) 100 mM phosphate at pH 6.8, b) 10 mM sodium acetate and 90 mM acetic acid at pH 4 and c) 10 mM sodium acetate and 90 mM acetic acid at pH 2. ALA concentration was determined using a Nanodrop 2000 UV/Vis absorbance spectrophotometer (Thermo Fisher Scientific) assuming an extinction coefficient of 20.1 $\mathrm{ml\,mg^{-1}\,cm^{-1}}$ at 280 nm.

S.3 ThT fluorescence and preparation of ALA prefibrillar species and fibrils

ALA samples ($4\,\mathrm{mg\,ml^{-1}}$) were prepared in 100 mM NaCl adjusted at pH 2 with 25% HCl. Samples were then placed in Eppendorf tubes and the fibril formation was thermally induced at 60°C on a temperature-controlled shaker running at 600 rpm (Eppendorf Thermomixer, UK) for approximately 24 hours. To monitor fibril formation over time the same self-assembly protocol was tested on a plate reader system (Polarstar, BMG Labtech), running at the same temperature and shaking-speed settings. 200-μL ALA samples were labeled by adding 20 μM Thioflavin T (ThT) and placed into 96-microwell polystyrene plates (Nalge Nunc). Wells were sealed using polyolefin sealing tape (Nalge Nunc) to avoid evaporation. Samples were excited at 450 nm and the emission intensity of ThT was recorded at 480 nm every 5 minutes over 24 hours in a bottom-bottom configuration. Four replicates of each sample were measured in parallel to check for reproducibility.

Extracting ALA fibrils.
After 24 hours of incubation at 60°C and 600 rpm shaking, samples were centrifuged (14000 rcf, 4°C, 30 min) in order to separate the mature fibrils from potential soluble species still present in the sample. Supernatant was then removed and the un-soluble species were re-suspended in acetate buffer at pH 2. Transmission Electron Microscopy shows the presence of fibrils (see main text, Figure 1e). Moreover, the SRCD spectra (main text, Figure 1b) do not show any α-helix component, ruling out the presence of any native-like protein or oligomer. The concentration of protein converted into fibrils was $3.1\,\mathrm{mg\,ml^{-1}}$ as indirectly estimated by UV-vis absorbance of the supernatant. The so-obtained sample was used for further characterization and experiments with DOPC GUVs. Note that before exposure to the GUV samples, amyloid-like fibrils were sonicated for 2-5 minutes to break up micron-scale aggregates into smaller objects. This procedure is necessary to create a population of small fibrils that can diffuse in the sample chamber uniformly and interact with the GUVs without the need of intense mixing procedures that may result in vesicle rupture.

Extracting ALA prefibrillar species.
To isolate ALA prefibrillar species the self-assembly process was stopped after 10 hours of incubation at 60°C (see Figure 1 in the main text). The so-obtained sample already contained a few visible aggregates (ALA fibrils). Fibrillar aggregates were then forced to sediment by centrifuging the samples at 14000 rcf for 30 minutes at 4°C. The supernatant containing ALA prefibrillar species was finally extracted. Dynamic light scattering confirms the presence of species with diameter of 80 nm) within the supernatant (main text, Figure 1d). The concentration of such species was $1.6\,\mathrm{mg\,ml^{-1}}$ as estimated by UV-vis spectroscopy. SRCD confirms that such mixture retains a predominant α-helical structure (main text, Figure 1b). This solution was then used for the experiments with DOPC GUVs.

S.4 ANS fluorescence

ANS stock solution (1mM) was previously prepared and its concentration was estimated by absorbance spectroscopy. For the kinetics experiments, ANS was added to the protein samples in the 96-well plate to a concentration of 5 μM. Samples were excited at 485 nm and the intensity of the emission at 520 nm was recorded every 5 minutes, using the same plate reader system as for the ThT measurements.

S.5 Thryptophan fluorescence

Aliquots of native-like ALA samples (1 ml) at pH 2, 4 and 7 (see preparation details above) were placed into disposable fluorescence cuvettes (Sigma Aldrich, Sweden) and excited at 290 nm with a spectrophotometer (Cary Eclipse Fluorescence Spectrophotometer, Agilent Technologies). The emission spectra were recorded between 300 and 440 nm at 25°C. We are aware of the potential inner filter effects due to the ALA concentration used, but these do not affect the position of the fluorescence bands.

S.6 Dynamic light scattering (DLS)

DLS was used to monitor the growth of soluble aggregates in the fibrillation process described above. Aliquots of 100 μl were taken at different times, centrifuged (14000 rcf, 4°C, 30 minutes) and the supernatants were placed in a low volume quartz cuvette (50 μl) and analyzed using a Zetasizer Nano ZSP, Malvern Instruments. Measurements were taken within 60 seconds at 25°C with a scattering angle of 173 degrees. For each sample, three sets of 10-15 averaged measurements were performed.

S.7 Transmission Electron Microscopy (TEM)

We used TEM to verify the presence of ALA fibrils in the samples prepared as described. Samples were diluted 1:50 in DI water and then 5 μl of such a solution were placed on a Copper 400 mesh grid (Electron Microscopy Sciences). After 60 s, 10 μl of distilled water were added to the grid and then excess water was removed. Then, 10 μl of 2% uranyl acetate (Agar Scientific) was placed on the grid and left for 30 s. Finally, two 10 μl-drops of distilled water were added and again excess water removed. The grid was then left to dry. Images were collected using a Phillips CM100 transmission electron microscope (Core Facility for Integrated Microscopy, Denmark)

S.8 Synchrotron radiation circular dichroism (SRCD)

SRCD spectra were collected on the AU-CD beamline at the ASTRID synchroton radiation source (Institute for Storage Ring Facilities, University of Aarhus, Denmark). The light from

the AU-CD beam line was polarized with a MgF_2 Rochon polarizer (B-Halle GmbH, Berlin) and a photo elastic modulator (Hinds, USA) produced alternating left and right handed circular polarized light. The light was then passed though the protein sample ($1\,\mathrm{mg\,ml^{-1}}$) and was detected by a photo multiplier tube (Type 9406B, ETL, UK). Camphor-sulfonic acid served as a calibration material for the instrument. Samples were loaded in a quartz cuvette (121.000 QS, 1 mm path, Hellma Analytics) and data were collected in the range 170-280 nm with a 1-nm step. High transmission voltage (HV) was also monitored. Measurements on buffers and protein samples were performed in duplicate and triplicate, respectively. Spectra were then averaged and smoothed with a Savitzky-Golay filter. Buffer-subtracted spectra of protein samples were normalized by the protein concentration.

S.9 Preparation of giant unilamellar vesicles (GUVs)

1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) GUVs were prepared by electroformation in a solution of 300 mM sucrose following the protocol described in Parolini et al. [1]. To enable fluorescent imaging, DOPC was mixed with 0.8% (molar) Texas Red 1,2-Dihexadecanoyl-sn-Glycero-3-Phosphoethanolamine (DHPE) or Oregon Green 488 DHPE. GUV samples were stored away from light at room temperature and used within a few days.

S.10 Protein labeling and GUV-protein sample preparation

To visualize native-like proteins in confocal microscopy, samples were conjugated with the fluorescent dye Atto 647 N. We prepared a solution with an approximate dye:protein molar ratio of 3:1. The prepared mixture was left under gentle stirring for 2 hours, protected from light. The labelled protein was separated from the unreacted dye by gel permeation chromatography using a Sephadex G-25 prepacked column (GE HealthCare Life Sciences) equilibrated with the same buffer used to prepare the protein samples. The same buffers were employed for the elution, and the conjugated protein solution was stored in the fridge before the final sample preparation. Labeled ALA was then prepared in solution at pH 2, 4 and 7. The final concentration of the labeled ALA solutions was $0.6\,\mathrm{mg\,ml^{-1}}$. These stock solutions were used for studying the interaction of DOPC GUVs.

Interaction of ALA monomers at pH 2, 4, 7 with the GUVs. Samples were prepared into sticky silicon rubber well-plates (each well measuring 6.5 mm × 6.5 mm × mm, Grace Biolabs) adhered onto hydrophobic polyethylene microscope coverslips. Each well was filled with 100 μl of a solution containing: 7 μl of solution containing electroformed GUVs labelled with Oregon Green DHPE, 90 μl of a 303 mM glucose solution, and 3 μl of the protein solutions labeled with Atto 647 N (see above). The final protein concentration was 18 $\mu\mathrm{g\,ml^{-1}}$. The glucose solution was prepared employing the same buffer used to prepare the relevant protein sample at 1 mM concentration, to match the pH (either 2, 4 or 7). Note that glucose is used to balance the osmotic pressure of the sucrose present inside the GUVs. The slight osmolarity excess of the

outer solution causes the vesicles to become slightly deflated (floppy) reducing membrane tension and facilitating the measurement of thermal fluctuation. The mismatch in density between the inside and outside solutions causes the GUVs to sink and settle to the bottom of the chamber, facilitating imaging.

Interaction of ALA prefibrillar species and fibrils with the GUVs. In these cases, 2 μl of GUVs labelled by Texas Red DHPE and 1 μl of the prepared prefibrillar species or fibril sample were added to 97 μl of a 303 mM glucose solution prepared with 1 mM acetate buffer at pH 2. The final prefibrillar species oligomers and fibrils were approximately 16 and 31 μg ml^{-1}, respectively. Bare GUV samples are prepared in a buffer identical to that used for the samples exposed to native-like proteins at pH 2.

S.11 Confocal microscopy

A Leica TCS SP5 confocal microscope, equipped with a HCX PL APO CS 63× 1.4 N.A. oil immersion objective, was used to image GUVs and assess the adhesion of proteins and induced morphological changes. The same machine was used to acquire high framerate videos of the fluctuating GUVs used for flickering spectroscopy. Emission lines at 488 nm and 514 nm from an Ar-Ion laser were used to excite Oregon Green DHPE and Texas Red DHPE respectively. An He-Ne laser emitting at 633 nm was used to excite Atto 647 N labelling the proteins.

S.12 Flickering Spectroscopy

Confocal videos of the GUV equators were recorded for about 90 s at 10-30 fps. A typical video for all the sample-types discussed in the main text is included in the SI (Supplementary Video 1-5, see following section for details). The videos were analyzed using a tailor-made MATLAB software to track the time-dependent contour of the equatorial sections with sub-diffraction precision. A detailed description of the contour-tracking code can be found in Shimobayashi et al. [2]. The squared amplitude of the thermal fluctuation modes was then extracted using Fast-Fourier Transform, and averaged over the entire video to obtain the mean-squared amplitude $\langle |h(q_n)|^2 \rangle$, where is the wave-vector of the n-th mode of equatorial fluctuation and is the length of the equatorial contour. Frames where the contour-tracking algorithm failed, typically due to substantial impurities on the membrane, were excluded from the averaging. To extract the bending modulus κ, $\langle |h(q_n)|^2 \rangle$ was fitted with the model [3]

$$\langle |h(q_n)|^2 \rangle = \frac{k_\mathrm{B}T}{2\ell\sigma}\left(\frac{1}{q_n} - \frac{1}{\sqrt{q_n^2 + \frac{\sigma}{\kappa}}}\right) \tag{S.12.1}$$

where σ is the membrane tension. The fit is perfomed in the n interval between 8 and 18, as lower-q modes are highly affected by the curvature of the membrane (not accounted for by the

theory behind the fitting formula) and higher-q modes are beyond the spatio-temporal resolution of the experimental setup. Figure S.1 shows typical experimental data and fitting curves for the samples discussed in the main text.

S.13 Supplementary Videos

Supplementary Videos 1-5 show examples of movies acquired for flickering spectroscopy measurements. Videos 1-5 are respectively relative to: 1) bare GUVs at pH 2; 2) GUVs exposed to native-like proteins at pH 2; 3) GUVs exposed to native-like proteins at pH 4; 4) GUVs exposed to ALA prefibrillar species; 5) GUVs exposed to ALA fibrils. Note that, as compared to raw data used for the analysis, the Videos have been compressed and the framerate reduced by a factor 10 to reduce file size

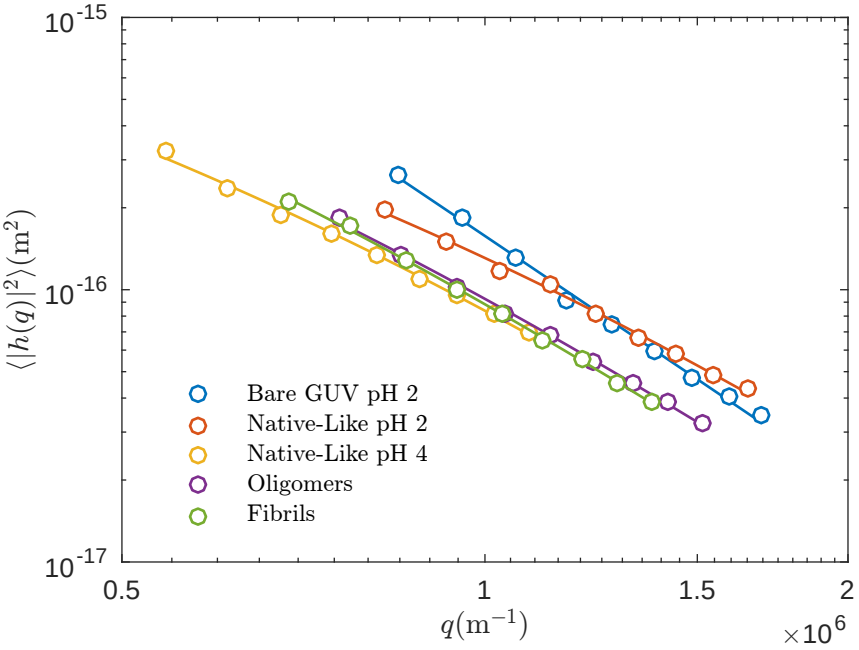

Figure S.1. Examples of fluctuation spectra for the five different samples discussed in the main text. Symbols indicate experimental data, solid lines fits with the model in Eq. S.12.1.

References

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