Liquid-liquid phase separation of type II diabetes-associated IAPP initiates hydrogelation and aggregation

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Short Title: Amylin hydrogelation and phase separation
Supplementary Information Appendix

Supplementary Materials and Methods

Peptides and reagents. Lyophilised synthetic full-length hIAPP, full-length rIAPP, biotinyl full-length hIAPP (biotinylated at the N-terminus), and biotinyl full-length rIAPP (biotinylated at the N-terminus) were purchased (Bachem) already purified by reverse-phase HPLC, and resuspended in DMSO at 512.4, 510.2, 128.1, and 145.9 μM, respectively. To remove any pre-aggregated species, the DMSO stock solution was sonicated and centrifuged for 1 h at 15,000 g at +4°C prior to use. DMSO maintains IAPP in a monomeric pool lacking any β-sheet secondary structures (1). Insulin (Sigma-Aldrich) was resuspended at 349 μM.

Fibrillisation. ThT fluorescence (excitation 450 nm, emission 480 nm) was measured in a 96-well plate (black wall, clear bottom; Greiner Bio-One Ltd), at 37°C on a BMG Polarstar plate reader (BMG labtech) using a bottom-bottom configuration (emission detected from the bottom of the well) and without shaking. Control wells contained ThT, buffers and avidin-TR when required. Control well values were subtracted from test well values. The kinetic parameters were calculated as follows; the lag phase from the intercept on the time axis of the line formed tangent to the inflection point; the elongation rate from the slope at the inflection point of the sigmoidal curve; and the plateau height by averaging the highest curve values attained at the end of the experiment. At least three independent assays were performed. Statistical analysis was performed with the two-sample t-test.

TEM. Plateaued ThT fibrillisation reactions, in absence of avidin fluorophore, were adsorbed onto 400 mesh formvar coated copper grids, incubated with 0.2% fish skin gelatine for 13 min, streptavidin 10 nm gold particles diluted 1 in 5 for 1 h (Sigma-Aldrich), biotin for 10 min (Vector Laboratories Inc), negatively stained with 2% aqueous uranyl acetate, before being viewed with a Tecnai electron microscope (Philips).

LLPS reactions (129 μM hIAPP in PBS) were pipetted as a drop on a coverslip and placed within a humidified chamber. After 90 or 180 min, a 400 mesh formvar coated copper grid was placed on top of the reaction drop and left for 2 min, before being negatively stained with 2% aqueous uranyl acetate and viewed with a Tecnai electron microscope.

LLPS. Different IAPP reactions were investigated: 4, 8,16, 32 and 129 μM hIAPP-100 μM ThS; 129 μM hIAPP-12.89 μM bIAPP-1.26 μM avidin D fluorescein (Vector Laboratories Inc); 129 μM hIAPP-100 μM ThS-12.89 μM bIAPP-1.26 μM avidin D Texas red (Vector Laboratories Inc); 129 μM hIAPP-100 μM ThS-12.89 μM bIAPP-1.26 μM avidin D Texas red in presence of 2.94, 12.9,
42.3 or 141.9 μM insulin; 129 μM rIAPP-12.89 μM brIAPP-1.26 μM avidin D fluorescein. Each experiment was repeated at least three times.

**LLPS image analysis.** Droplet number and mean circle area were measured using the ‘analyze particle’ plugin in FIJI (ImageJ 1.51n) using a background subtraction of 20 and an autothreshold ‘moments dark’. Sphericity measurements were calculated in Imaris (Bitplane). Maximum intensity z-projections were made in Zen black, and 3D projections in Imaris. Colocalisation (fluorescence overlap) was measured using the FIJI plugin JACoP. To determine the mean fluorescence intensity within the droplets, a region of interest (ROI) was assigned manually, in FIJI, to the inside of each droplet (avoiding the droplet surface, i.e. circles were fitted on their phase border) on the DIC images. Then the DIC droplet ROIs were applied on the green or red channel and the mean green fluorescence intensity within the droplet ROI was measured. The violin plots of fluorescence intensity within the droplet ROIs and the difference between mean values, were done using the webtool at https://huygens.science.uva.nl/PlotsOfDifferences. Surface rendering was performed in Imaris, after manual thresholding for background subtraction and Gaussian surface smoothing.

**FRAP.** The flow of IAPP molecules within the droplet, or from the bulk into the droplet, was measured in Zen Black by quantifying the recovery of the bleached area at the cost of the unbleached region. The bleached region was corrected for general photobleaching. The fractions of mobile and immobile molecules were quantified in Zen Black.

**Particle tracking, aggregate speed, aggregate velocity and connectivity.** Different experimental conditions, in H$_2$O or D$_2$O, were tested: 4 μM hIAPP-9.5±0.4x10$^5$ 0.2 μm fluoresbrite carboxylate YG beads (PolySciences Inc); 3.6 μM hIAPP-0.4 μM bIAPP-0.08 μM avidin D-TR and 9.5±0.4x10$^5$ 0.2 μm fluoresbrite carboxylate YG beads; 4 μM hIAPP-5.56 μM BG-BODIPY; 3.6 μM hIAPP-0.4 μM bIAPP-0.08 μM avidin D-TR and 5.56 μM BG-BODIPY. 3D reconstruction was performed in Imaris. Maximum intensity z-projections were made in FIJI. Colocalisation was measured using the FIJI plugin JACoP. Particle tracking was generated without gap closing, setting the minimum length of track segments from first step in a way to disregard particles that are not at AWI, and frame-to-frame linking was set with a search radius less than the intra-particle distance. Static particle tracks were colour coded for time (from green to blue and red). Tracking results (coordinates of tracked particles over time) were exported to matrices for further analysis. The mean aggregate speed corresponds to the average speed of all the tracked particles within the imaging view. The velocity of tracked particles at time $t$ is computed as $\vec{v}(t) = (v_x(t), v_y(t))$ where $v_x = \frac{x(t)-x(t-\Delta t)}{\Delta t}$ and $v_y = \frac{y(t)-y(t-\Delta t)}{\Delta t}$, where $\Delta t$ is the time increment in s. To determine the aggregate connectivity, for any pairs of particles within a distance $d$
= 138.4 nm (50 pixels), we computed the dot product of their velocities as follows: \( \vec{v} \cdot \vec{v}' = v_x v'_x + v_y v'_y \) where \( \vec{v} \) is the first particle velocity and \( \vec{v}' \) is the second particle velocity (the order does not matter). If the dot product is positive (i.e. the angle between the vectors is <90° and therefore the vectors point in similar directions), it means that the particles are moving more or less in the same direction, we thus connect the two particles by a red line. If the dot product is negative (i.e. the angle between the vectors is >90°), it means that the particles are moving more or less in the opposite direction, we thus connect the two particles by a black line instead.

**FLIM.** FLIM images (256×256 pixels) were obtained using an Olympus FV 1000 scanning confocal microscope, with a 10x objective (NA = 0.40), coupled with a Becker & Hickl time-correlated single photon counting card SPC-150. The excitation source was obtained with a pulsed diode laser at 488 nm at 80 MHz (BDL-488-SMC) with a pulse duration of 60-90 ps and a repetition rate of 80 MHz. A cooled HPM100-40 detector (Becker & Hickl) and a 525±25 nm band pass filter were used to collect emission. The acquisition time was 120 s for each image.

An exponential decay was fitted to the fluorescence decay curve in each pixel of the image using the SPCImage software 4.9.7 (Becker & Hickl) and an instrumental response function generated by the fitting software from the rising edge of the decay. Each fluorescence lifetime value was assigned a colour, from red (short lifetime) to blue (long lifetime).

**Supplementary Information References**

1. C. L. Shen, R. M. Murphy, Solvent effects on self-assembly of beta-amyloid peptide. *Biophys J* **69**, 640-651 (1995).
Supplementary Information Figures

Fig. S1. Lower concentrations of hIAPP also undergo LLPS. Merged DIC and ThS labelling (100 μM) (top panels), and DIC (bottom panels) images of hIAPP phase-separated droplets. Scale bar = 5 μm.
**Fig. S2. Insulin as a modulator of hIAPP.** (a) 129 μM hIAPP-12.9 μM bIAPP-1.28 μM avidin TR was incubated in PBS with 100 μM ThS and various concentrations of insulin (2.94, 12.9 or 141.9 μM). hIAPP droplet accumulation and size increase over time (DIC, brIAPP and ThS labelling). Insulin by itself does not undergo LLPS (right panel). (b) hIAPP droplets in presence of 2.94, 12.9 or 141.9 μM insulin fuse (white arrowheads) and relax into larger droplets (black arrowhead). (c) Droplets internal rearrangement of hIAPP molecules with 12.9 μM insulin over time. At different times after LLPS onset, the fluorescence intensity recovery of ThS-hIAPP molecules within a droplet bleached region, and the fraction of mobile and immobile molecules have been quantified. Shown are droplet number analysed from at least three independent replicates. (d) In presence of 12.9 μM insulin, hIAPP molecule exchange between droplets and bulk over time. At different times after LLPS onset, the fluorescence intensity recovery of ThS-hIAPP molecules within the whole bleached droplets, and the fraction of mobile and immobile molecules have been quantified. Shown are the droplet number analysed from at least three independent replicates. (e) 141.9 μM insulin does not prevent hIAPP aggregates development on the droplet surface (top panel) and the formation of an interconnected meshwork of aggregates (bottom panels). Shown are consecutive z slices acquired with the airyscan mode.
Figure a: Images showing the effects of various concentrations of insulin on the fusion of droplets. Controls are shown at the top, and different concentrations of insulin are added at specific time points.

Figure b: Partial FRAP of droplets at different time points, showing the movement and fusion over time.

Figure c: Normalized fluorescence over time, with bars indicating the percentage of mobile and immobile fractions.

Figure d: FRAP of the whole droplet at different time points, showing the fluorescence over time.

Figure e: Images showing the growth of aggregates, with aggregates growing from the droplet surface over time.
Fig. S3. Microscopy experimental setup. Due to the working distance of the 10x objective and the high curvature of the meniscus of the IAPP surface active solution, it was not possible to get a volume close enough to the bottom of the well for imaging without getting the middle part of the meniscus touching the well. By inverting the plate, it was then possible to get a large enough volume into the well to get the AWI at a distance sufficient for the objective without having to worry about the meniscus touching the bottom of the well. (a) A 345 µl hIAPP reaction was pipetted in a well of a 96-well plate (with a total working volume of 360 µl), nearly filling up the well. The well was sealed with grease and a borosilicate glass coverslip. The plate was placed upside down onto the stage plate holder of Zeiss LSM 880 Confocal Microscope, within the controlled chamber set-up at 25°C. The well was imaged with a 10x objective, and images acquired using ZEN operating software. z stacks encompassing the AWI and an area spanning 1417x1417 µm², from the edge of the well, were collected with a 5 µm interval between z slices and with no time gap between stacks for at least 18 h. 3D reconstruction of the z stacks was performed in Imaris v8.4.1, and visualised either with the AWI at the front facing the experimentalist and the bulk solution behind it (b), or the AWI flat viewed from the side with the bulk solution above (c).
Fig. S4: Aggregation and immobility are solely due to hIAPP and not to the fluorophores themselves aggregating. The fluorophores were incubated individually in PBS in a well of a 96-well plate. The well, within the environmentally controlled stage chamber of a Zeiss LSM 880 Confocal Microscope set-up at 25°C, was imaged in confocal mode using a 10 x objective, and images were acquired using ZEN black. Z stacks encompassing the AWI and an area spanning 1417x1417 µm², from the edge of the well, were collected with a 5 µm interval between z slices and with no time gap between stacks for 18 h. 3D projections were performed in Imaris and visualised with the AWI at the front facing the experimentalist and the bulk solution behind it. Presented are snapshots of the Imaris 3D projections for each control at the 18 h time point.
H₂O | D₂O
---|---
0.08 μM avidin D fluorescein
0.08 μM avidin D Texas Red
5.56 μM benzylguanine Bodipy
5.56 μM benzylguanine Bodipy with 0.08 μM avidin D Texas Red
9.5±0.4x10⁵ 0.2 μm green beads
9.5±0.4x10⁵ 0.2 μm green beads with 0.08 μM avidin D Texas Red
Fig. S5. At the AWI, hIAPP aggregates move less and over shorter distances in D$_2$O than in H$_2$O. IAPP, with biIAPP or BG-BODIPY or 0.2 μm fluoresbrite carboxylate YG beads, was incubated in PBS in a well of a 96-well plate. The well was imaged and z stacks encompassing the AWI were collected for at least 18 h. Maximum intensity projections of the time course, tracking of the aggregates and their trajectories were performed as described in Methods. Shown are the trajectories of the aggregates, in H$_2$O or D$_2$O, for several replicates of 3.6 μM hIAPP with 0.4 μM biotinyl IAPP-0.08 μM avidin D-TR (a), or 4 μM hIAPP incubated with 5.56 μM BG-BODIPY (b), or 4 μM hIAPP incubated with 9.5±0.4x10$^5$ 0.2 μm fluoresbrite carboxylate YG beads (c).
Fig. S6. 'Connectedness' between hIAPP aggregates at the AWI - IAPP with BG-BODIPY in H$_2$O. 4 µM hIAPP was incubated with 5.56 µM BG-BODIPY in PBS and H$_2$O in a well of a 96-well plate. The well was imaged, z stacks encompassing the AWI collected for at least 18 h, maximum intensity projections of the time course and tracking of the aggregates performed as described in Methods. From the aggregate tracking, movement vectors and connectivity between aggregates were determined. In (a) are shown the Image J-derived maximum intensity projections of relevant time points of an 18 h time course (top panels), the connectivity between aggregates (a red line depicts aggregates moving in the same direction, i.e. connectedness; and a black line depicts aggregates moving in opposite direction, i.e. non-connectedness)(middle panels), and the movement vectors (bottom panels). (b) shows one example of the evolution of the connectivity between aggregates over relevant time points. The numbers indicate individual clusters of connected aggregates that evolve over time.
Fig. S7. 'Connectedness' between hIAPP aggregates at the AWI - bIAPP with avidin D fluorescein in D₂O. 3.6 μM hIAPP was incubated with 0.04 μM bIAPP-0.08 μM avidin D fluorescein in PBS and D₂O in a well of a 96-well plate. In (a) are shown the Image J-derived maximum intensity projections of relevant time points of an 18 h time course (top panels), the connectivity between aggregates (a red line depicts aggregates moving in the same direction, i.e. connectedness; and a black line depicts aggregates moving in opposite direction, i.e. non-connectedness)(middle panels), and the movement vectors (bottom panels). (b) shows one example of the evolution of the connectivity between aggregates over relevant time points. The numbers indicate individual clusters of connected aggregates that evolve over time.
Fig. S8. 'Connectedness' between hIAPP aggregates at the AWI - IAPP with 0.2 μm beads in D₂O. 4 μM hIAPP was incubated with 9.5±0.4x10⁵ 0.2 μm fluoresbrite carboxylate YG beads in PBS and D₂O in a well of a 96-well plate. In (a) are shown the Image J-derived maximum intensity projections of relevant time points of an 18 h time course (top panels), the connectivity between aggregates (a red line depicts aggregates moving in the same direction, i.e. connectedness; and a black line depicts aggregates moving in opposite direction, i.e. non-connectedness)(middle panels), and the movement vectors (bottom panels). (b) shows one example of the evolution of the connectivity between aggregates over relevant time points. The numbers indicate individual clusters of connected aggregates that evolve over time.
Fig. S9: The fluorescence lifetime of BG-BODIPY, associated with the hIAPP adsorbed layer at the AWI, increases with time, with the increase being higher and more homogeneous in D$_2$O than H$_2$O. (a) FLIM image of 5.56 μM BG-BODIPY incubated in PBS for 19 h at 25°C. (b) Typical histograms of lifetime obtained for the AWI region of 4 μM hIAPP incubated with 5.56 μM BG-BODIPY, in H$_2$O or D$_2$O, at 25°C for 5 or 21 h. (c) Typical decay traces obtained for the AWI region of 4 μM hIAPP incubated with 5.56 μM BG-BODIPY, in H$_2$O or D$_2$O, at 25°C for 5 or 21 h. The curve fitting to the decay traces is shown by a black line.
**Fig. S10. Model for initiation of IAPP LLPS, droplet hydrogelation, aggregation, and eventually percolation of the whole system.** (a) In a reaction drop, disordered amphiphilic IAPP precursors rapidly adsorb to the AWI. This promotes a local concentration increase, peptide chain alignment, and a change into a $\beta$-sheet conformation (conf.). Further AWI adsorption (ads.) increases sufficiently the concentration of $\beta$-sheet precursors into localised ‘pockets’ to trigger LLPS. Droplet fusion occurs and the AWI-catalysed LLPS then spreads into the bulk. Eventually the phase-separated liquid droplets undergo hydrogelation before fibrillar aggregation occurs at their surface. The blue double line arrows indicate the processes/steps undertaken by rIAPP, however these are slower than that of hIAPP. The red arrows indicate the processes/steps delayed by insulin. (b) At the onset of LLPS, recruitment of hIAPP molecules from the bulk is permitted until an impermeable coat forms at the droplet surface, by recruitment of $\beta$-sheet precursors, precludes it. Further precursor recruitment to the droplet surface, from within the droplets, leads to saturation and formation of aggregation-prone stacked $\beta$-sheet species (green, ThS positive). Propagation of stacked $\beta$-sheet species leads to hydrogelation within the droplets. Bulk precursors recruited to the droplet surface undergo a conformational change into $\beta$-sheets, rapidly incorporates into the pre-existing stacked $\beta$-sheets, and promote aggregation on the droplet surface. Aggregation then spreads to create a fibrillar network interconnecting droplets and aggregates, which grows to form aggregate clusters. These clusters provide a microenvironment to spatially organise aggregates and increase their local concentration, which supports further aggregation. Eventually, clusters fuse, fibril entanglement occurs to create a fibrillar meshwork, which leads to percolation of the whole system. The blue double line arrows indicate the processes/steps undertaken by rIAPP, however these are slower than that of hIAPP. The red arrows indicate the processes/steps delayed by insulin.
Movies

Movie 1. Liquid-liquid phase separation of hIAPP. A drop of a 129 µM hIAPP reaction, in a 24.81 µl reaction volume, was pipetted in an uncoated glass bottom MatTek dish. The dish was placed within the environmentally controlled stage chamber of a Zeiss LSM 880 Airyscan Confocal Microscope set-up at 25°C. LLPS was visualised over time by acquiring images using ZEN operating software and a 63x oil objective.

The AWI (outer edge of the reaction drop) is on the left-hand side of the video. The arrowheads indicate droplets that will undergo fusion with one another.

Movie 2. hIAPP droplet fusion. A drop of a 129 µM hIAPP reaction in PBS was placed on a MatTek dish and imaged.

The AWI (outer edge of the reaction drop) is on the left-hand side of the video. The arrowheads indicate droplets that will undergo fusion with one another.

Movie 3. hIAPP liquid-liquid phase separation is catalysed by the AWI. A drop of a reaction containing 129 µM hIAPP, 100 µM ThS, 12.89 µM bIAPP, 1.28 µM avidin-TR, in PBS was placed on a MatTek dish and imaged.

The AWI (outer edge of the reaction drop) is on the left-hand side of the video.

Movie 4. LLPS of rIAPP. A drop of a 129 µM rIAPP-12.89 µM brIAPP-1.28 µM avidin fluorescein reaction, in a 24.81 µl reaction volume, was pipetted in an uncoated glass bottom MatTek dish.

The AWI (outer edge of the reaction drop) is on the left-hand side of the video. The arrowheads indicate droplets that will undergo fusion with one another.

Movie 5. Insulin delays hIAPP LLPS. A drop of a 129 µM hIAPP-12.89 µM bIAPP-1.28 µM avidin-TR-100 µM ThS reaction in PBS and 42.3 µM insulin was placed on a MatTek dish and imaged.

The AWI (outer edge of the reaction drop) is on the left-hand side of the video. The arrowheads indicate droplets that will undergo fusion with one another.

Movie 6. hIAPP aggregation at the AWI - bIAPP with avidin D fluorescein in H2O. 3.6 µM hIAPP was incubated with 0.4 µM bIAPP-0.08 µM avidin D fluorescein in H2O in a well of a 96-well plate. The well was imaged, z stacks encompassing the AWI and an area spanning 1417x1417 µm² were collected for at least 18 h. 3D projections were performed in Imaris and visualised with the AWI at the front facing the experimentalist and the bulk solution behind it.
Movie 7. hIAPP aggregation at the AWI - bIAPP with avidin D fluorescein in D$_2$O. hIAPP aggregates accumulate at the AWI, forming ‘clusters’ that become immobile. 3.6 μM hIAPP was incubated with 0.4 μM bIAPP-0.08 μM avidin D fluorescein in D$_2$O in a well of a 96-well plate. The well was imaged, z stacks encompassing the AWI and an area spanning 1417x1417 μm$^2$ were collected for at least 18 h. 3D projections were performed in Imaris and visualised with the AWI at the front facing the experimentalist and the bulk solution behind it.

Movie 8. hIAPP aggregation at the AWI - BODIPY in H$_2$O. 4 μM hIAPP was incubated with 5.56 μM BG-BODIPY in H$_2$O in a well of a 96-well plate. The well was imaged, z stacks encompassing the AWI and an area spanning 1417x1417 μm$^2$ were collected for at least 18 h. 3D projections were performed in Imaris and visualised with the AWI at the front facing the experimentalist and the bulk solution behind it.

Movie 9. hIAPP aggregation at the AWI - BODIPY in D$_2$O. 4 μM hIAPP was incubated with 5.56 μM BG-BODIPY in D$_2$O in a well of a 96-well plate. The well was imaged, z stacks encompassing the AWI and an area spanning 1417x1417 μm$^2$ were collected for at least 18 h. 3D projections were performed in Imaris and visualised with the AWI at the front facing the experimentalist and the bulk solution behind it.

Movie 10. hIAPP aggregation at the AWI - beads in H$_2$O. 4 μM hIAPP was incubated with 9.5±0.4x10$^5$ 0.2 μm fluoresbrite carboxylate YG beads in H$_2$O in a well of a 96-well plate. The well was imaged, z stacks encompassing the AWI and an area spanning 1417x1417 μm$^2$ were collected for at least 18 h. 3D projections were performed in Imaris and visualised with the AWI at the front facing the experimentalist and the bulk solution behind it.

Movie 11. hIAPP aggregation at the AWI - beads in D$_2$O. 4 μM hIAPP was incubated with 9.5±0.4x10$^5$ 0.2 μm fluoresbrite carboxylate YG beads in D$_2$O in a well of a 96-well plate. The well was imaged, z stacks encompassing the AWI and an area spanning 1417x1417 μm$^2$ were collected for at least 18 h. 3D projections were performed in Imaris and visualised with the AWI at the front facing the experimentalist and the bulk solution behind it.

Movie 12. hIAPP aggregation spreads into the bulk solution. 4 μM hIAPP was incubated with 9.5±0.4x10$^5$ 0.2 μm fluoresbrite carboxylate YG beads in H$_2$O, in a well of a 96-well plate. The well was imaged, z stacks encompassing the AWI and an area spanning 1417x1417 μm$^2$ were collected for
at least 18 h. 3D projections were performed in Imaris and visualised with the AWI flat viewed from the side with the bulk solution above.