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Influence of a Curcumin Derivative on hIAPP Aggregation in the Absence and Presence of Lipid Membrane

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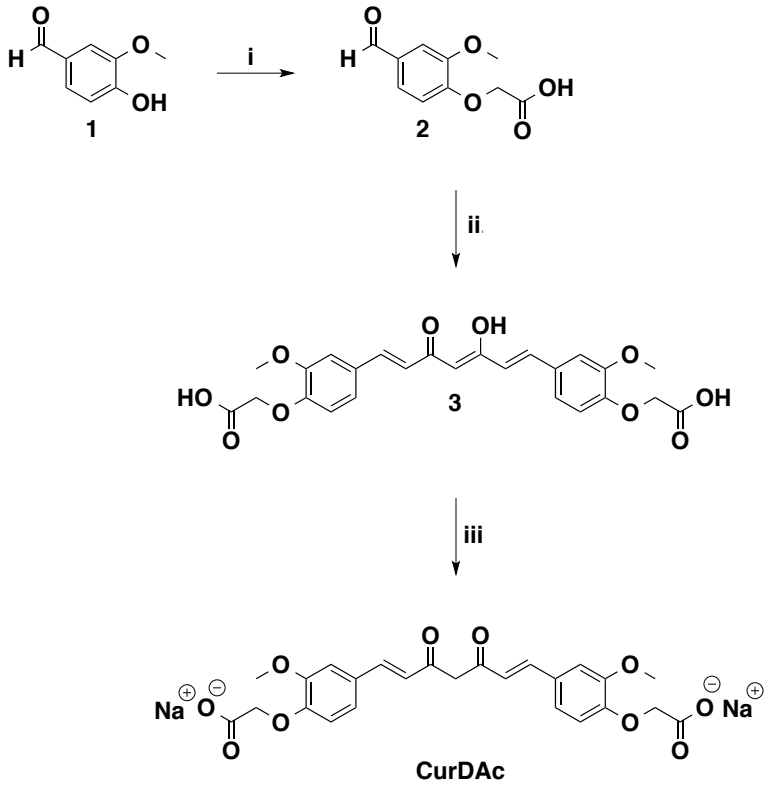
MATERIALS AND METHODS

Vanillin, 2,4-pentadione and n-butylamine were purchased from Sigma Aldrich. Tri-n-butyl borate was purchased from Alfa Aesar. All compounds were used without further purification. NMR characterizations for synthetic intermediates and **CurDAc** were performed using a Bruker Avance 300 MHz spectrometer. Mass spectra were recorded on a LCQ Fleet Mass Spectrometer (Thermo-Fisher Ltd., U.S.A.)

Synthesis of CurDAc



Reagent Conditions: (i) $ClCH_2COOH$; 1 N NaOH; H_2O; reflux, 12 h. (ii) 2,4-pentadione; boron oxide; tri n-butyl borate; n-butylamine; DMF; 70 ^{O}C, 4 h. (iii) ACN, 1 N NaOH, 0 ^{O}C, 5 min.

Synthetic procedure for 2-(4-formyl-2-methoxyphenoxy)acetic acid ***(2):***[1]

The vanillin (1.0g, 0.0065 mol) was dissolved in 1 N NaOH (16 mL) solution. The solution was refluxed with chloroacetic acid (0.68g, 0.0072 mol) for 12 hrs. Then the reaction mixture was diluted with water (10 mL) washed with ethyl acetate (2 x 25 mL), then the aqueous layer was separated and acidified with conc HCl. Then the mixture was extracted with ethyl acetate (3 x 50 mL) (in the ethyl acetate layer the product was dissolved along with unreacted vanillin). The combined organic layers were

extracted with saturated sodium bicarbonate solution (2 x 50 mL) and separated. The sodium bicarbonate solution was acidified with conc. HCl which gave the product as a white precipitate. The product was subsequently filtered and dried in hot air oven at 60 °C for 24 hrs (yield: 86.2%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.86 (s, 1H), 7.44 – 7.42 (m, 2H), 6.92 (d, *J* = 8.7 Hz, 1H), 4.76 (s, 2H), 3.95 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$+DMSO-D_6) δ 190.52, 169.57, 152.52, 130.57, 125.77, 112.14, 109.79, 65.39, 55.74. ESI-MS calculated. m/z 210.04. found 209.03 (M^--1).

Synthetic procedure for 2,2'-((((1E,6E)-3,5-dioxohepta-1,6-diene-1,7-diyl)bis(2-methoxy-4,1-phenylene))bis(oxy))diacetic acid ***(3):***[1]

To a 2,4-pentadione (0.099 mL, 0.95 mmol), boron oxide (0.065 g, 0.95 mmol), tri-n-butyl borate (0.50 mL, 1.89 mmol) and vanillin acetic acid (0.4g, 1.89 mmol) were added. The mixture was dissolved in dimethylformamide (2 mL) and heated to 70 °C. When the reaction temperature reaches 70 °C, n-butylamine (0.096 mL, 0.95 mmol) was added drop wise over 5 min. Then the reaction mixture was stirred at 70 °C for 4 hrs. Then the reaction mixture was added to 5% hot acetic acid solution (4 mL) and stirred for 3 hrs. It was diluted with water (25 mL) and extracted with ethyl acetate (2 x 50 mL). The combined organic layers were again washed with water (50 mL) and extracted with 10% sodium bicarbonate solution (2 x 25 mL). The aqueous layer was separated and neutralized with 2 N HCl (the pH of the solution should be 6.0-6.5). The yellow precipitate obtained was filtered and dried in vacuum. The filtered solid was washed with ethyl acetate afforded product **3** as a yellow solid (yield: 62.5%). ^{1}H NMR (300 MHz, DMSO-D_6) δ 7.64 (d, *J* = 15.6 Hz, 2H), 7.43 (s, 2H), 7.29 (d, *J* = 7.8 Hz, 2H), 7.96 – 6.86 (m, 4H), 6.18 (s, 1H), 4.79 (s, 4H), 3.90 (s, 6H). ESI-MS calculated. m/z 484.14. found 485.04 (M^++1).

Synthetic procedure for sodium 2,2'-((((1E,3Z,6E)-3-hydroxy-5-oxohepta-1,3,6-triene-1,7-diyl)bis(2-methoxy-4,1-phenylene))bis(oxy))diacetate ***(CurDAc)***

The compound **3** (0.1 g, 0.20 mmol) was taken in the round bottom flask and cooled to 0 °C. To that solid 1 N NaOH (0.413 mL) was added and stirred for 5 min at 0 °C. Then to that solution acetonitrile (3mL) was added, the precipitate was obtained as

the free acid. The product was filtered and dried in vacuum to give the sodium salt as a reddish brown solid (yield: 90.1%). ^{1}H NMR (300 MHz, D_2O) δ 7.13 (d, *J = 15.8 Hz*, 2H), 6.87 (d, *J = 8.0 Hz*, 2H), 6.81 (s, 2H), 6.57 (d, *J = 8.0 Hz*, 2H), 6.24 (d, *J = 15.8 Hz*, 2H), 4.29 (s, 4H), 3.74 (s, 6H). ESI-MS calculated. m/z 528.10. found 529.25 (M^++1).

Peptide Preparation. To remove preformed aggregates, hIAPP was dissolved in hexafluoroisopropanol (HFIP) followed by removal of the solvent by lyophilization for 48 hours in aliquots of either 0.1 - 0.3 mg. The peptide aliquots were then stored at -20 ^{O}C. hIAPP monomer sample was prepared by dissolving the lyophilized peptide in chilled, dilute HCl (pH 4) to stock concentration between 150 – 200 μM, kept at 0 ^{O}C (ice), vortexed for 15 s. and bath sonicated (at 0 ^{O}C) for 1 min. Fresh hIAPP monomer samples were diluted into the appropriate buffer for each subsequent experiment.

Bicelle and Vesicle Preparation: All lipids were purchased from Avanti Polar Lipids (Alabaster, AL). DMPC/DHPC (2:1) bicelles were prepared by lypholizing the lipid film overnight. Lipids were rehydrated in phosphate buffered saline (10 mM, 100 mM NaCl, pH 7.4) and vortexed at 4 ^{O}C until lipids were completely dissolved. Lipid solutions underwent five freeze-thaw cycles to generate bicelles.[2] Lipid vesicles were prepared by rehydration and micro-extrusion through a 100 nm membrane 21 times[2]. Vesicles were measured by dynamic light scattering (DLS) to confirm their size (data not shown). Dye-filled vesicles were prepared at 10 mg/mL in a similar fashion, however rehydrated with a 6-carboxyfluorescein solution. After extrusion, dye-filled vesicles were separated on a size-exclusion column (GE Sephadex G50) to remove any free carboxyfluorescein. The concentration of dye-filled vesicles was measured using the Stewart assay.[3]

Thioflavin T (ThT) Fluorescence Assays. ThT fibril formation was measured by increased fluorescence emission upon binding of amyloid fibers to the commonly used amyloid-specific dye, ThT.[4] ThT was added to a chilled phosphate buffered saline (10 mM phosphate, 100 mM NaCl, pH 7.4) to make a final dye concentration of 20 μM. For experiments without lipids, **CurDAc** (10 μM) was added. To solutions containing bicelles and vesicles, lipids were added to a final concentration of 100 - 1000 μM. hIAPP monomer solution was added to each well to a final peptide concentration of 10 μM. Fluorescence emission was measured in microplate formate on a BioTek Synergy 2

microplate spectrofluorometer using excitation and emission wavelengths of 440 and 480 nm, respectively.

Dye Leakage Fluorescence Assay: POPC/POPG (7:3) vesicles were prepared with 6-carboxyfluorescein as describe above. The final concentration of dye-filled vesicles used in each experiment was 0.2 mg/mL. Vesicles were added to a solution of PBS (10 mM, 100 mM NaCl, pH 7.4) and to this **CurDAc** (10 μM) was added. An initial fluorescence scan (λ_{ex} = 490 nm; λ_{em} = 520 nm) was taken to ensure no free dye was present in solution. To each well hIAPP (10 μM) was then added. After 24 h, vesicles were treated with 0.1% Triton-X to burst the vesicles for the maximum fluorescence measurement. Control fluorescence scans (λ_{ex} = 490 nm) were run in the presence of 0.2 mg/mL carboxyfluorescein and 10 μM **CurDAc** to demonstrate no signal overlap between the dye and the small molecule.

Circular Dichroism (CD). CD measurements were performed on JASCO J-815 Spectropolarimeter using a 0.1 cm path length cell. Freshly prepared monomer solution was added to PBS (10 mM, 100 mM NaF, pH 7.4) at a final concentration of 20 μM. Samples containing **CurDAc** were co-incubated with 20 μM compound. Molar CD per residue values were calculated using $\varepsilon = \theta_{obsd}/(3298lcn)$, where θ_{obsd} is the observed ellipticity measured in millidegrees, c is the molar concentration, l is the cell path length in centimeters, and n is the number of residues in the peptide.

Dynamic Light Scattering (DLS). Light scattering experiments were performed on hIAPP (25 μM) and hIAPP with **CurDAc** (25 μM) using a DynaPro Nanostar instrument from Wyatt Technology (Santa Barbara, CA). Light scattering was measured at 90°. The intensity correlation function and the distribution of the hydrodynamic radii (R_{hyd}) of the particles contributing to the scattering were determined using DYNAMICS software (Wyatt Technology).

Transmission Electron Microscopy (TEM). Samples for negative stain TEM analysis were deposited on continuous carbon films on copper rhodium 100 mesh grids (Electron Microscopy Sciences, EMS Hatfield PA.). Prior to adding samples, the grids were charged using a glow discharger for 15 s at 30 mA negative discharge. Aliquots from ThT experiments incubated for 4 h were adsorbed to the grids for 2 min prior to rinsing with two 10 μL drops of water for 10 s. Samples were blotted using No. 2 Whatman filter

paper. Samples for TEM were then stained with a 10 μL drop of freshly filtered 2% uranyl acetate (EMS) for 15s before blotting excess stain. Samples were analysed using a Philips CM-100 microscope operating at 80 kV.

Saturation Transfer Difference Nuclear Magnetic Resonance (STD NMR).[5] Saturation transfer difference (STD) NMR experiments for a sample containing **CurDAc** (250 μM) and hIAPP monomers (25 μM) were carried at 25 ºC for 18 hours consecutively with an interval of 15 min using a Bruker 600 MHz NMR spectrometer equipped with a cryo probe. The peptide, hIAPP or its fibril was RF-irradiated at -1 (on-resonance) and at 40 ppm (off-resonance) for a duration of 2 s and 128 scans were co-added to get the spectrum. The selective RF-irradiation was achieved by a train of Gaussian pulses with 1% truncation for a duration of 49 ms with an interval of 1 ms at 50 dB.

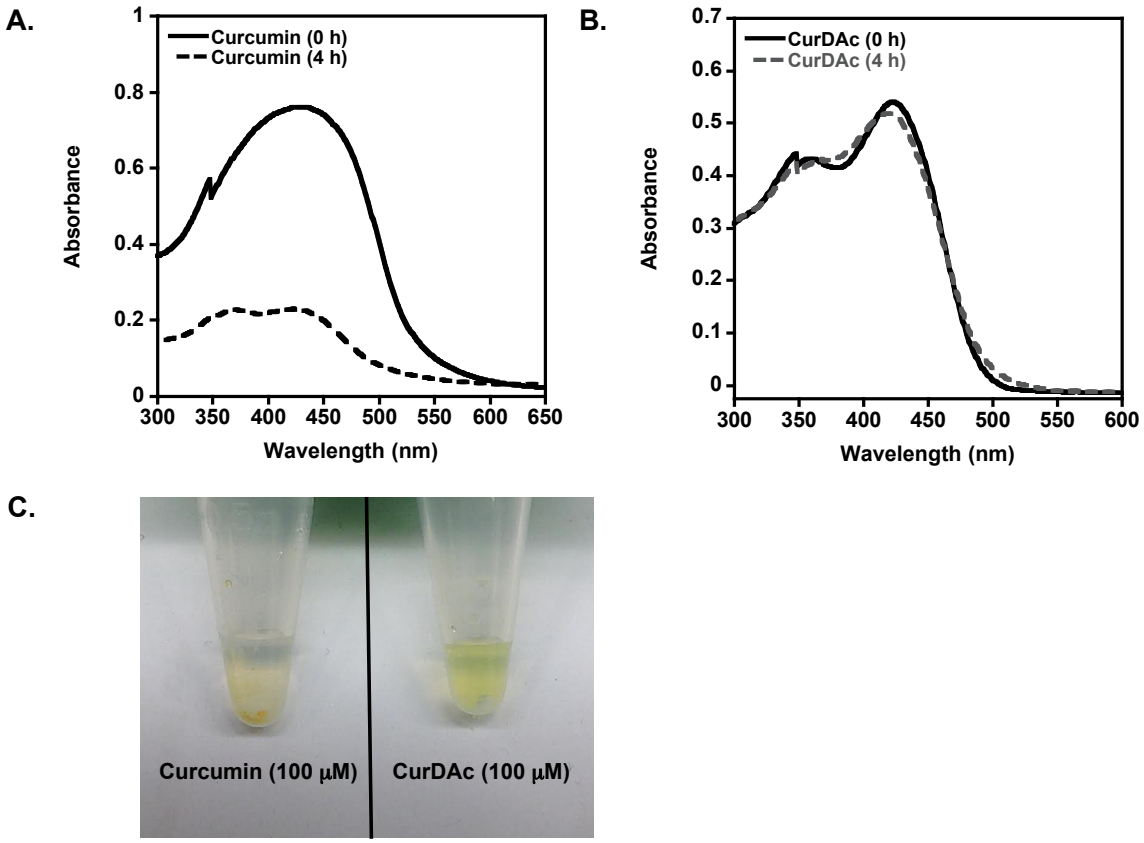


Fig. S1: UV-Vis spectra of A) curcumin and B) **CurDAc** (right). C) 100 μM curcumin (left) and 100 μM **CurDAc** (right). A solid pellet is formed from insoluble curcumin in water after centrifugation. Under identical conditions, **CurDAc** remains as a homogenous solution.

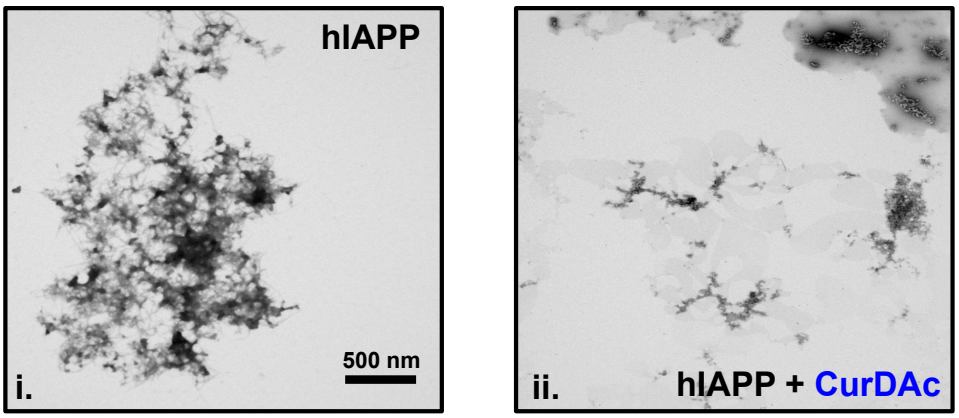


Fig. S2: Transmission electron micrographs of hIAPP (10 μM) alone (i) and incubated with **CurDAc** (ii). Samples visualized after 4 h. Upon incubation with **CurDAc** (10 μM), smaller, more amorphous hIAPP species are visible by TEM, unlike the fibril network seen with peptide alone.

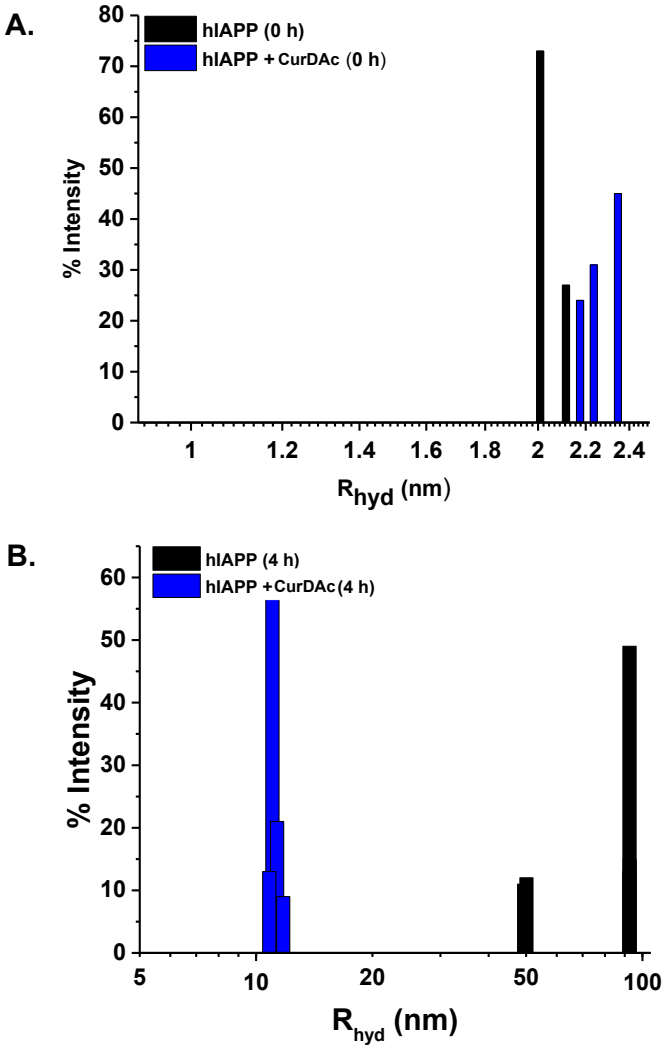


Fig. S3: Dynamic light scattering (DLS) graphs representing hydrodynamic radius (r_H) of hIAPP (black, 25 μM) and hIAPP with **CurDAc** (blue, 25 μM) at 0 h (a) and 4 h (b). Possible small hIAPP oligomer assemblies (r_H = 10 nm) are detected by DLS in the presence of **CurDAc** whereas larger oligomeric or protofibrillar aggregates are seen for peptide alone after 4 h.

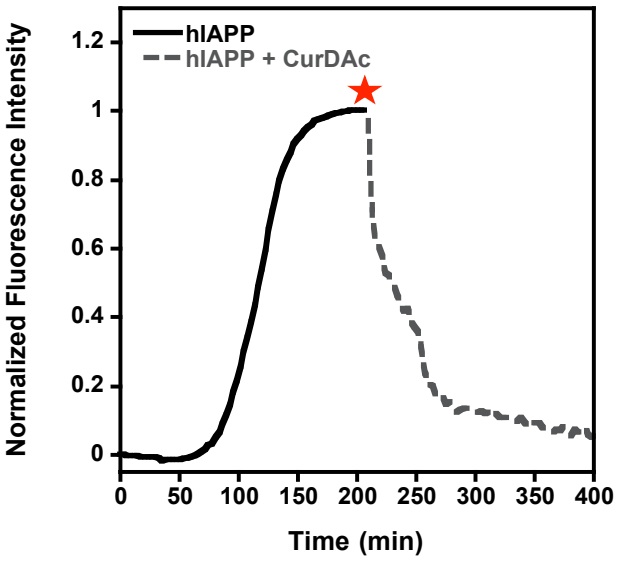


Fig. S4: ThT assay used to measure the disaggregation of preformed hIAPP (10 μM, black) fibers with **CurDAc** (10 μM, grey). The red star indicates the time when **CurDAc** was added. As discussed in the main text, these results indicate that **CurDAc** can depolymerize preformed hIAPP fibrils in solution over time.

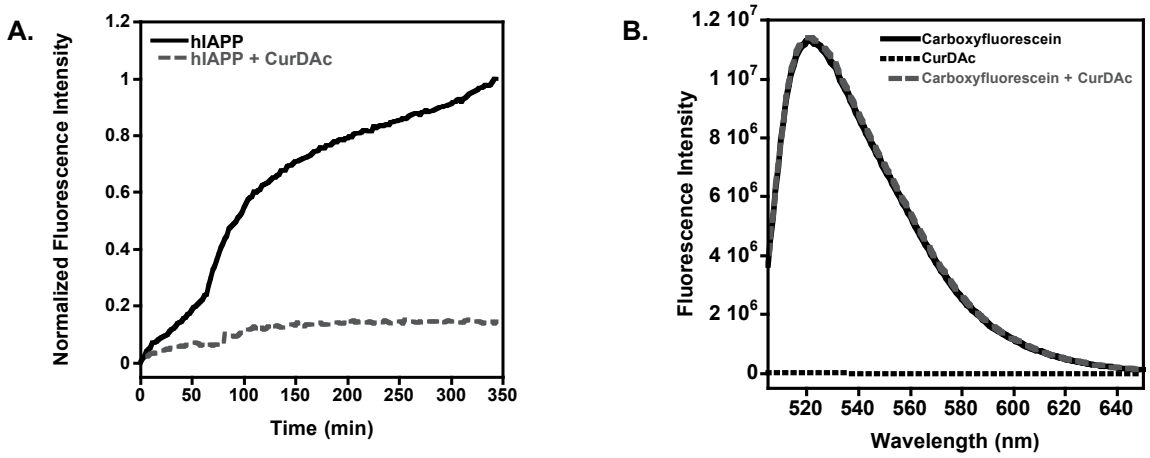


Fig. S5. Dye leakage assay of POPC:POPG (7:3) LUVs (0.2 mg/mL) encapsulated with 6-carboxyfluorescein. hIAPP alone (black, 10 μM) shows an increase in dye release which is mitigated when **CurDAc** is present (grey, 10 μM). There is no overlap in the fluorescence signal when **CurDAc** is present (B) indicating no interference from the small molecule.

References

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