Supporting Information

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SI Materials and Methods

Materials. The 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3 phospho-(1′-*rac*-glycerol) (DOPG), total brain extract lipids (TBE-lipids), and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) were obtained from Avanti Polar Lipids. Carboxyfluorescein, DMSO, thioflavin-T (ThT), cholesterol, and hexafluoroisopropanol (HFIP) were obtained from Sigma-Aldrich.

Peptide Synthesis and Purification. Human islet amyloid polypeptide (IAPP), rat IAPP, and the mutants were synthesized on a 0.25 mmol scale with a CEM microwave peptide synthesizer, using 9-fluornylmethoxycarbonyl (Fmoc) chemistry. Fmoc-protected pseudoproline dipeptide derivatives were incorporated to facilitate the synthesis (1, 2). The 5-(4'-fmoc-aminomethyl-3′, 5-dimethoxyphenol) valeric acid resin was used to generate an amidated C terminus. Double couplings were performed for the pseudoprolines, the residues following each pseudoproline, Arg, and all of the β-branched residues. Peptides were cleaved from the resin through the use of standard trifluoroacetic acid (TFA) methods. Crude peptides were dissolved in 20% acetic acid (vol/vol), frozen in liquid nitrogen, and lyophilized to increase their solubility before additional work-up. The disulfide bond was formed via oxidation in DMSO (3). The peptides were purified by reverse-phase HPLC using a Vydac C18 preparative column (10 mm × 250 mm). A two-buffer system was used: buffer A consists of 100% H_2O and 0.045% HCl (vol/vol), and buffer B comprises 80% acetonitrile, 20% H_2O, and 0.045% HCl (vol/vol). HCl was used as the ion-pairing agent, as residual TFA can cause problems with cell toxicity assays and has been shown to influence the aggregation kinetics of some IAPP-derived peptides (4). The identity of the pure products was confirmed by mass spectrometry using a Bruker MALDI-TOF MS: human IAPP, expected 3,903.3, observed 3,903.1; rat IAPP, expected 3,921.3, observed 3,921.6; 3XL-IAPP, expected 3,785.2, observed 3,785.9; I26P-IAPP, expected 3,887.2, observed 3,887.3; double mutant (DM)-IAPP, expected 3,927.3, observed 3,927.1; pramlintide (PM), expected 3,949.4, observed 3,949.7; H18R-PM, expected 3,968.4, observed 3,968.1; and *N*-methyl–IAPP, expected 3,931.3, observed 3,931.1. Analytical HPLC was used to check the purity of the peptides before each experiment. This is an important control because deamidation can be a complicating factor with studies of IAPP amyloid formation (5, 6).

Sample Preparation. Peptide stock solutions were prepared by dissolving peptides at 1.6 mM in 100% HFIP and incubating for at least 6 h. Aliquots of the stock solutions were freeze dried to remove organic solvents and redissolved in 20 mM Tris·HCl, 100 mM NaCl, pH 7.4, buffer at the desired concentration.

Preparation of LUVs. The large unilamellar vesicles (LUVs) were prepared from a 75:25 mixture of DOPC and DOPG, or 100% DOPG, or 100% TBE-lipids or a mixture of DOPC, DPPC, and cholesterol at 1:2:1. Stock solutions were dissolved in chloroform in a glass tube, evaporated with a stream of nitrogen gas, and then were dried under a vacuum overnight to completely remove the residual organic solvent. The resulting lipid film was hydrated in 20 mM Tris·HCl and 100 mM NaCl, pH 7.4, buffer for 1 h. The multilamellar vesicles were then subjected to 10 freeze-thaw cycles and extruded 15 times through 100 nm pore size filters (Whatman, GE). The phospholipid concentration was determined by the method of Stewart (7). For the dye leakage experiments, carboxyfluorescein-containing LUVs were prepared using the same protocol except that the dried lipid film was rehydrated with a buffer containing 70 mM carboxyfluorescein, 20 mM Tris·HCl, and 100 mM NaCl, pH 7.4. Nonencapsulated carboxyfluorescein was removed from carboxyfluorescein-filled vesicles via size exclusion chromatography using a PD-10 column (GE Healthcare Life Sciences) and elution with 20 mM Tris·HCl 100 mM NaCl buffer, pH 7.4. A fresh vesicle solution was used for each experiment.

ThT Fluorescence Assays. Fluorescence experiments were performed using a Beckman model D880 plate reader. An excitation wavelength of 430 nm and emission wavelength of 485 nm were used. Samples were incubated at 25 °C in 96-well plates. Experiments in the presence of membranes were initiated by adding a solution of 32 μM ThT, 400 μM, or 0.32mg/mL LUVs, 20 mM Tris·HCl, and 100 mM NaCl, pH 7.4, to dry lyophilized peptide. Each experiment was repeated three times using different IAPP stock solutions.

Membrane Leakage Experiments. An Applied Phototechnology fluorescence spectrophotometer was used for the leakage experiments. Aliquots of peptide stock solutions were lyophilized and then redissolved in a mixture of carboxyfluorescein-filled LUVs, 20 mM Tris·HCl, and 100 mM NaCl, pH 7.4, to the final desired peptide concentration (2–60 μM). Immediately after resolubilization, the cuvette was gently shaken for 3 s. Fluorescence was measured using an excitation wavelength of 492 nm and an emission wavelength of 517 nm. The excitation and emission slits were set at 2 nm. Peptide was incubated with vesicles for 10 min and for 48 h at 25 °C. For each experiment, the baseline fluorescence of the carboxyfluorescein-filled LUVs was measured, and the maximum leakage induced by total disruption of the lipid vesicles was determined by the addition of Triton X-100 to a final concentration of 0.2%. The percent leakage of the dye is calculated as:

$$\text{Percentage leakage} = 100 \times (\text{Ft} - \text{Fbaseline})/(\text{Fmax} - \text{Fbaseline}).$$

All of the leakage experiments were repeated three times using different IAPP stock solutions. All values represent means ± SEM ($n = 3$).

CD. Aliquots of peptide stock solutions were lyophilized and then redissolved in a solution of 400 μM or 0.32 mg/mL LUVs, 20 mM Tris·HCl, and 100 mM NaCl, pH 7.4, to 60 μM IAPP. CD experiments were performed using an Applied Photophysics Chirascan CD spectrometer and a 0.1 cm quartz cuvette. Spectra were recorded at 25 °C, over a range of 190–260 nm, at 1 nm intervals with an averaging time of 0.5 s, and are the result of three repeats. Background spectra (buffer and LUVs without peptides) were subtracted from the collected data.

Transmission Electron Microscopy (TEM). TEM was performed at the Life Science Microscopy Center at Stony Brook University. TEM samples were prepared from the same solutions used for the CD experiments. We blotted 15 μL of peptide and LUV solution on a carbon-coated Formvar 300 mesh copper grid for 1 min and then negatively stained it with saturated uranyl acetate for 1 min.

Cell Culture. Transformed rat insulinoma-1 (INS-1) pancreatic β cells were grown in RPMI 1640 supplemented with 10% FBS,

11 mM glucose, 10 mM Hepes, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol, 100 U/mL penicillin, and 100 U/mL streptomycin. Cells were maintained at 37 °C under 5% CO_2.

AlamarBlue Cell Viability Assays. Cytotoxicity was measured by AlamarBlue reduction assays. INS-1 β cells were seeded at a density of 30,000 cells per well in 96-well plates and cultured for 24 h before stimulation with wild-type and mutant IAPP peptides. Peptides were dissolved and incubated in 20 mM Tris·HCl (pH 7.4, 25 °C) before addition to cells. Peptide solutions were incubated on cells for 5 h, followed by 5 h cell incubation with 10% AlamarBlue in culture media. Fluorescence (excitation, 530; emission, 590 nm) was measured on a Beckman Coulter DTX880 fluorescent plate reader. Values were calculated relative to those of control cells treated with buffer only. All values represent means ± SEM (n = 3).

1. Abedini A, Raleigh DP (2005) Incorporation of pseudoproline derivatives allows the facile synthesis of human IAPP, a highly amyloidogenic and aggregation-prone polypeptide. *Org Lett* 7(4):693–696.
2. Marek P, Woys AM, Sutton K, Zanni MT, Raleigh DP (2010) Efficient microwave-assisted synthesis of human islet amyloid polypeptide designed to facilitate the specific incorporation of labeled amino acids. *Org Lett* 12(21):4848–4851.
3. Abedini A, Singh G, Raleigh DP (2006) Recovery and purification of highly aggregation-prone disulfide-containing peptides: Application to islet amyloid polypeptide. *Anal Biochem* 351(2):181–186.
4. Nilsson MR, Raleigh DP (1999) Analysis of amylin cleavage products provides new insights into the amyloidogenic region of human amylin. *J Mol Biol* 294(5):1375–1385.
5. Nilsson MR, Driscoll M, Raleigh DP (2002) Low levels of asparagine deamidation can have a dramatic effect on aggregation of amyloidogenic peptides: Implications for the study of amyloid formation. *Protein Sci* 11(2):342–349.
6. Dunkelberger EB, et al. (2012) Deamidation accelerates amyloid formation and alters amylin fiber structure. *J Am Chem Soc* 134(30):12658–12667.
7. Stewart JCM (1980) Colorimetric determination of phospholipids with ammonium ferrothiocyanate. *Anal Biochem* 104(1):10–14.

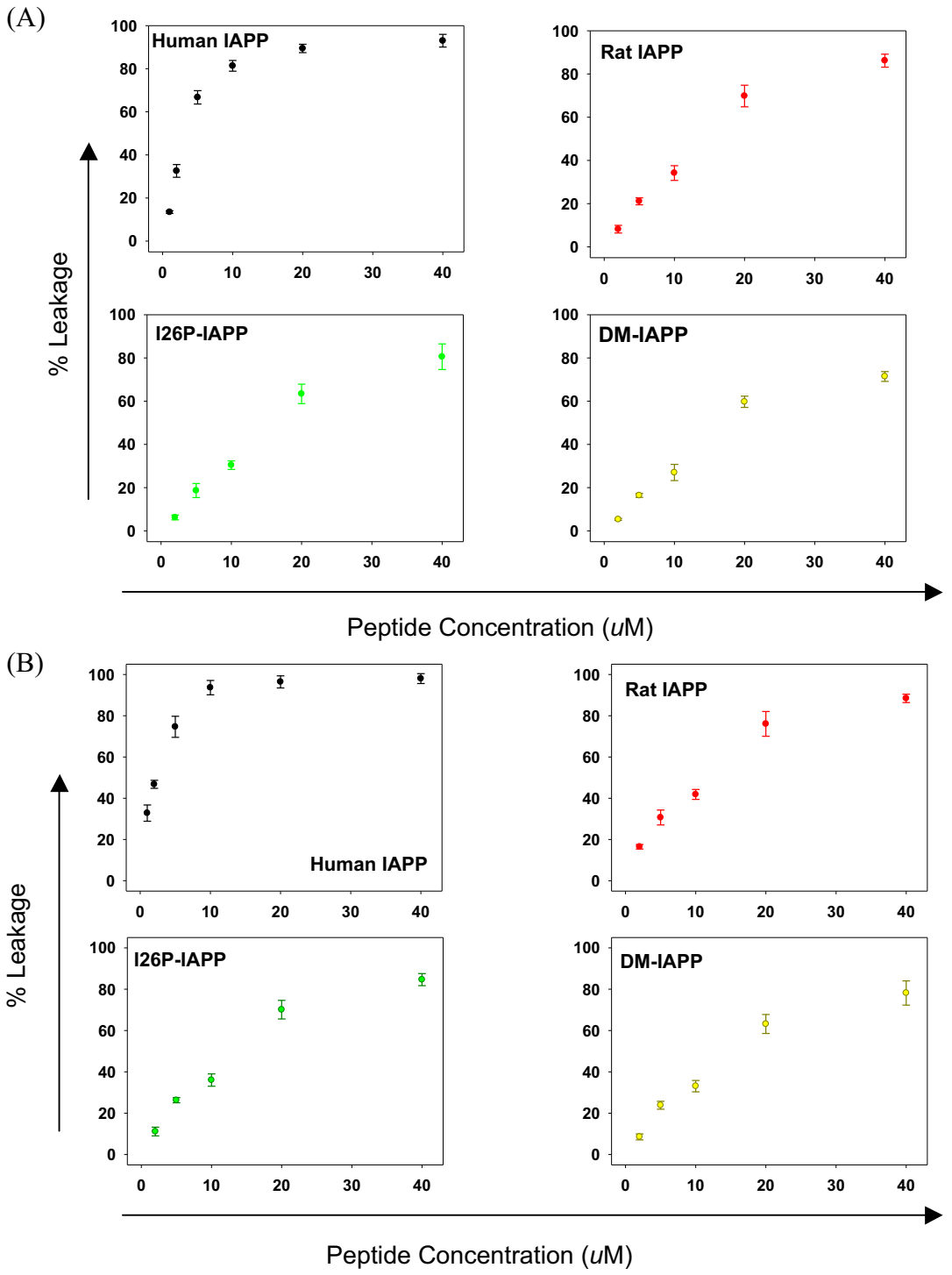

Fig. S1. Membrane leakage induced by human IAPP and IAPP variants was tested after incubating with 400 μM pure anionic model membranes (100% DOPG) for 10 min (*A*) and 48 h (*B*). Data were plotted for the individual peptides from Fig. 3 to clearly show the error bars. Peptide concentrations range from 2 μM to 40 μM. Experiments were performed at 25 °C in 20 mM Tris·HCl, 100 mM NaCl, pH 7.4 buffer.

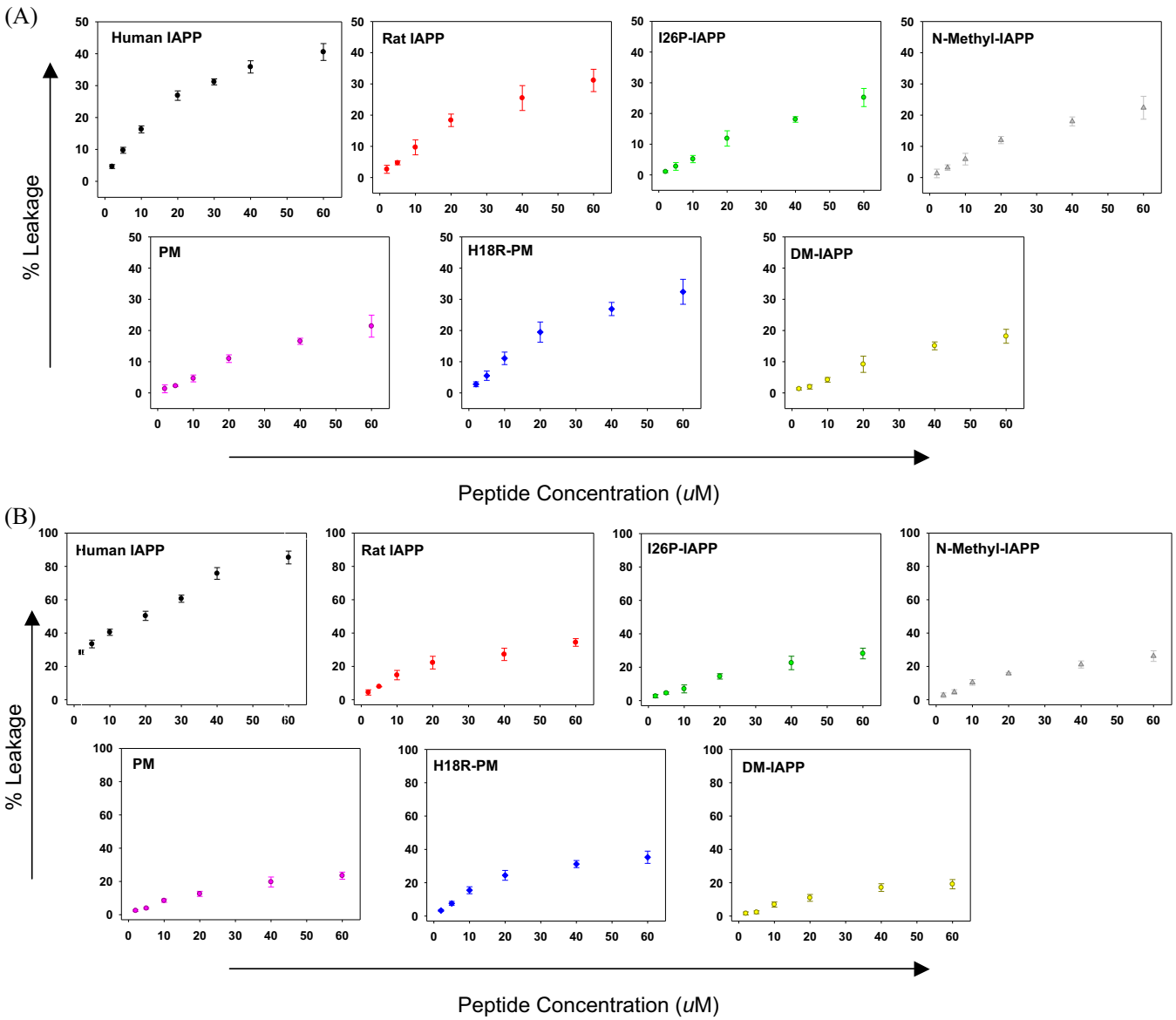

Fig. S2. Membrane leakage was measured after incubating 400 μM 25% anionic model membranes (25% DOPG + 75% DOPC) with peptides for 10 min (*A*) and 48 h (*B*). Data were plotted for the individual peptides from Fig. 4 to clearly show the error bars. Peptide concentrations range from 2 μM to 60 μM. Experiments were performed at 25 °C in 20 mM Tris·HCl, 100 mM NaCl, pH 7.4 buffer.

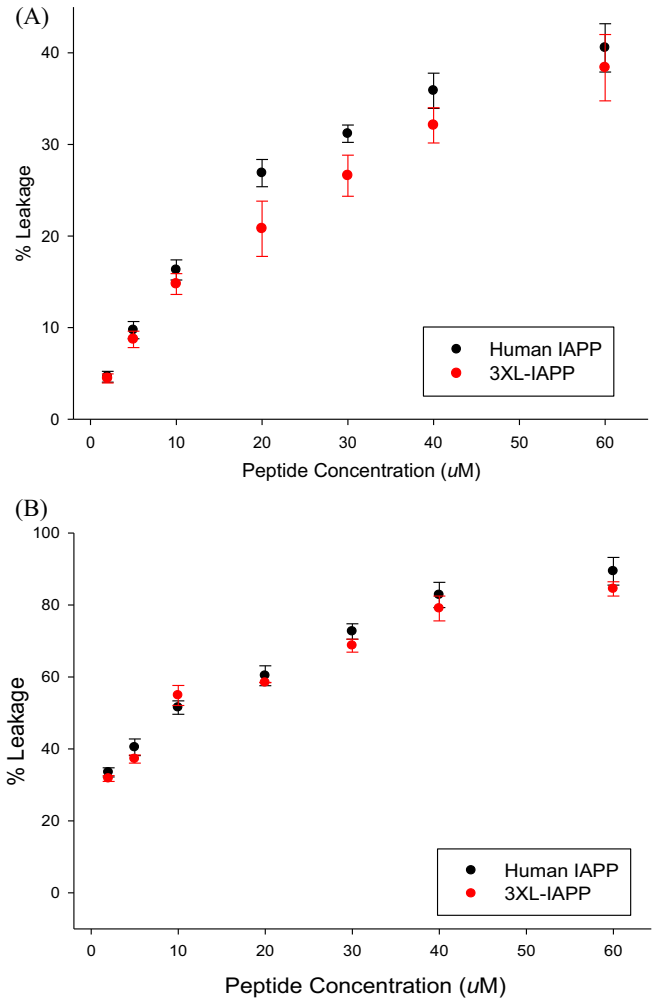

Fig. S3. Aromatic residues are not required for membrane leakage. The percent leakage induced by human IAPP and 3XL-IAPP is shown. Peptides were incubated in the presence of 400 μM 25% anionic model membranes (25% DOPG + 75% DOPC) for 10 min (*A*) and 48 h (*B*). Experiments were performed at 25 °C in 20 mM Tris·HCl, 100 mM NaCl, pH 7.4 buffer.

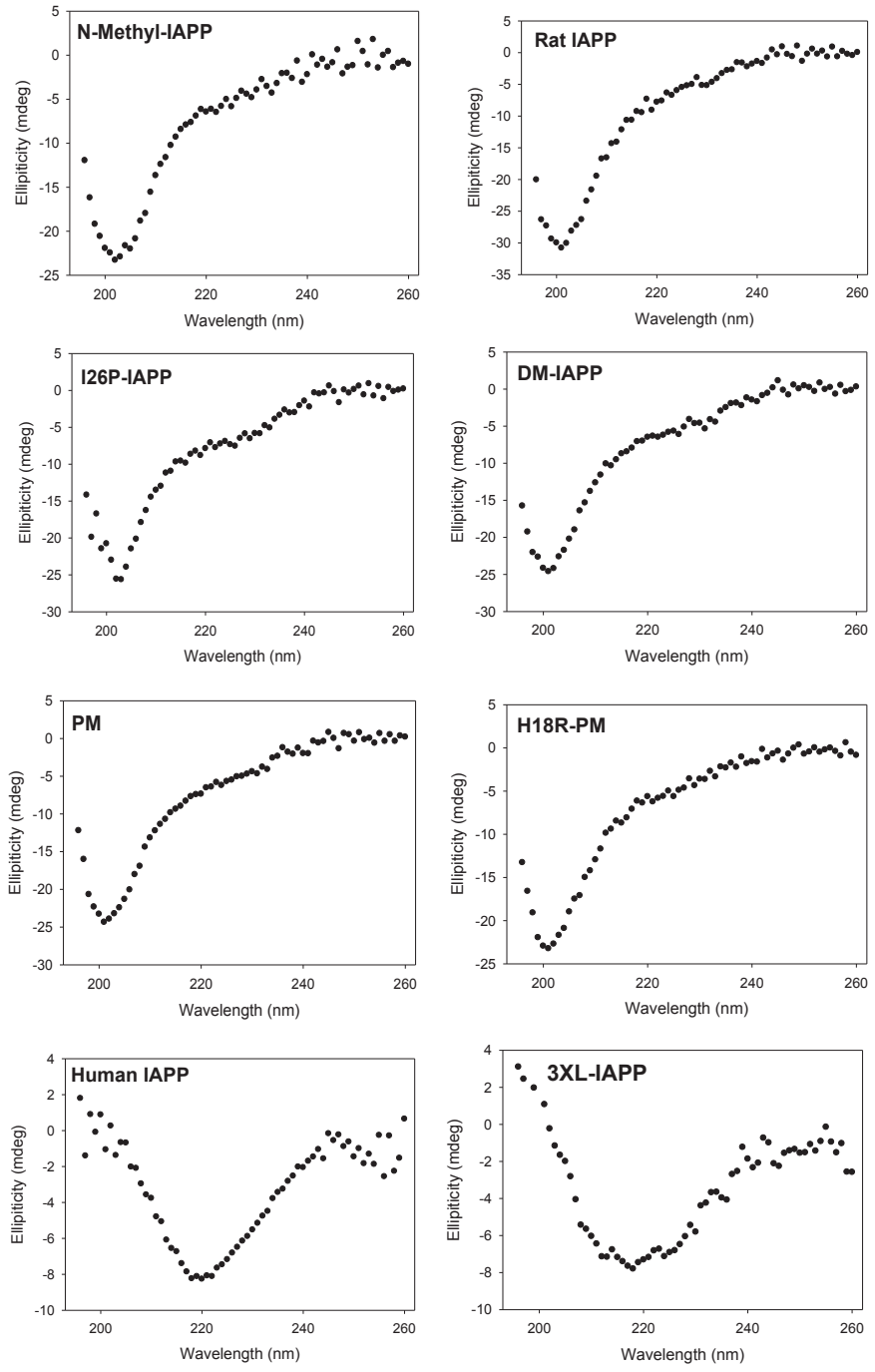

Fig. S4. Membrane disruption does not require formation of α-helix or β-sheet structure. CD spectra of IAPP and IAPP mutants after incubating with 400 μM 25% anionic model membranes (25% DOPG + 75% DOPC) for 48 h. Note the lack of α-helical or β-sheet signal for the IAPP mutants with the exception of the 3XL peptide. The peptide concentration was 60 μM, and experiments were performed at 25 °C in 20 mM Tris·HCl, 100 mM NaCl, pH 7.4 buffer.

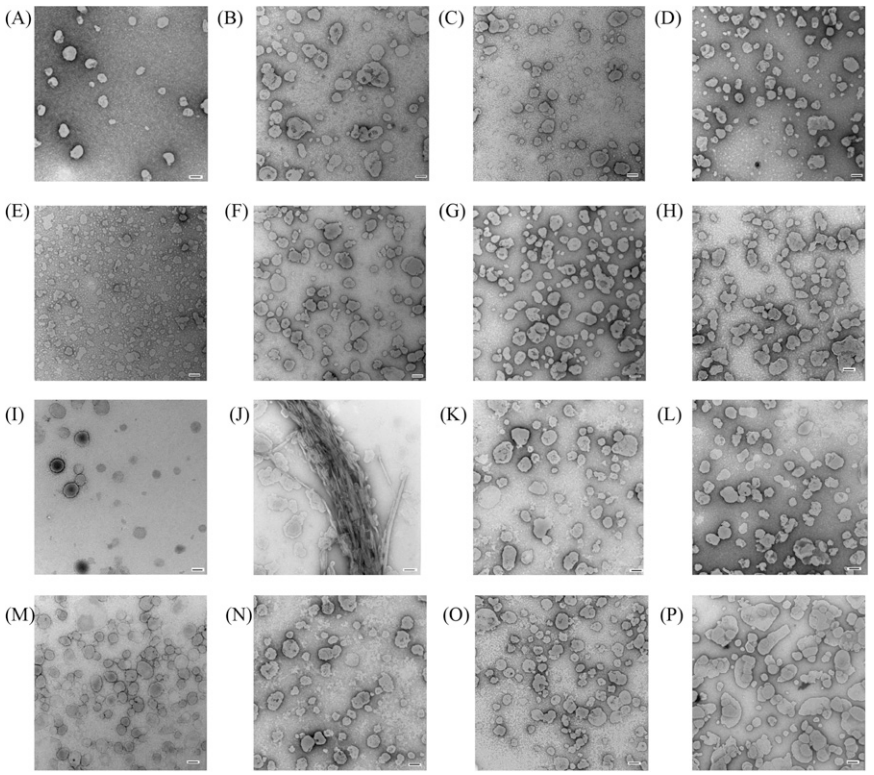

Fig. S5. TEM images of human IAPP and the full-length IAPP variants after incubating with 400 μM 25% anionic model membranes (25% DOPG + 75% DOPC) for 10 min (*A*–*H*) and 48 h (*I*–*P*). (*A* and *I*) Control, 400 μM model membranes without peptide; (*B* and *J*) human IAPP; (*C* and *K*) rat IAPP; (*D* and *L*) I26P-IAPP; (*E* and *M*) DM-IAPP; (*F* and *N*) *N*-methyl–IAPP; (*G* and *O*) PM; (*H* and *P*) H18R-PM. Note that after 48 h incubation, none of the IAPP variants formed amyloid fibrils, whereas human IAPP formed dense mats of fibrils. The peptide concentration was 60 μM, and experiments were performed at 25 °C in 20 mM Tris·HCl, 100 mM NaCl, pH 7.4 buffer. (Scale bar, 100 nm.)

(A)

1 11 21 31 41 51

MKVIFLKDVKGKGKKGEIKNVADGYANNFLFKQGLAIEATPANLKALEAQKQKEQR

(B)

41 51 61 71

MLSDEDFKAVFGMTRSAFANLPLWKQQNLKKEKGLF

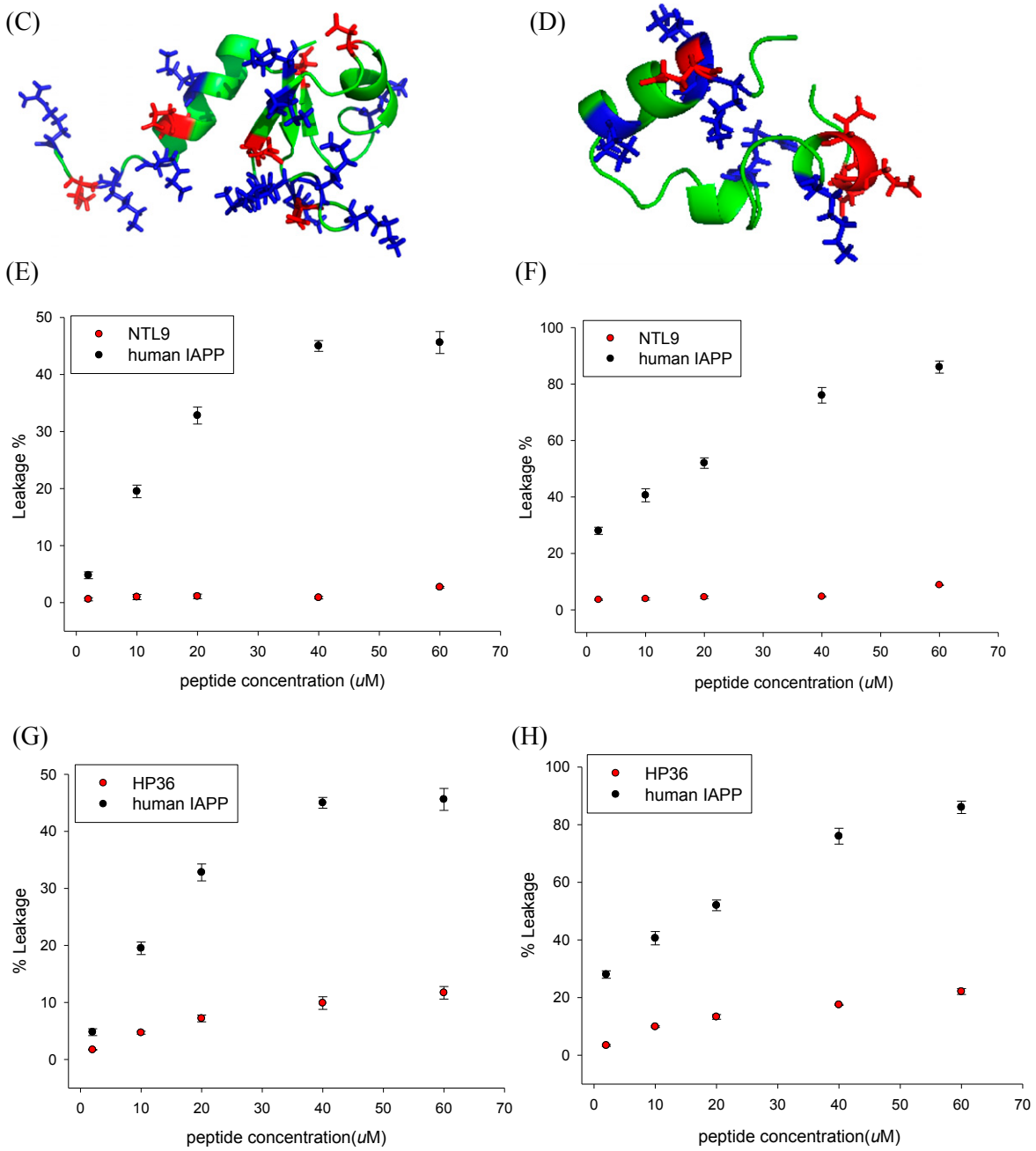

Fig. S6. Comparison of the leakage induced by the N-terminal domain of the ribosomal protein L9 (NTL9) or the leakage induced by the protein villin headpiece helical subdomain HP36 to that induced by human IAPP. (*A*) Primary sequence of NTL9 with acidic residues highlighted in red and basic residues in blue. (*B*) Primary sequence of HP36 with acidic residues highlighted in red and basic residues in blue. (*C*) A ribbon diagram of the structure of NTL9 is shown with acidic and basic residues displayed in stick format. (*D*) A ribbon diagram of the structure of HP36 is shown with acidic and basic residues displayed in stick format. (*E*) Leakage induced by NTL9 after 10 min of incubation. (*F*) Leakage induced by NTL9 after 48 h of incubation. (*G*) Leakage induced by HP36 after 10 min of incubation. (*H*) Leakage induced by HP36 after 48 h of incubation. Experiments were conducted using 100 nm vesicles prepared from 75% DOPC, 25% DOPG. Total lipid concentration is 400 μM. Samples were at 25 °C, pH 7.4, 20 mM Tris·HCl, 100 mM NaCl.

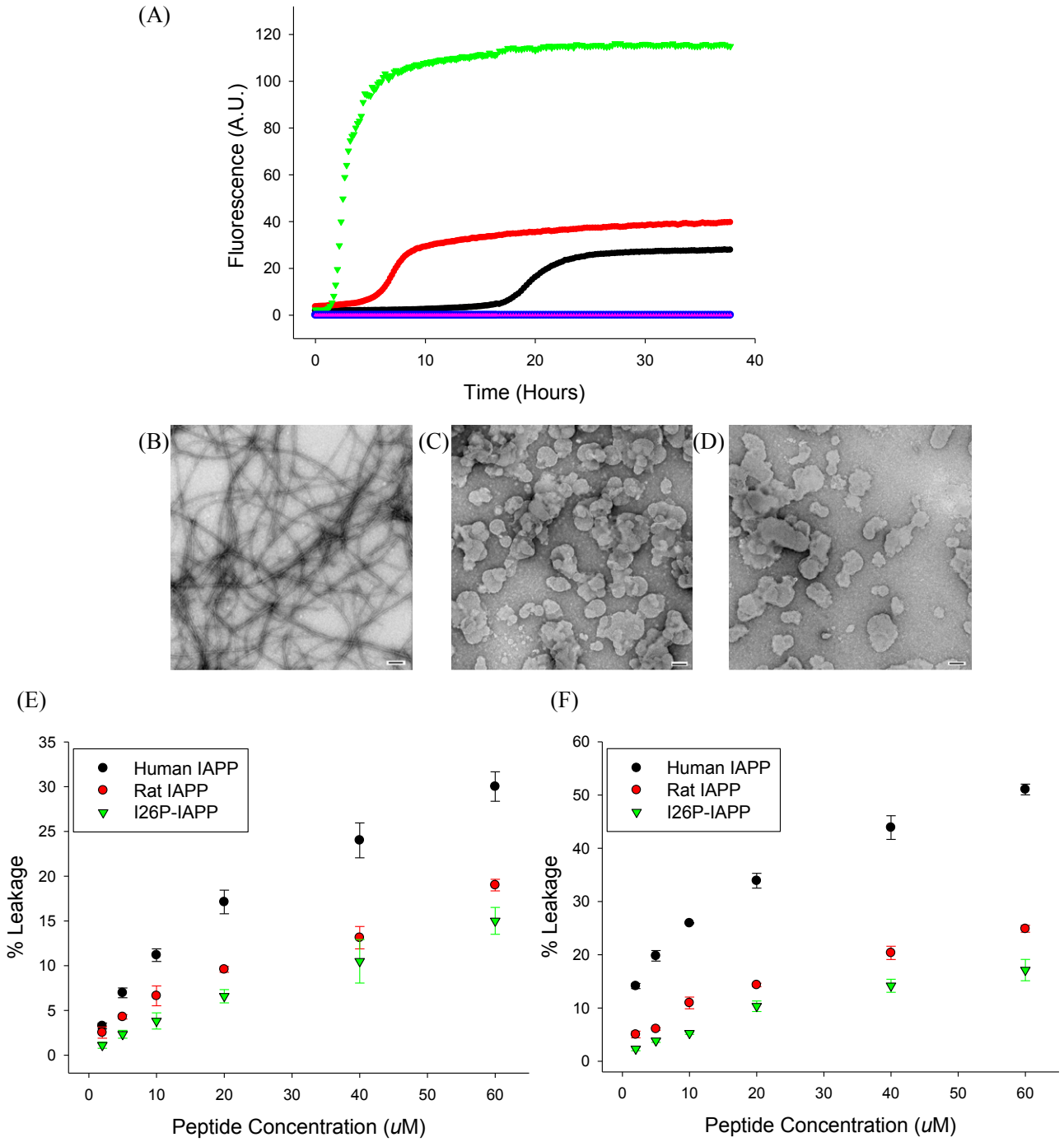

Fig. S7. (*A*) TBE-lipids catalyze amyloid formation by human IAPP. ThT monitored kinetics of amyloid formation: black, human IAPP in the absence of membranes; green, human IAPP in the presence of 0.32 mg/mL 25% anionic model membranes (25% DOPG + 75% DOPC); red, human IAPP in the presence of 0.32 mg/mL TBE-vesicles; blue, rat IAPP in the presence of 0.32 mg/mL TBE-vesicles; pink, I26P-IAPP in the presence of 0.32 mg/mL TBE-vesicles. TEM images of human IAPP (*B*), rat IAPP (*C*), and I26P-IAPP (*D*) after incubating with 0.32 mg/mL TBE-vesicles for 48 h. Note no amyloid fibrils were observed for rat IAPP and I26P-IAPP. The peptide concentration is 60 μM. Membrane leakage induced by human IAPP, rat IAPP, and I26P-IAPP was tested after incubating 0.32 mg/mL TBE-vesicles with peptides for 10 min (*E*) and 48 h (*F*). Peptide concentrations range from 2 μM to 60 μM. All of the experiments were performed at 25 °C in 20 mM Tris·HCl, 100 mM NaCl, pH 7.4 buffer.

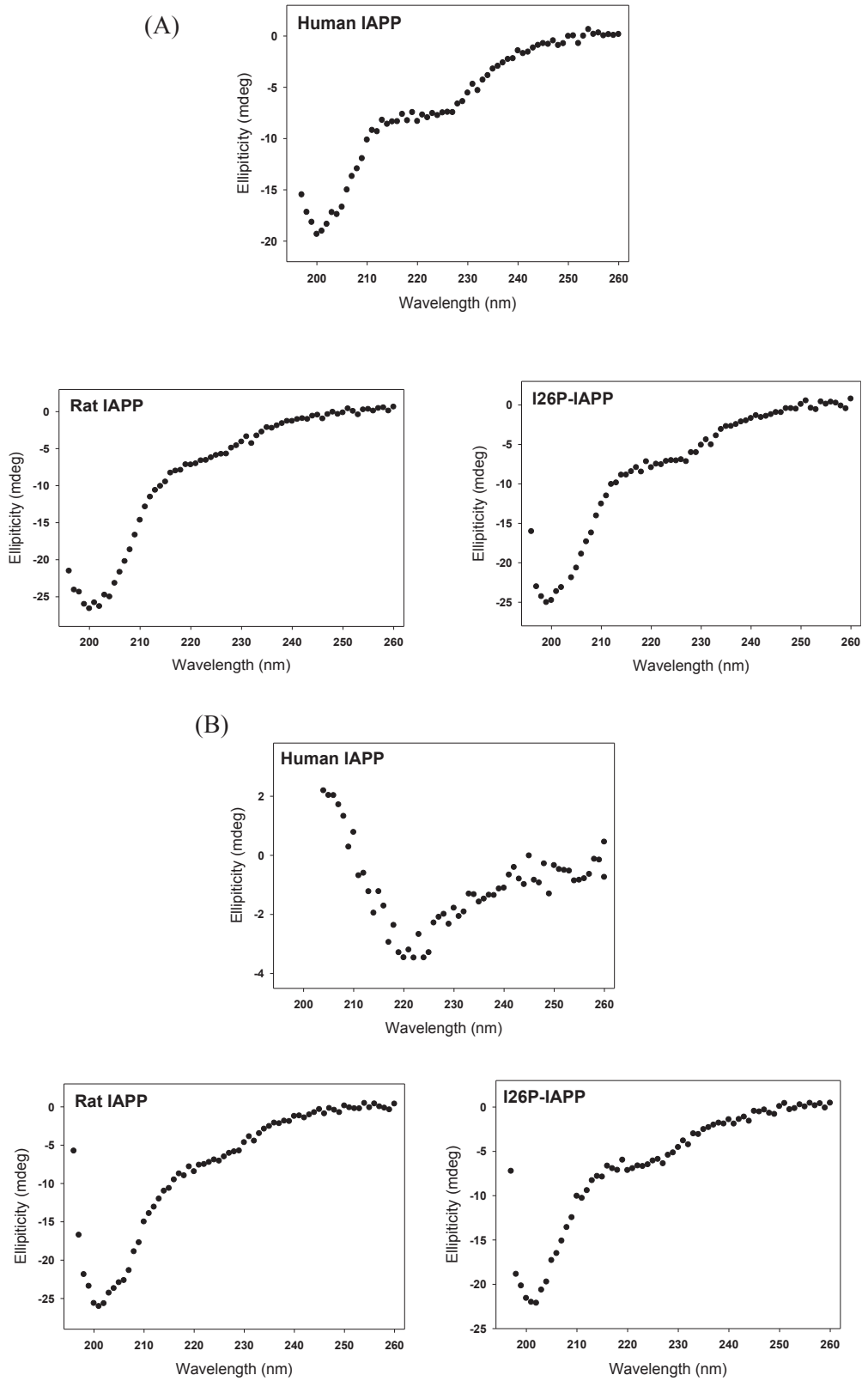

Fig. S8. CD spectra of human IAPP, rat IAPP, and I26P-IAPP after incubating with 0.32 mg/mL TBE-vesicles for 10 min (*A*) and 48 h (*B*). Note the lack of β-sheet or α-helical structure for rat and I26P-IAPP. The peptide concentration was 60 μM. Experiments were performed at 25 °C in 20 mM Tris·HCl, 100 mM NaCl, pH 7.4 buffer.

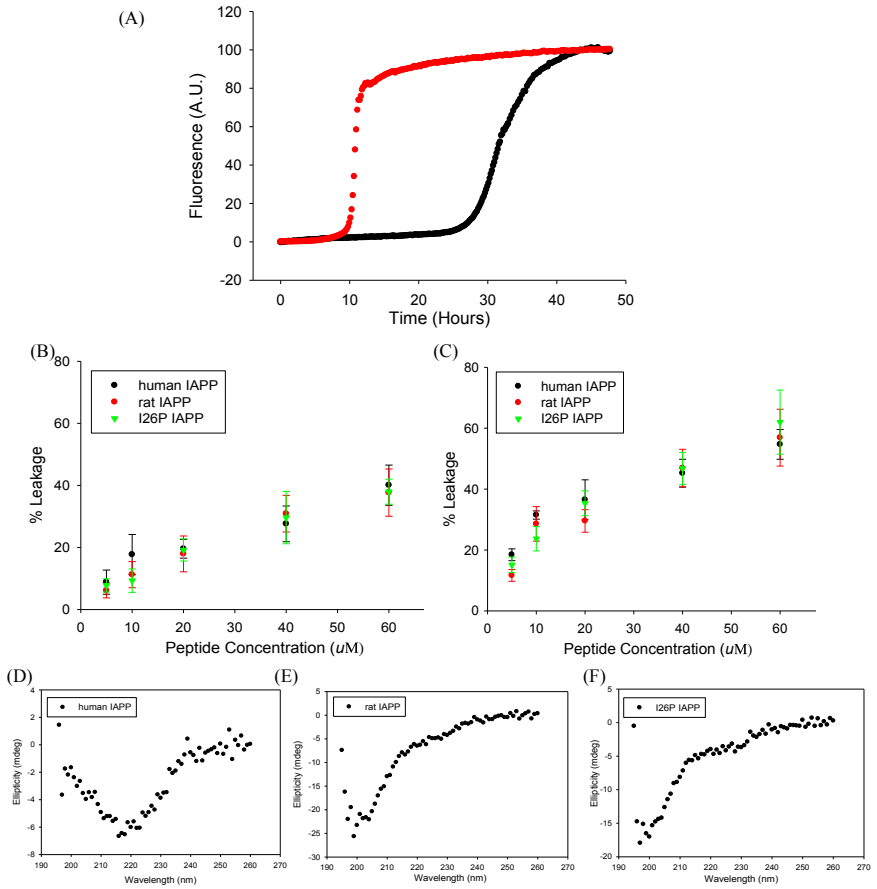

Fig. S9. (*A*) Amyloid formation by human IAPP was accelerated in the presence of 400 μM of the model membrane DOPC/DPPC/cholesterol 1:2:1. Human IAPP in the absence of membrane, black; human IAPP in the presence of membrane, red. Membrane leakage induced by human IAPP, rat IAPP, and I26P IAPP was tested after incubating with 400 μM DOPC/DPPC/cholesterol 1:2:1 vesicles for 10 min (*B*) and 48 h (*C*). Peptide concentrations range from 5 μM to 60 μM. CD spectra of human IAPP (*D*), rat IAPP (*E*), and I26P IAPP (*F*) after incubating with 400 μM DOPC/DPPC/cholesterol 1:2:1 vesicles for 48 h. Note the lack of β-sheet or α-helical structure for rat IAPP and I26P IAPP. The peptide concentration was 60 μM. Experiments were conducted at 25 °C in 20 mM Tris·HCl, 100 mM NaCl, pH 7.4 buffer.