Supporting Information

In vivo mitigation of amyloidogenesis through functional-pathogenic double protein coronae

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MATERIALS & METHODS

Syntheses of b_aCNT and b_mCNT

β-lactoglobulin (bLg) amyloids were first prepared by overnight heating of bLg (Sigma-Aldrich, MW: 18.4 kDa) solution (2%) at 80 °C and pH 2, and then probe-sonicated at 40% of full intensity (20 kHz, 750 W) for 2 h to obtain bLg amyloid fragments (b_a). Aqueous dispersion of multi-walled carbon nanotubes (Nanostructured & Amorphous Materials, Inc.; purity 95+ %; outer diameter: 10-20 nm; length: 0.5-2 μm) at a concentration of 0.2 mg/mL were first probe-sonicated at 20% of full intensity for 10 min. b_a (0.2%) was added into CNTs immediately after sonication, and the dispersion was adjusted to pH 4.3 and further sonicated

for 30 min (ice bath for 4 °C) to obtain b_sCNT. The dispersion was then centrifuged (9,200 RCF; 30 min; 4 °C) to separate large aggregates, and the supernatant was adjusted to pH 4.3 and subjected to heating at 70 °C for 20 min to obtain b_aCNT. In addition, b_mCNT was synthesized by the same procedures, except replacing b_a with monomeric bLg.

Thioflavin T (ThT) assay

A ThT kinetic assay was used to monitor IAPP fibrillization in the presence of pristine and functionalized CNTs. 200 µg of human islet amyloid polypeptide (IAPP1-37; MW: 3904.5 Da; AnaSpec) was weighed on a microbalance and dissolved in 200 µL of deionized water to make a stock solution, which was used to make further dilutions for experiments. In a 96 well plate, 100 µL (50 µM) of IAPP, ThT dye (100 µM), and b_aCNT/b_mCNT (50 µM with respect to bLg) were incubated for 13 h at 28 °C. ThT fluorescence was monitored (excitation/emission: 440 nm/485 nm) at 1 h intervals (PerkinElmer EnSpire 2300). Controls were performed with IAPP alone, or with pristine CNTs and b_a at equivalent concentrations. ThT dye was incubated with pristine CNTs, b_aCNT/b_mCNT, bLg amyloids and b_a under comparable conditions, but without IAPP, as controls. The effect of human plasma proteins on b_aCNT inhibition of IAPP aggregation was measured by an additional ThT assay. The molar ratio of IAPP to plasma proteins was adjusted from 1:1 to 2:1 and 4:1. Details of the blood protein collection method are described in a previous publication.[1] Blood was collected from a healthy donor after obtaining informed consent for any experimentation in this study, in accordance with the University of Melbourne Human ethics approval 1443420 and the Australian National Health and Medical Research Council Statement on Ethical Conduct in Human Research. All experiments were performed in compliance with the relevant laws and institutional guidelines of Monash University Occupational Health & Safety.

FTIR, TGA, CD and DLS measurements

Fourier transformed infrared spectroscopy (FTIR) and thermogravimetric analysis (TGA) were performed with freeze-dried b_aCNT, IAPP, b_aCNT and IAPP incubated for 13 h at comparable concentrations as for the ThT assay. IAPP incubated with b_aCNT was purified from un-adsorbed IAPP by centrifugation. FTIR was performed with a Shimadzu IRtracer-100 with a GladiATR-10 accessory. Around 1 mg of sample was placed in the holder and FTIR spectra were recorded in the 1600-1700 cm^{-1} amide I band. The peak was de-convoluted with LabSolutions IR and peak fitting was performed to quantify the percentage secondary structures. For TGA, 1 mg of sample was placed in the holder (PerkinElmer Pyris 1) and analysis was performed from 50 °C to 700 °C with a heating rate of 10 °C/min, under a continuous flow of nitrogen (1 mL/min). Circular dichroism (CD) spectroscopy was performed in addition to FTIR, to analyze the secondary structural changes in the corona of b_aCNT, before or after IAPP adsorption. IAPP, b_aCNT and IAPP incubated with b_aCNT were pipetted into CD cuvettes at a concentration of 0.5 mg/mL with respect to the protein contents and CD spectra were recorded from 190 to 240 nm with a 0.5 nm step size at room temperature. The data was analyzed via Dichroweb and Contin/reference set 4 was used to estimate the percentage secondary structures.[2] The zeta potential and hydrodynamic size of the samples were measured by dynamic light scattering (DLS) under ambient conditions (Malvern Instruments).

Small- and wide-angle X-ray scattering (SAXS, WAXS)

Simultaneous SAXS and WAXS experiments were performed using a Rigaku MicroMax-002$^+$ microfocused beam (4 kW, 45 kV, 0.88 mA) to obtain direct information on the SAXS and WAXS reflections. The Cu K_α radiation ($\lambda_{Cu\ K\alpha}$ = 1.5418 Å) was collimated by three pinhole (0.4, 0.3, and 0.8 mm) collimators. The scattered X-ray intensity was detected by a Fuji Film BASMS 2025 imaging plate system (15.2 × 15.2 cm^2, 50 μm resolution) and a two-dimensional Triton-200 X-ray detector (20 cm diameter, 200 μm resolution), for WAXS and SAXS regions, respectively. An effective scattering vector range of 0.05 $nm^{-1} < q < 20\ nm^{-1}$ was obtained,

where q is the scattering wave vector defined as $q = 4\pi\sin\theta/\lambda_{\text{Cu K}\alpha}$, with a scattering angle of 2θ.

X-ray photoelectron spectroscopy

The elemental composition of pristine CNTs was analyzed by X-ray photoelectron spectroscopy (XPS; ESCA LAB 220i-XL Thermo VG Scientific U.K.). XPS data files were processed using the application CasaXPS software (version 2.3.13). Mean values +/- deviations were calculated based on three measurements on different spots.

Zebrafish embryos as a toxicity model for screening amyloidogenesis

The AB wild-type zebrafish (*Danio rerio*) was maintained at 28 ± 0.5 ºC on a 14 h:10 h light/dark cycle in a fish breeding circulatory system (Haisheng, Shanghai, China). Embryos were produced by adult spawning triggered by first light in the morning. All experiments with zebrafish embryos were performed in Holtfreter's buffer[3]. Microinjections were performed at 5 nL each time, unless specified, under a 20 psi injection pressure by a pneumatic microinjection system (PV830 Pneumatic Picopump, WPI). First, imaging of ThT-tagged amyloids was performed in the green fluorescence protein (GFP) channel by a fluorescence microscope (EVOS FL Auto, Life Technologies). IAPP or $A\beta_{42}$ (Human Aβ1-42; MW: 4514.1 Da; AnaSpec) and bLg (50 μM) were fibrillized into amyloids in the absence and presence of ThT dye (50 μM). A drop of fibrillized amyloid solution was placed in a 96 well plate and visualized. ThT tagged amyloids were visible in the GFP channel, while no fluorescence was observed for ThT or amyloid alone. IAPP (100 μM, fibrillized with 100 μM of ThT) was injected (5 nL) inside embryos at 3 h post fertilization (hpf). The embryos were placed in 100 μL of Holtfreter's buffer inside a 96 well plate and their development was monitored for 3 consecutive days in bright field and the GFP channel. Control embryos were injected with 5 nL of buffer or ThT dye (100 μM) with a microinjector (PV830 Pneumatic Picopump, WPI). The

experiment first involved immersion of embryos inside IAPP or Aβ solutions. Different concentrations of IAPP or Aβ (12.5 to 200 μM) were dissolved in buffer and 200 μL of each protein solution was placed in a 96 well plate. Embryos at 3 hpf with and without chorions were then immersed in the solutions. The chorionic membranes were removed under a microscope by tweezers inside buffer.

A ThT kinetic assay of IAPP fibrillization was performed in the presence of the embryos, with and without chorionic membranes, observed through the GFP channel (excitation/emission: 488/540 nm). 200 μL of solution containing one embryo, 50 μM of IAPP and 100 μM of ThT was incubated at 28 °C and the fluorescence was recorded for 13 h at a 1 h interval. IAPP alone in buffer and ThT with embryos, with and without chorionic membranes, were measured as controls. For the ThT assay inside the embryos, 5 nL of IAPP (50 μM) and ThT (100 μM) were injected in the chorionic fluids, and ThT fluorescence was recorded with the EVOS microscope and analyzed by ImageJ for corrected fluorescence.

Amyloid toxicity mitigation in an embryonic zebrafish model

Minimum inhibitory concentrations (MIC) for IAPP and Aβ against zebrafish embryos were obtained by dissolving different concentrations of IAPP and Aβ (2 to 100 μM) in buffer and 5 nL of the solution was injected inside the perivitelline space of 3 hpf embryos. For the sequestration experiment, 5 nL of buffer containing 10 or 15 μM of IAPP or Aβ and CNTs of equivalent concentrations (with respect to b_a or b_m on the CNT surfaces) were injected inside the chorionic fluids. The treated embryos were placed in 200 μL of buffer in a 96 well plate and toxicity mitigation was studied in the context of hatching survival of embryos on the 3rd day of fertilization. Embryos injected with 5 nL of buffer were used as controls. For imaging, 5 nL of buffer with 10 μM of IAPP and 20 μM of ThT dye was injected with and without b_aCNT and ThT fluorescence images were recorded with the EVOS fluorescence microscope.

Reactive oxygen species (ROS) generation

ROS generation assay was performed with 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) dye. 5 nL of buffer, containing 10 μM of IAPP (with or without 10 μM of b_aCNT or b_mCNT) and 20 μM of H_2DFCDA dye was injected inside chorionic fluids of zebrafish embryos. The embryos were incubated in 200 μL of buffer in a 96 well plate at 28 °C for 12 h. ROS generation was characterized by imaging the embryos in the GFP channel and images were analyzed in ImageJ for corrected total ROS fluorescence.

Helium ion microscopy (HIM) and transmission electron microscopy (TEM)

HIM was used to image the interaction of fibrillizing IAPP and zebrafish embryos. Embryos with chorionic membranes were incubated inside 100 and 25 μM of IAPP monomers dissolved in buffer, at 28 °C. After 6 h of incubation, the buffer was replaced by 2.5 % paraformaldehyde to fix the embryos. The embryos were left in paraformaldehyde solution for 2 h under ambient conditions and then at 4 °C overnight. Afterwards, the embryos were transferred into ethanol by gradually replacing the paraformaldehyde solution with 20, 40, 60, 80 and 100 % ethanol, with 2 h incubation at room temperature for each step. Embryos preserved in 100 % ethanol were further subjected to critical point drying with liquid CO_2. Dried embryos were positioned on a carbon tape and imaged with HIM (Zeiss Orion NanoFab), operating at a 0.6 to 0.8 pA beam current and a 0.2 s dwell time. To image the interaction of IAPP with lipid membranes of embryonic cells, chorionic membranes of the embryos treated with 25 μM of IAPP were raptured with tweezers under an optical microscope, prior to HIM imaging.

TEM imaging of CNTs, b_aCNT, b_mCNT, and b_aCNT or b_mCNT with IAPP (24 h incubation) was performed by drying a drop of sample on farmvar-coated copper grids and negative staining was done with 1 % uranyl acetate. TEM images were captured by a Tecnai G2 F20 transmission electron microscope (FEI, Eindhoven, The Netherlands) operated at 200 kV.

Cell culture and in vitro viability assay

Pancreatic βTC-6 (ATCC) beta cells were cultured in complete Dulbecco's modified Eagle's medium (DMEM) with 15% fetal bovine serum (FBS). A 96 well plate (Costar black, clear bottom) was coated with 70 µL Poly-L-lysine (Sigma, 0.01%), incubated at 37 °C for 30 min and cells at a density of ~70,000 cells per well in 200 µL DMEM with 15% FBS were added to the wells. Cells were incubated for 48 h at 37 °C and 5% CO_2 to reach ~80% confluency. The cell culture medium was then refreshed with 1 µM propidium iodide dye (PI, excitation/emission: 535 nm/617 nm) in DMEM and incubated for 30 min. Fresh IAPP, b_aCNT and IAPP in the presence of b_aCNT (20 µM final concentration of each sample) were added into the wells. All samples were examined in triplicate and measured by Operetta (PerkinElmer) in a live cell chamber (37 ºC, 5% CO_2) after 7 h of treatment. The percentage of dead cells (PI-positive) relative to total cell count was determined by a built-in bright-field mapping function of Harmony High-Content Imaging and Analysis software (PerkinElmer). The measurement was conducted at 5 reads per well and performed in triplicate. Untreated cells were recorded as controls.

Statistical analysis

The data was presented as mean ± standard deviation. The experiments were performed in triplicate. The level of significance was determined by one-way ANOVA followed by Turkey's test and p value < 0.05 was considered as statistically significant. The group size was 20 embryos and three groups per sample were used for experiments.

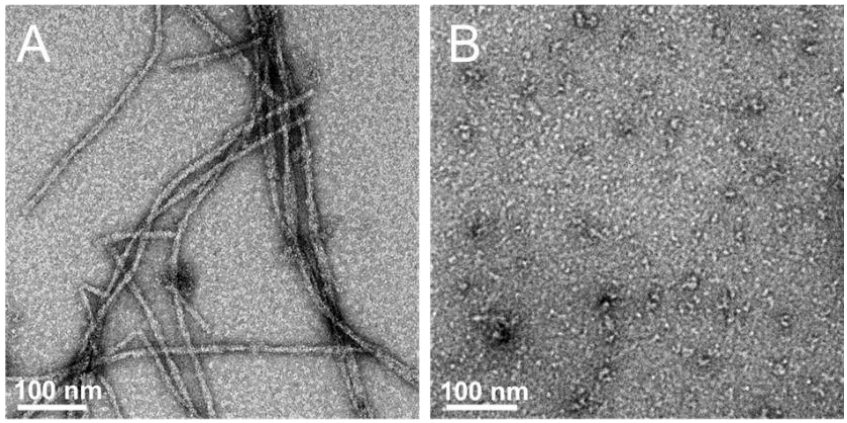

Figure S1. (A) bLg amyloids and (B) sonicated fragments of bLg amyloids.

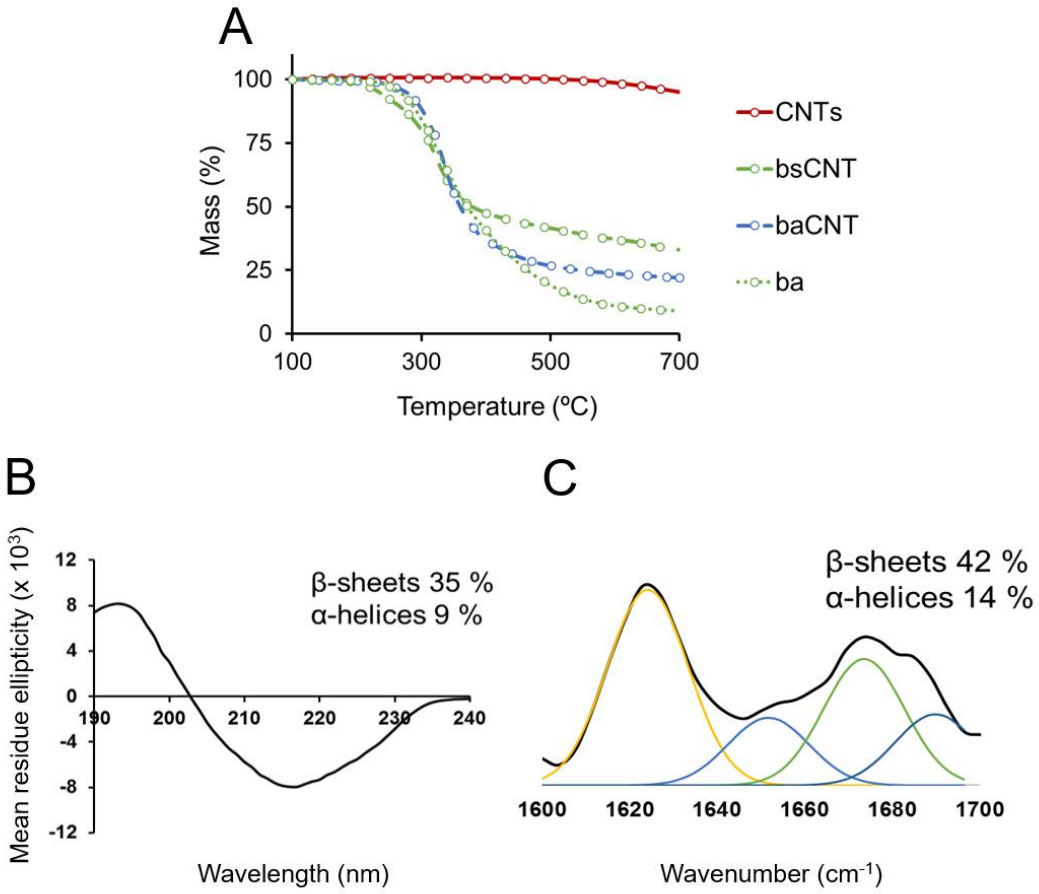

Figure S2. (A) TGA analysis of b_sCNT (CNTs coated with an initial thin layer of b_a via sonication) and b_aCNT (heating of b_sCNT to obtain a b_a corona on CNT), pristine CNTs and b_a as the controls. (B) CD spectroscopy and (C) FTIR amide I band deconvolution for the secondary structure of mature IAPP fibrils.

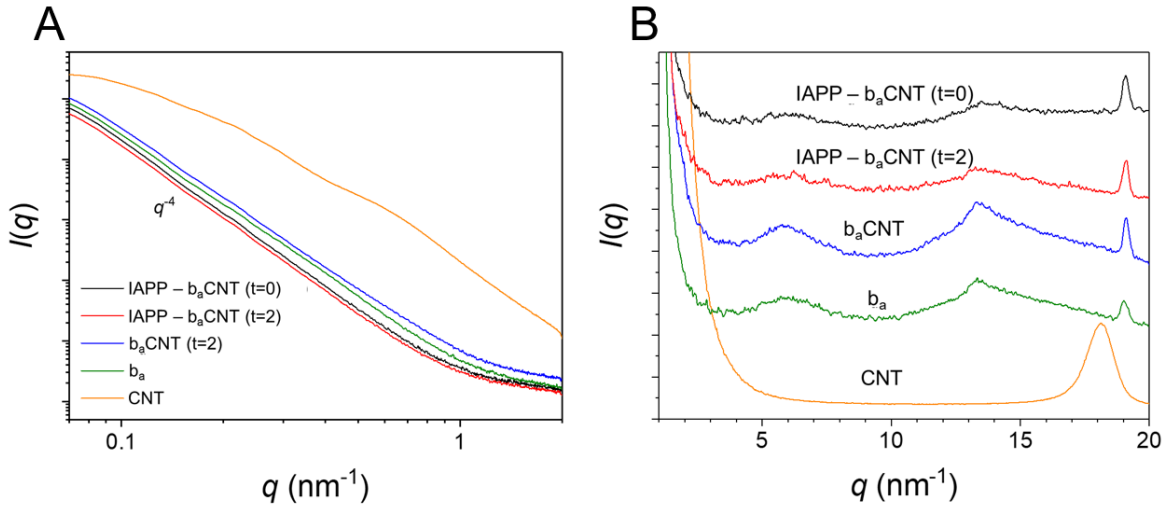

Figure S3. (A) The WAXS intensity profiles of IAPP-b_aCNT (t=0) (black), IAPP-b_aCNT (t=2) (red), b_a@CNTs (blue), b_a (green), and CNTs (orange). (B) The SAXS intensity profiles of IAPP-b_aCNT (t=0) (black), IAPP-b_aCNT (t=2) (red), b_aCNT (blue), b_a (green), and CNTs (orange). The presence of CNTs showed no effect on the β-sheet secondary structure.

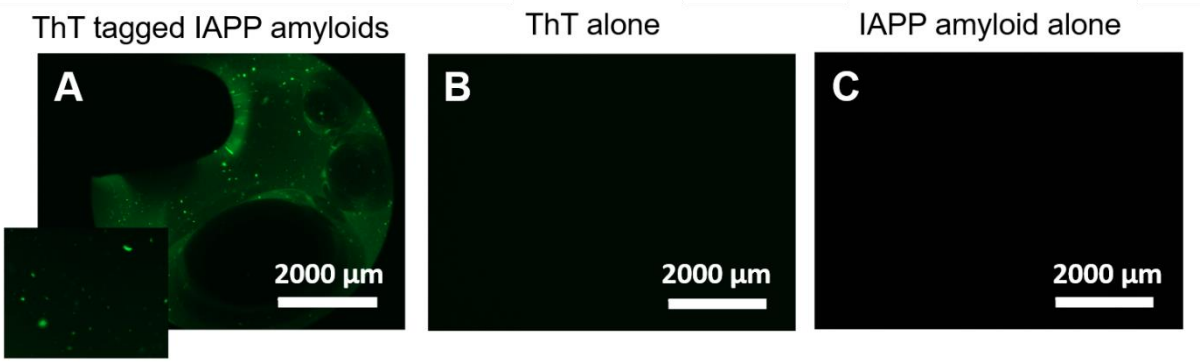

Figure S4. (A) ThT tagged IAPP amyloids (100 µM of ThT and IAPP; incubated for 24 h) were visible under the GFP channel, while ThT alone (B) or IAPP alone (C) were invisible.

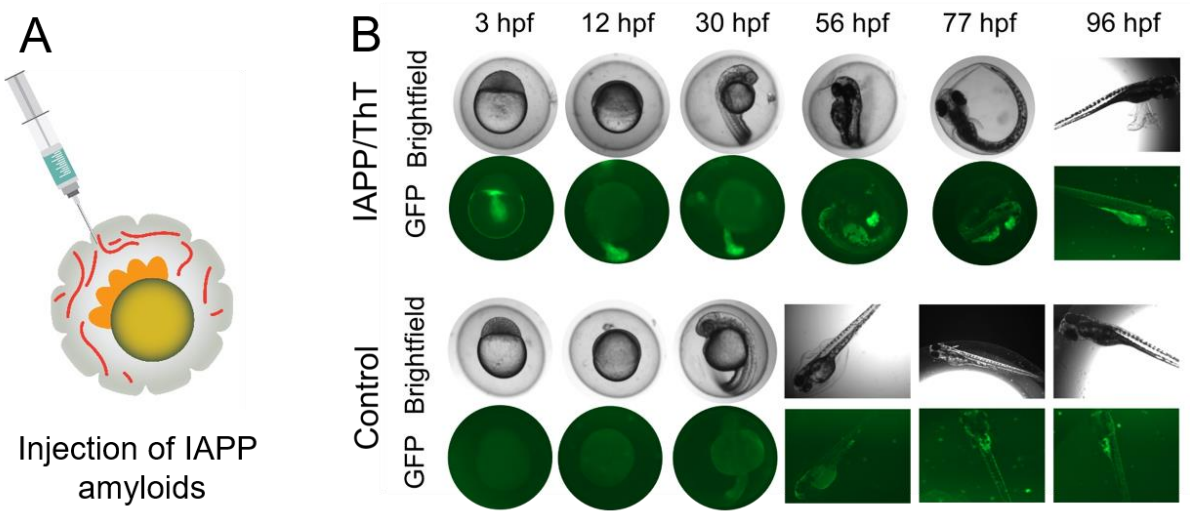

Figure S5. Toxicity of mature IAPP in zebrafish embryos. (A) Illustration of mature IAPP (100 µM peptide, with 100 µM of ThT dye) injected inside 3 hpf zebrafish embryos. (B) Controls of embryos injected with equal amounts of ThT presented no fluorescence. Hatching in IAPP amyloid treated embryos was significantly delayed compared to the control.

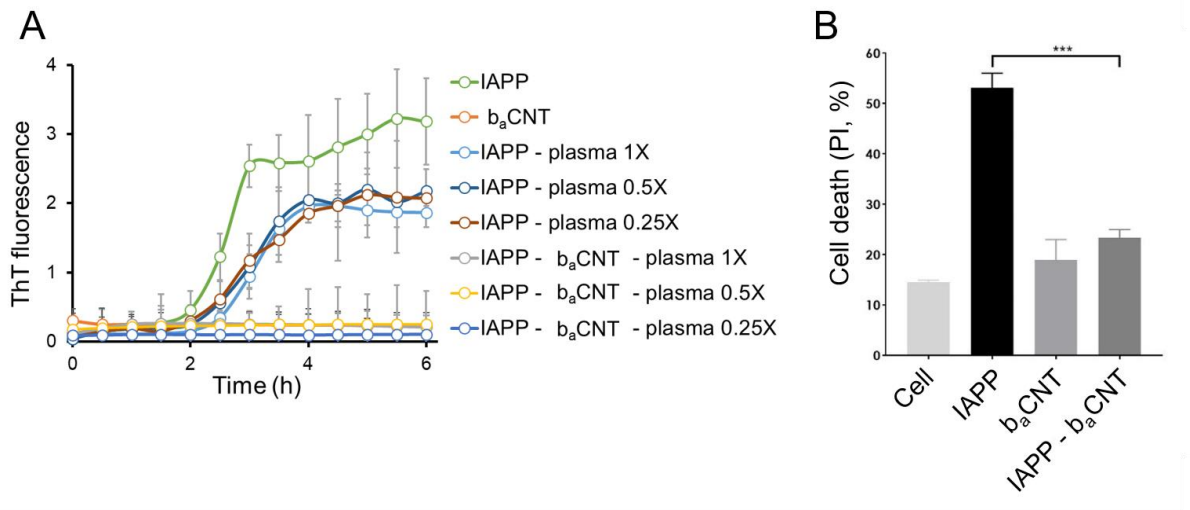

Figure S6. (A) ThT kinetic assay on b_aCNT inhibition of IAPP aggregation, in the presence of plasma proteins. IAPP/plasma protein molar ratio: 1:1, 2:1 and 4:1. IAPP concentration: 50 µM. (B) βTC6 pancreatic beta cell mortality induced by IAPP, in the presence of b_aCNT. IAPP final concentration: 20 µM. b_a/IAPP molar ratio: 1:1. Incubation: 7 h.

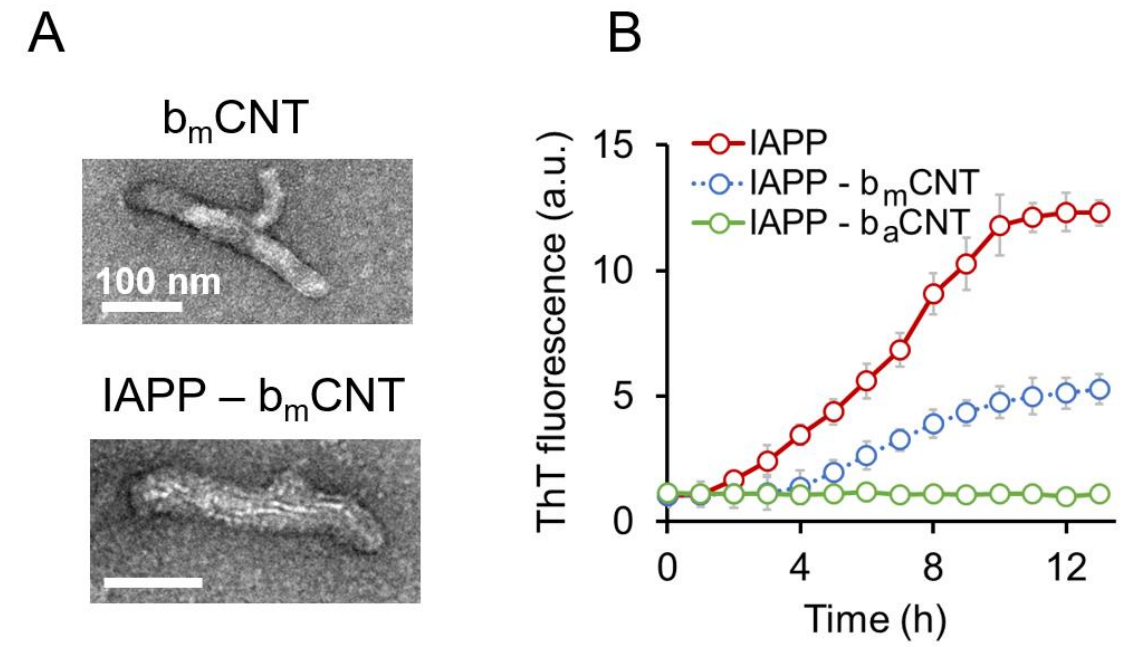

Figure S7. (A) TEM imaging of bLg monomer-coated CNTs (b_mCNT) and b_mCNT incubated with IAPP for 24 h. Scale bars: 100 nm. (B) ThT assay of IAPP fibrillization in the presence of b_mCNT vs. b_aCNT. IAPP: 50 µM. b_m: 50 µM. b_a: 50 µM.

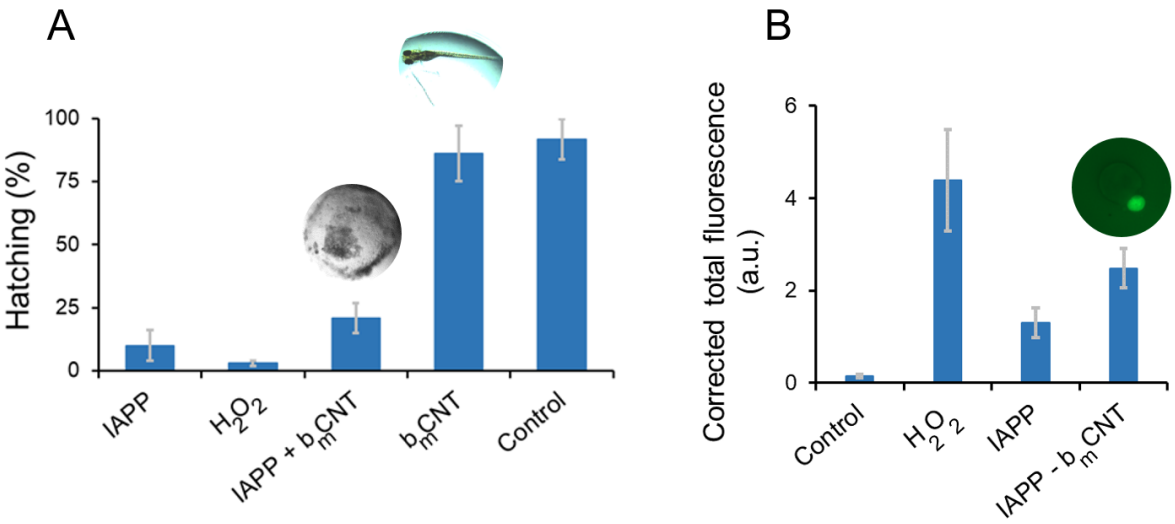

Figure S8. (A) IAPP and b_mCNT were injected inside zebrafish embryos and no significant mitigation of IAPP toxicity was observed in terms of hatching survival of embryos on the 3rd day post fertilization (72 hpf). (B) No significant reduction of IAPP-elicited ROS production was observed with b_mCNT after 12 h of incubation. IAPP: 10 µM. b_m: 10 µM.

Table S1. Zeta potential and hydrodynamic size of b_a@CNT before and after IAPP incubation.

Sample	Hydrodynamic diameter (nm)	Zeta potential (mV)
b_sCNT	165.8 ± 13.4	-11.8 ± 3.2
b_aCNT	184.1 ± 15.2	-18.4 ± 2.4
IAPP + b_aCNT	516.1 ± 53.9	-12.5 ± 2.1
IAPP monomer	46.1 ± 5.3	15.8 ± 4.2
IAPP amyloid	568.9 ± 87.3	65.3 ± 3.6
b_a	11.5 ± 2.1	-9.5 ± 1.8
Pristine CNTs	3236 ± 438.2	-15.1 ± 2.2

*Subscripts: s - sonication, and a - amyloid fragments.

References

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(3) Lin, S.; Zhao, Y.; Xia, T.; Meng, H.; Ji, Z.; Liu, R.; George, S.; Xiong, S.; Wang, X.; Zhang, H. *ACS Nano* **2011,** 5, (9), 7284-7295.