Supplementary Material (ESI) for Chemical Communications

Copper-induced cytotoxicity: reactive oxygen species or islet amyloid polypeptide oligomer formation

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Working Hypothesis

By studying the effects of copper on hIAPP aggregation and recreating this effect using nickel, we found evidence that copper-mediated hIAPP cytotoxicity is related to the formation of granular oligomers by hIAPP. First, we observed that copper increased hIAPP-induced cytotoxicity (Fig. 1a, 1b). Second, the addition of copper affected the kinetics of hIAPP aggregation: copper not only inhibited fibril formation, but its binding to hIAPP also changed hIAPP conformation (Fig. S2, S8d). We characterized the size and morphology of copper-induced hIAPP oligomers (Fig. 2, S3). Similar to copper, nickel also inhibited hIAPP fibril formation (Fig. S4b), increased small granular oligomer formation by hIAPP (Fig. 3a), and increased the cytotoxicity of hIAPP (Fig. 3b). Importantly, unlike copper, nickel did not increase oxidative stress (Fig. S4a). Taken together, these results suggested that copper-induced hIAPP cytotoxicity was due mainly to the formation of small granular oligomers of hIAPP rather than to ROS-mediated cytotoxicity (Scheme S1).

Copper Quenching Tyrosine Intrinsic Fluorescence of hIAPP

Copper-induced reduction in hIAPP tyrosine fluorescence is dose-dependent as well as ThT results (Fig. S2c). Similar studies on Aβ and copper have shown that copper induces Aβ dimer formation by tyrosine coupling (D. P. Smith, G. D. Ciccotosto, D. J. Tew, M. T. Fodero-Tavoletti, T. Johanssen, C. L. Masters, K. J. Barnham and R. Cappai, *Biochemistry*, 2007, **46**, 2881-2891.), suggesting that copper interacts with hIAPP and thereby influences its conformation and its aggregation characteristics.

Probing Copper-hIAPP Binding by Circular Dichroism and Raman Spectroscopy

We further studied conformation changes by copper through far-UV circular dichroism (CD) spectroscopy (see Supplementary Fig. S8d online). hIAPP alone shows a β-sheet structure, the classic conformation for amyloid. In contrast, when incubated with various concentration of copper hIAPP adopts significantly different conformation. They displayed slightly negative ellipticities from 200 to 210 nm but showed no negative peak in 216 nm, which indicates the formation of α-helix conformation and reduction of β-sheet structure. The differences in the spectroscopic patterns suggest that in the presence of copper hIAPP adopts a non-β-sheet conformation.

To explore copper binding sites of hIAPP, we used Raman spectroscopy (see Supplementary Fig. S8c online), which spectra provide information on strong interactions between copper and binding-related amino acids.(T. Miura, K. Suzuki, N. Kohata, H. Takeuchi, *Biochemistry* **2000**, *39*, 7024-7031; J. Dong, C. S. Atwood, V. E. Anderson, S. L. Siedlak, M. A. Smith, G. Perry, P. R. Carey, *Biochemistry* **2003**, *42*, 2768-2773) Firstly, at 1173 cm^{-1} and 1505 cm^{-1}, increasing intensities with the increase of copper concentration indicate copper-tyrosinate binding. (T. Miura, K. Suzuki, N. Kohata, H. Takeuchi, *Biochemistry* **2000**, *39*, 7024-7031) And the intensity ratio of the "tyrosine doublet" at 850/830 cm^{-1} considered as symptomatic of tyrosinate, is slightly less than 1 when IAPP incubated with copper.(T. Miura, K. Suzuki, N. Kohata, H. Takeuchi, *Biochemistry* **2000**, *39*, 7024-7031) A feature at 1618 cm^{-1} that is characteristic of the tyrosine side chain in its OH form was appeared with higher concentration of copper.(T. Miura, K. Suzuki, N. Kohata, H. Takeuchi, *Biochemistry* **2000**, *39*, 7024-7031) A new peak appeared in high ratio of copper and IAPP samples at 1290 cm^{-1}, suggests His-copper complex was formed, shift from the original peak of His at 1277 cm^{-1}.(J. Dong, C. S. Atwood, V. E. Anderson, S. L. Siedlak, M. A. Smith, G. Perry, P. R. Carey, *Biochemistry* **2003**, *42*, 2768-2773) Besides, the features of S-S changed from 'gauche-gauche-gauche' conformation (550 cm^{-1}) to 'trans-gauche-trans' conformation (509 cm^{-1}) and finally disappeared with the feature at 672 cm^{-1} correspond to C-S raised, which indicate Cys may play a role in copper-binding. A list of assignments for the major bands showed in Fig. S8c can be found in Table S1. In addition, tyrosine fluorescence data support our identification (see Figure S2*c*).

Materials and Methods

Preparation of Gly-Copper

The $CuCl_2$ solution for all the experiments is protected by glycine (6 eq mol to $CuCl_2$). The stock solutions were shaked at room temperature for 12 hours before use.

Fresh hIAPP Stock Solution Preparation

hIAPP (1-37) (American Peptide Company, INC., USA) stocks were prepared by dissolving in dimethyl sulfoxide (DMSO, AMRESCO, USA) to 10 mg/ml for cellular assay and to 1 mg/ml for the other, sonicating at room temperature for 15 min. Stock solutions were stored in aliquots and frozen at -20 ^{o}C prior to use. Prior to use all buffers and solutions were filtered through a 0.20 μm filter to remove any particulate matter.

Peptide Aggregation

hIAPP aggregation was induced by adding hIAPP stock solution to PBS (phosphate buffered saline, containing NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM, KH_2PO_4 2 mM), pH 7.4, to the final concentration and incubated at 37 °C.

Cell Culture

INS-1 rat insulinoma cell line was a gift from Prof. Tao Xu (Institute of Biophysics, Chinese Academy of Science, China). Cells

were grown in monolayer cultures in RPMI-1640 (GIBCO Invitrogen, USA) medium supplemented with 10% FBS (GIBCO Invitrogen, USA), 2 mmol/l l-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin, at 37 °C in a humidified (5% CO_2, 95% air) atmosphere. The cells were seeded in 96-well microplate (Corning Costar Corporation, USA) at a density of 1×10^4 cells per well for MTT assays. For TUNEL/DAPI assays, cells were seeded in 6-well plates (Corning Costar Corporation, USA) at a density of 4×10^4 cells per well.

MTT Assay

INS-1 cells seeded in 96-well microplate were cultured at 37 °C in a humidified atmosphere for 24 h, and then the cells were exposed to different treatments for 48 h. After incubation, medium was changed and Hank's Balancing Buffer (SIGMA, USA) was added followed by adding 20 μl/well of MTT (AMRESCO, USA) buffer (5 mg/ml in PBS) and incubated for 4 h. The medium was aspirated and replaced with 100 μl/well of DMSO to dissolve the formazan salt. The color intensity of the formazan solution, which reflects the cell viability, was measured at 560 nm using a Synergy 4 Plate Reader (Bioteck Company, USA).

TUNEL Assay and DAPI Staining

DNA fragmentation is one of the hallmarks of cell apoptosis which can be detected by TUNEL assay. This assay was performed according to the manufacturer's (Promega Corporation, USA) instructions. Briefly, treated INS-1 cells cultured on cover glass in 6-well plates were fixed with 4 % paraformaldehyde for 30 min at 4 ^oC. After washed in PBS, cells were permeabilized with 0.2% Triton X-100 for 20 min, and then incubated with 50 μl TUNEL reaction mixture for 60 min at 37 °C. The nuclei of the cells were double stained with 1 μg/ml of DAPI (Promega Corporation, USA) for 30 min. Stained cells were washed with PBS and observed on a fluorescence microscopy (Olympus IX71, Japan)

Thioflavin T (ThT) Fluorescence assay

Thioflavin T fluorescence assay was performed by combining 10 μl of incubated solution with 190 μl solution of 10 μM ThT in 12 mM phosphate buffer, pH 7.4. Fluorescence measurements were recorded in 96-well black microplate (Corning Costar Corporation, USA) using a Synergy 4 Plate Reader at room temperature. Ex=440 nm (slit width = 5 nm), em=482 nm (slit width = 10 nm).

Tyrosine Intrinsic Fluorescence Assay

Fluorescence spectra were collected using a FP-4500 fluorescence spectrophotometer (Hitachi, Japan). Ex= 280 nm (slit width = 5 nm), and emission was monitored over 290–400 nm (slit width = 10nm). Samples were placed in a four-sided quartz fluorescence cuvette and data were recorded at room temperature. The total peptide concentration was 10 μM in 4% DMSO. The copper concentrations change from 0 to 250 μM respectively. And data was collected after 12 h incubation.

High Resolution Transmission Electron Microscopy (HRTEM)

The HRTEM samples were prepared by placing 8 μl of the incubated solution in ThT assay on 300 mesh formvar-coated copper grids for 2 mins before removing excess solution. Then the sample was stained with 1% fresh tungstophosphoric acid for another 2 mins. The grid was blotted on filter paper and allowed to dry before observing the specimen in a JEM-2010 electron microscope (JEOL, Japan) at 120 kV.

Atom Force Microscopy (AFM)

AFM experiments were performed in tapping mode under ambient conditions using Si cantilevers with nominal resonance frequency of 330 kHz and nominal spring constant 35 N/m (Nano-scope IIIa SPM system, Veeco, USA).

Dynamic Light Scattering (DLS)

The DLS experiments were performed using the Zetasizer Nano instrument (Malvern Instruments, Worcestershire, UK). 1ml of 10 μM hIAPP solution with varies concentrations of copper was incubated at 37 °C in PBS for 12 h, centrifuged at 20000 rpm at 4 °C for 30 min to remove large precipitates, and the supernatants were transferred to a fluorescence cuvette and temperature-controlled at 25 °C within a tolerance of ±0.02 °C. A 4 mW He-Ne laser (633 nm wavelength) with a fixed detector angle of 173 ° was used for the measurements.

Cross-Linking and NuPAGE Analysis

hIAPP solution of 50 μM with/without copper (250 μM) were incubated in 10 mM PBS buffer, pH 7.4, at RT for 3 h or 48 h. Cross-linking was performed according to standard protocols. Briefly, peptide solutions were incubated with aqueous glutaraldehyde solution (25%) (SIGMA, USA) for 2 min at room temperature. The reaction was then quenched by the addition of a freshly made 2 M $NaBH_4$ in 0.1 M NaOH. Following incubation for 20 min at RT solutions were placed on ice and titrated to pH 2.5-3.0 using aqueous trichloroacetic acid (10%). Solutions were allowed to stand on ice for 20 min, and following centrifugation (10 min, 12000 rpm) pellets were dissolved and heated to 70 °C with NuPAGE sample buffer for 10 min. Then the samples were subjected to NuPAGE electrophoresis in 12% Bis-Tris gels with MES running buffer according to the manufacturer's (Invitrogen) recommendations. Silver stain kit (Beyotime Company, China) was used to demonstrate the protein content.

Hydrogen peroxide assay

The generation of H_2O_2 was detected by H_2DCF-DA assay according to an existing protocol. H_2DCF-DA (5 mM in DMSO) (Molecular probes) is deacetylated in the presence of 0.25 M NaOH for 30 min and neutralized at pH 7.4 to a final concentration of 1 mM. Reactions are carried out in PBS (pH 7.4) in a 96-well plate. They contain hIAPP (0.1 μM to 10 μM), Cu-Gly (1 μM, a reducing agent (dopamine (Sigma, USA), 5 mM), deacetylated H_2DCF 100 mM, and horseradish peroxidase (1 mM, pH 7.4, Biodee Biotechnology, China). The reaction mixture is incubated at 37 °C for 60 min. Fluorescence is recorded using a Synergy 4 Plate Reader (Bioteck Company, USA) with excitation and emission at 485 nm and 530 nm, respectively.

MALDI-TOF/MS

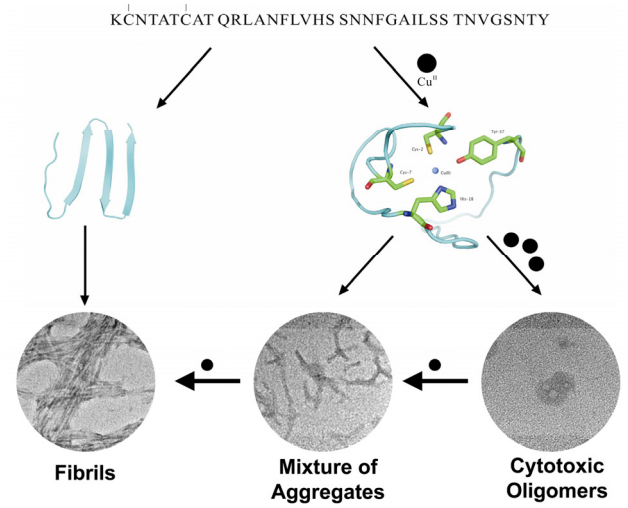
Samples were first diluted 1:1 with matrix solution (a saturated solution of 3, 5-dimethoxy-4-hydroxycinnamic acid in 50% acetonitrile with 0.1% trifluoroacetic acid in water), loaded onto a plate, and allowed to dry. The sample was then analyzed on an Applied Biosystems 4700 Proteomics Analyzer MALDI-TOF/TOF (USA) operated in reflection mode. Myoglobin was used as an internal standard.

Electron Paramagnetic Resonance (EPR) spectroscopy

The ESR spectra were recorded on a Bruker ER200D-SRC X-band spectrometer equipped with a 100 kHz field modulation. The magnetic fields were calibrated by using a Bruker ER 035M NMR gaussmeter. The microwave frequencies were measured with a Nanjing Sample Instrument SP 3382A frequency counter. The temperature was controlled using a Bruker ER 4111VT variable temperature unit.

Raman Spectroscopy

hIAPP (50 μM) and different concentrations of copper (0 μM, 25 μM, 37.5 μM, respectively) were incubated for 24 h at room temperature before test. The tubes were then centrifuged at 20000 rpm, 4 °C for 30 mins. Depositions were used for Raman spectroscopy. The samples were placed in the focused laser beam with the help of a Raman microscope system (Renishaw RM-1000, Renishaw, UK). Ten milliwatts of 633 nm laser excitation from a krypton laser was used to generate the Raman scattering under a 50X objective with a data acquisition time of 50 s for 3 times accumulation. The intensities of Raman spectra were normalized by using the Phe bending band at 1001 cm^{-1}.



Scheme S1. Putative model for copper-induced aggregation of hIAPP. Both models of aggregation are sketch map.

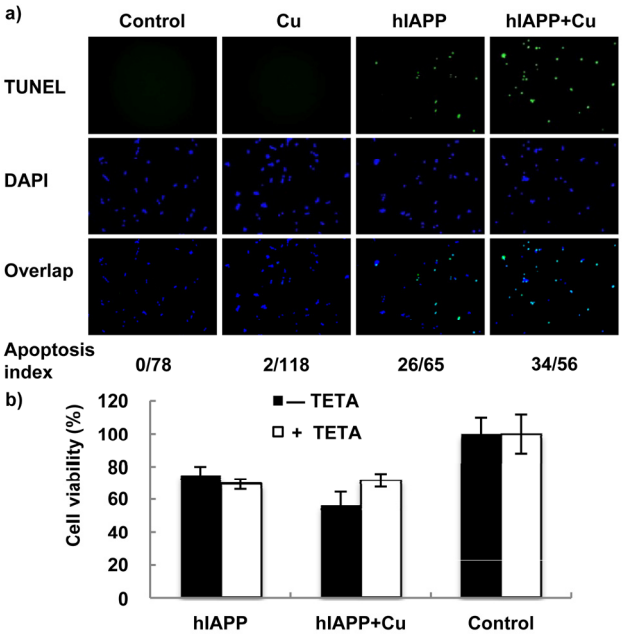


Fig. S1 hIAPP cytotoxicity increases in the presence of copper. (a) INS-1 cells incubated with 10 μM hIAPP alone or in the presence of 20 μM copper for 48 h were double-stained with DAPI (blue) and TUNEL (green) to evaluate apoptosis. Control groups mean cells treated with vehicle addition. The magnification is 200. **: $p < 0.01$ compared to hIAPP (by two-tailed Student's T-test). (b) The role of copper in the cytotoxicity of hIAPP was determined though adding the chelator trientine (TETA) in MTT assay. In the presence of TETA, the cytotoxicity of hIAPP with or without copper showed no difference.

Circular Dichroism (CD)

30 μM hIAPP with varies concentration of copper was incubated in PBS for 12h at 37 °C, centrifuged at 20000 rpm at 4 °C for 30 min to remove large precipitates. Then the supernatants were transferred for testing to ensure the CD spectra would not interfered by the aggregates. CD spectra were recorded on a Jasco model J-720 spectropolarimeter (JASCO, Tokyo, Japan) at room temperature under a constant flow of nitrogen gas. Typically a cell with a 0.1 cm path length was used for spectra recorded between 190 and 250 nm with sampling points every 0.5 nm. The spectra represent the average of 4 scans. 200 μl samples were added each time.

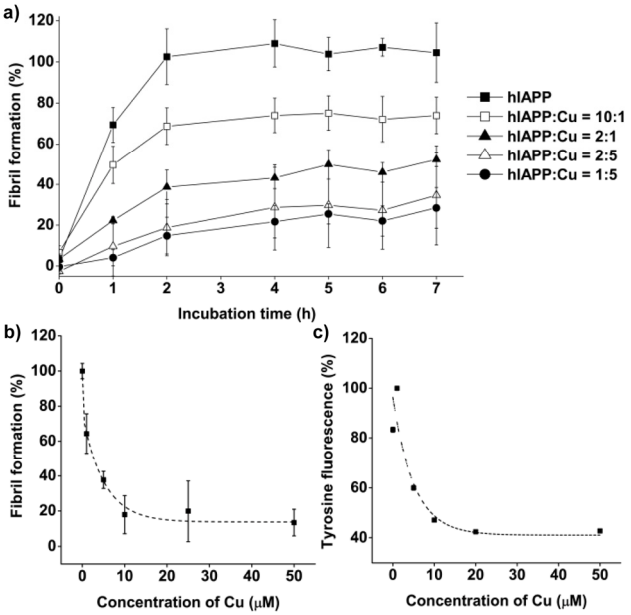


Fig. S2 hIAPP fibrillogenesis is inhibited by copper. (a) Effects of copper on hIAPP fibril formation as assessed by the ThT fluorescence assay over 7 h. hIAPP was added at 10 μM (■) along with copper at the following concentrations: 1 μM (□), 5 μM (▲), 25 μM (Δ), 50 μM (●). The data are shown as means ± SD; n = 3. (b) Dose-dependent inhibition of hIAPP fibrillogenesis by copper. hIAPP fibril formation was monitored by the ThT fluorescence assay after incubation of 10 μM hIAPP with the indicated concentrations of copper for 4 h at 37 °C. The data are shown as means ± SD; n = 3. An exponential decay curve fits the data (dotted line). (c) The tyrosine fluorescence signal of hIAPP was quenched in a dose-dependent manner when 10 μM hIAPP was co-incubated for 12 h at 37 °C with the indicated concentrations of copper. The data are shown as the mean changes in fluorescence against the fluorescence reading of phosphate buffer alone ± SD; n = 3. An exponential decay curve fits the data (dotted line).

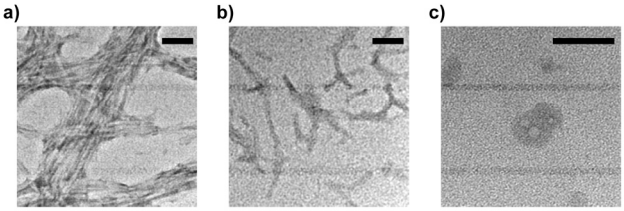


Fig. S3 Copper increases the formation of smaller and more granular hIAPP oligomers. The morphological changes of hIAPP aggregates formed upon incubation with increasing concentrations of copper were monitored by high-resolution transmission electron microscopy (HRTEM). HRTEM images of (a) hIAPP fibrils; (b) hIAPP aggregates formed upon incubation with copper (1:1) or (c) copper (1:2) for 48 h at 37 °C.

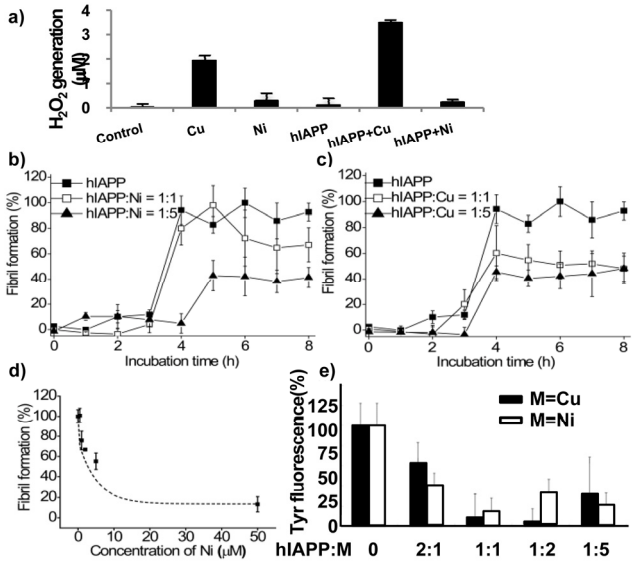


Fig. S4 hIAPP fibrillogenesis is inhibited by nickel. (a) The H_2DCF-DA test for oxidative stress was performed on solutions of copper (20 μM) and nickel (20 μM) alone or plus hIAPP (10μM). The ThT fluorescence assay was used to assess the effects of (b) nickel and (c) copper [1 μM (□), 5 μM (▲)] on hIAPP (10 μM) fibril formation (■) over 8h. The data are shown as means ± SD; n = 3. (d) Nickel inhibits hIAPP fibrillogenesis in a dose-dependent manner. The ThT fluorescence assay was used to assess the effects of the indicated concentrations of nickel on hIAPP (10 μM) fibril formation upon incubation at 37 °C for 4 h. The data are shown as means ± SD; n = 3. An exponential decay curve fits the data (dotted line). (e) The tyrosine fluorescence signal of hIAPP was quenched in a dose-dependent manner when 10 μM hIAPP was co-incubated for 12 h at 37 °C with the indicated concentrations of nickel. The data are shown as the mean changes in absorbance against the absorbance reading of phosphate buffer alone ± SD; n = 3. An exponential decay curve fits the data (dotted line).

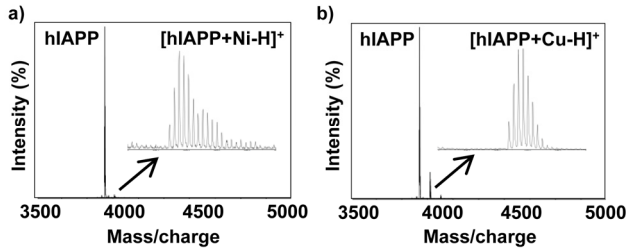


Fig. S5 Nickel binds to hIAPP similar as copper. MALDI-TOF/MS results of hIAPP (10 μM) incubated with either (a) nickel (1000 μM) or (b) copper (100 μM). Insert is the zoomed in spectra to show hIAPP-Ni/hIAPP-Cu peak clearly.

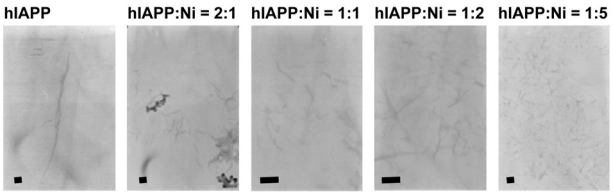


Fig. S6 Nickel enhances smaller and granular oligomer formation. The morphologic transition of hIAPP aggregation with increased concentration of nickel as monitored by TEM: hIAPP aggregation alone; hIAPP co-incubation with nickel (2:1, 1:1, 1:2, 1:5) at 37 °C for 48 h. The concentrations of hIAPP are all 10 μM. The scale bars correspond to 200 nm.

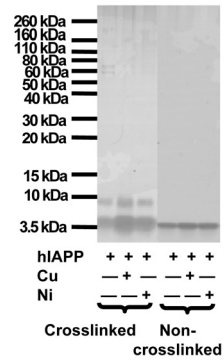


Fig. S7 Similar to copper, nickel increases hIAPP oligomer formation. The size changes of hIAPP aggregates formed upon incubation with nickel and copper were monitored. hIAPP (10 μM) was incubated alone or with 50 μM nickel or copper for 1 h at 37 °C. Subsequently, the proteins were cross-linked with glutaraldehyde (or not, as indicated) and analyzed by gel electrophoresis (NuPAGE) and silver staining.

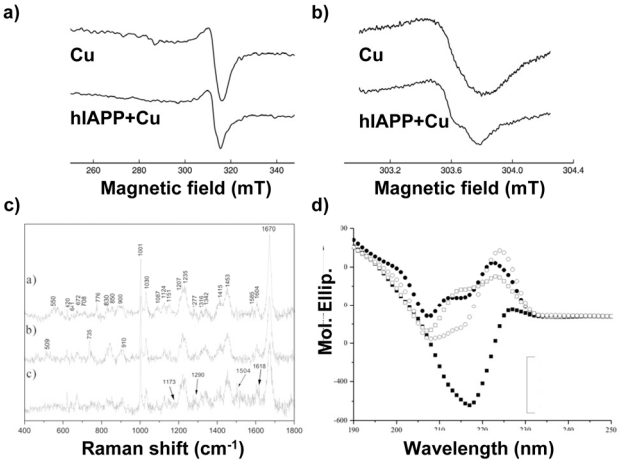


Fig. S8 (a) EPR spectra of Cu (100 μM) alone or combined with 100 μM hIAPP at pH 7.4 in Hepes buffer at -78 °C. (b) EPR spectra of copper are focused on to show the differences between copper alone and copper binding to hIAPP. (c) Raman spectra of copper ions incubated with hIAPP. The spectra are at a [Cu^{II}]: [hIAPP] mole equivalent ratio of a) 0, b) 0.5, and c) 0.75. The Raman intensities of the spectra are normalized by using the 1001 cm^{-1} Phe band as an internal intensity reference. (d) CD spectra of copper-induced hIAPP conformational changes. hIAPP was incubated at 37 °C for 12 h at 30 μM (■) along with copper at the following concentrations: 15 μM (●), 30 μM (□), 150 μM (○).The concentration of hIAPP is 30 μM. Curves are against blank and repeated for 4 times.

Table S1. Raman Frequencies and Assignments for hIAPP aggregates.

Frequency (cm^{-1})	Assignment
509	S-S (gauche-gauche-gauche)
550	S-S (trans-gauche-trans)
620	Phe
641	Tyr
672	ν(C-S) of C-S-S-C
708	ν(C-S)
735	Skeletal bending
776	Skeletal bending
830	Tyr
850	Tyr
900	ν(C-C)
910	ν(C-C)
1001	Phe
1030	Phe
1087	[C-C and C-N stretch], Lys, Arg, Gln, Asn
1124	Val, Ile + [C-Cα and C-N stretch]
1151	[C-N] + Ile
1173	Tyr, Phe
1207	Tyr, Phe
1235	Amide III in β-sheet
1277	His
1290	Cu-histidine
1316	Amide III and CH_2 twist/wag
1342	CH deformation
1415	[C(=O)N-] + Cu
1453	CH_2 deformation
1504	Tyr^-
1585	Phe
1604	Phe
1618	C=O bound to Cu (Tyr)
1670	Amide I in β-sheet