Supplementary Information (SI) for Journal of Materials Chemistry B.

Smart adaptable metal sequestering peptide conjugate to modulate Aβ fibrillar aggregation

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Reagents and solvents:

Rink amide MBHA resin (Loading 0.7 mmol/g), all Fmoc (N- terminus protected) amino acids, BOP [(Benzotriazole-1-yloxy) tris (dimethylamino) phosphonium hexafluorophosphate] and human Alzheimer's β-amyloid ($A\beta_{1-40}$) peptide were purchased from GL Biochem (Shanghai). N, N-Diisopropylethylamine (DIPEA) was purchased from Spectrochem Pvt. Ltd. Acetic anhydride (synthesis grade), N-methyl imidazole (extra pure), Trifluoroacetic acid (TFA) of extra pure grade were purchased from SRL (India). Milli-Q water at 18.2 Ω was used. Dimethylformamide (DMF, extra pure grade), dichloromethane (extra pure grade), and acetonitrile solvents of HPLC grade were purchased from Merck (India).

The procedure of peptide synthesis:[1]

The peptide AMSP was synthesized by standard Fmoc/tBu solid-phase peptide synthesis (SPPS) method. Rink amide MBHA resin (200 mg, loading 0.7 mmol) was taken in a 5 ml frit-fitted plastic syringe and allowed to swell with DCM solvent followed by DMF solvent. Next, the Fmoc group attached to the resin was cleaved with 20% piperidine in DMF, followed by washing with DMF. Subsequently, 3.0 equivalents of Fmoc amino acid, 3.5 equivalents of BOP (coupling reagent) and 6.0 equivalents of DIPEA (base) was dissolved in DMF and added to the syringe which was then allowed to rotate for coupling. After the reaction, it was acetylated (capping) with 2.0 equivalents of acetic anhydride and N-methyl imidazole in DCM. The cycle of the Fmoc cleavage of the terminal amino acid and coupling of the anticipated amino acids was continued as stated above. The sidechain of Glu was cleaved with $ZnBr_2$ in DCM followed by coupling (twice) with taurine using 4.0 equivalents of taurine, 6.0 equivalents of BOP (coupling reagent), 8.0

equivalents of DIPEA (base) in DMF/ DCM for 6h. Finally, the C-terminus of the peptide was cleaved from the Rink amide resin with 2ml of TFA: DCM (8.5:1.5) and one drop of H_2O for 3h. The reaction mixture was precipitated by cold diethyl ether to get the crude peptide that was purified and characterized.

Purification and characterization of the peptides:

The crude peptides were dissolved in acetonitrile/water mixture and purified by RP-HPLC (Waters 600E) using a C18-μ Bondapak column at a flow rate of 4 mL/ min. A binary solvent system [solvent A (0.1 % TFA in water) and solvent B (0.1 % TFA in acetonitrile)] were used. The Waters 2489 UV detector with dual detection at 214 and 254 nm was used. To purify the peptide, A total run time of 20 min. was used, and the gradient was set as 5-100 % acetonitrile for 18 min, followed by 100% acetonitrile till 20 min. The purity of the peptides was checked by a Waters 600E analytical HPLC system. An Ascentis C18 analytical column, a flow rate of 1 ml/min, a linear gradient of 5-100% acetonitrile over 18 minutes in a total run time of 20 min were used. ESI-Mass of the peptide was analyzed on Agilent-Q-TOF 6500 instrument equipped with Mass hunter workstation software.

Density Functional Theory (DFT) Calculation:

The most stable conformation of the intermediates was obtained from DFT based methods using B3LYP as energy functional and 6-31G as a basis set through Gaussian 5.0.9 program.

Molecular docking studies:

AutoDock Vina version 1.1.2 software was used for molecular docking study as described in the protocols.[2,3]

Biophysical methods used for the studies:

Aβ sample preparation. $A\beta_{1-40}$ was procured from GL Biochem Shanghai, China. Required amount of $A\beta_{1-40}$ was dissolved in 20 μL of TFA which was evaporated using nitrogen gas. HFIP was added and evaporated by purging nitrogen gas to remove TFA completely and get disaggregated $A\beta_{1-40}$. This procedure was repeated for another two times. 2.0 ml of PBS (50 mM, pH 7.4) was added into the disaggregated Aβ, followed by sonication and vortex to get a transparent solution. The total solution was divided equally (as required) followed by addition of 800 μL of PBS to each portion to obtain a final concentration of 50 μM.

Metal ion chelation effect of AMSP by Isothermal Titration Calorimetry (ITC):

ITC experiments were conducted on a MicroCal iTC200 (GE Healthcare) at 37 °C. The measurements were carried out in 50 mM PBS (pH 7.4). All the solutions were degassed properly to remove dissolved gases before titration. $A\beta_{1-40}$ peptide and AMSP was loaded distinctly in the cell, and metal chloride salts were taken in the syringe. The stirring speed for Aβ was kept at 250 rpm, pre-titration delay was 150 seconds, an initial volume of 0.4 μL and others of 2 μL were implemented (for AMSP, the values are 300 rpm, 150 seconds, 0.4 μL, and 1.3 μL respectively). The ITC data were corrected for the heat of dilution of the titrant and subtracted by injecting the metal ion solution into the sample buffer. Finally, the sequential binding sites model was selected to analyse data using Origin 7.0 software.

Thioflavin T (ThT) fluorescence assay:[3,4] Thioflavin T (ThT) was purchased from Sigma Aldrich and a stock of 50 μM in PBS (50 mM, pH 7.4) was prepared and stored at 4 °C with an aluminium-foil cover. Purified AMSP was dissolved in PBS (50 mM, pH 7.4) to obtain a solution of variable concentrations (100 μM and 500 μM of AMSP) and co-incubated with $A\beta_{1-40}$ at 37 °C along with the controls on a water bath. To execute the fluorescence study, 40 μL of peptide sample from the reaction mixtures was mixed with 200 μL of ThT solution (50 μM) and the final volume of 400 μL was made up with PBS (50 mM, pH 7.4). Three different replicate solutions were

prepared for each data point. The ThT assay was performed on a Fluoromax-4 Horiba instrument, emission was measured at 485 nm and excitation at 440 nm using a slit of 5 nm. The OriginPro 8 software was used to plot the graph of three different sets of replicate solutions and the average was taken with observed standard deviation.

Circular Dichroism (CD) Spectroscopy:[5,6] Peptide stock solutions for CD study were prepared like the ThT fluorescence assay. To perform the assay, the reaction mixtures was diluted with PBS (50 mM, pH 7.4) to get a final concentration of 100 μM (50 μM for $A\beta_{1-40}$). 400 μL of the sample was taken in a cuvette having a bandwidth of 1 mm. Spectra were recorded from 190 nm to 260 nm on a JASCO (Model J-1500) instrument. Three measurements were averaged. Observed ellipticity (mDeg) from Spectra Manager was transformed to mean residue molar ellipticity by the following equation:

$[\theta]$ (deg. cm^2 .$dmol^{-1}$) = Ellipticity (mdeg). 10^6 / Pathlength (mm). [Protein] (μM). N

Transmission Electron Microscopy (TEM):[3-4] To improve the contrast of TEM images and reduce image noise, negative staining was used. A 10 μL aliquot from the reaction mixtures was drop casted on the carbon-coated copper grid after a certain time intervals and allowed to float for 1 min followed by removing excess solution using blotting paper. Then 10 μL of 2% uranyl acetate was added on the grid and allowed to float for a further 1 min and then removed the excess solution. The sample was air-dried at room temperature and kept in a desiccator. TEM analyses were carried out on a JEOL instrument (Model: JEM 2100) at 200 kV.

Dynamic Light Scattering (DLS) spectroscopy:[7,8] The size of $A\beta_{1-40}$ aggregates and co-incubated peptide samples were determined using Zetasizer Nano-ZS90 (Malvern Instrument). To execute the DLS studies, reaction mixtures were diluted to obtain a final concentration of 10 μM. All the DLS results were reported as the average of three measurements.

Large unilamellar vesicles (LUVs) leakage study:[9,10] Large unilamellar vesicles (LUVs) were synthesized by mixing three lipids, DPPC, Cholesterol, and GM1 with 68:30:2 molar ratio, and the vesicle leakage studies was carried out as reported earlier.[3]

Characterization of AMSP:

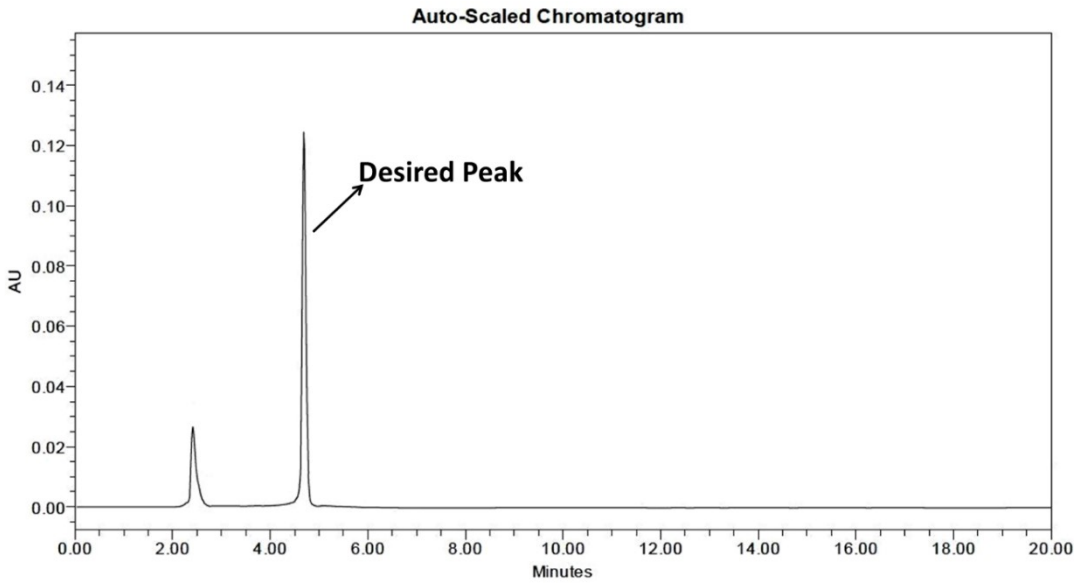

Figure S1. HPLC profile picture of the adaptable metal scavenger peptide (AMSP).

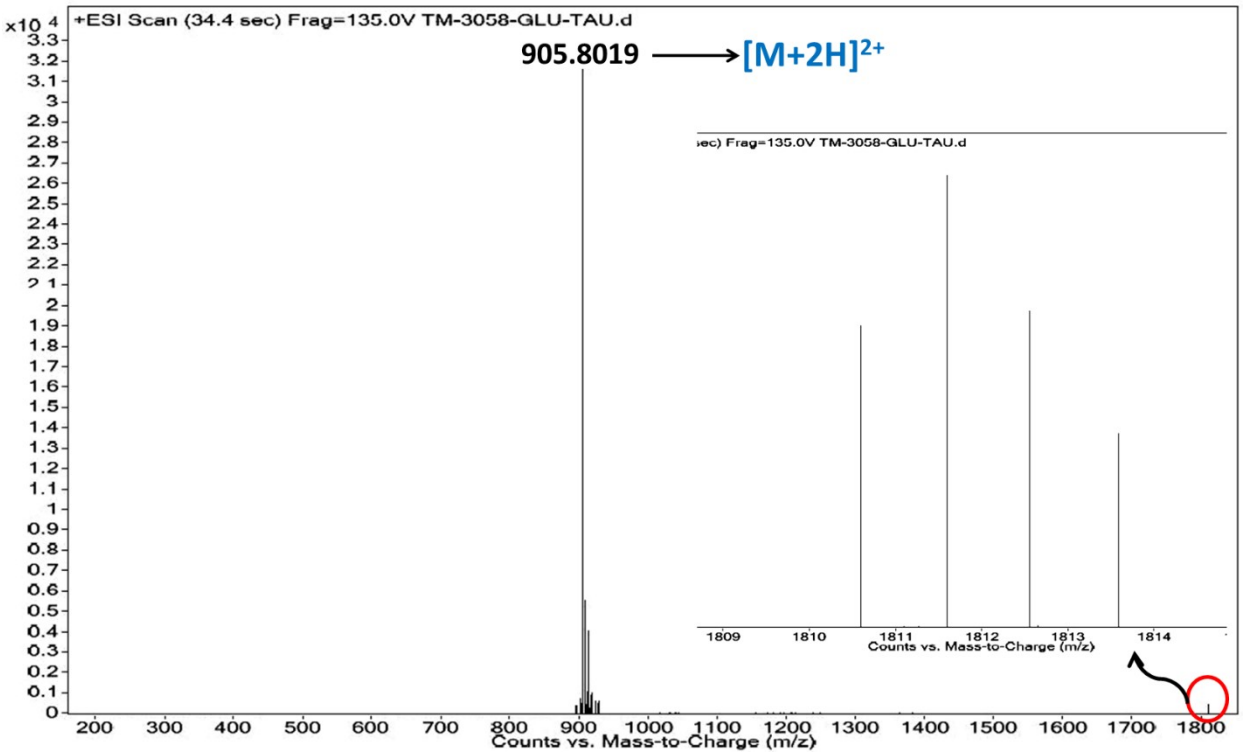

Figure S2. ESI-MS profile picture of purified AMSP. Calculated m/z for $C_{68}H_{103}N_{19}O_{31}S_4$ $[M+H]^+$ is 1810.5950, observed 1810.7 (inset); and $C_{68}H_{103}N_{19}O_{31}S_4$ $[M+2H]^{2+}$ is 905.7975, observed 905.8019.

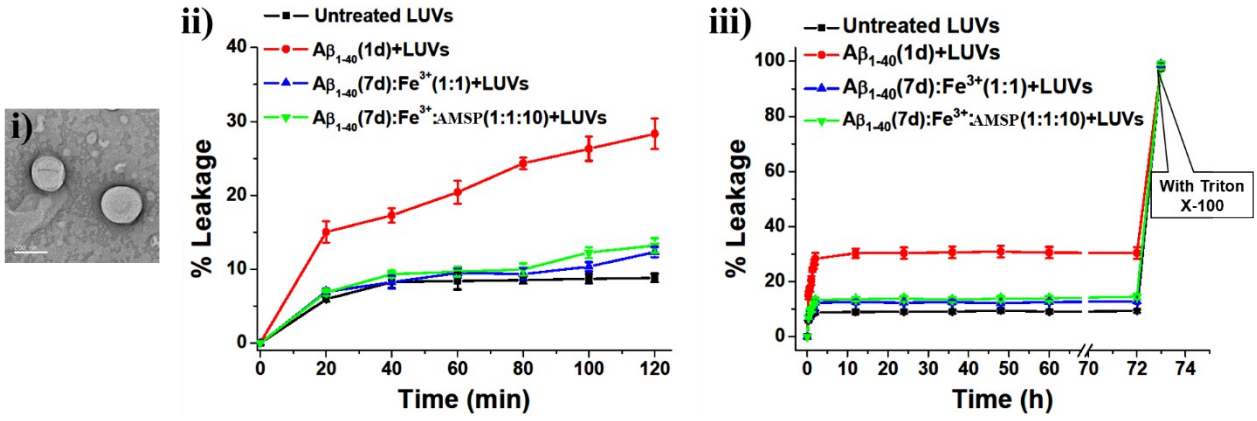

Figure S3. The TEM image (i) of negatively stained LUVs. Scale bar is indicated as 200 nm. The % of dye (carboxyfluorescein) leakage from LUVs (ii) up to 120 min and (iii) up to 72 h. The % of dye leakage by untreated LUVs (black), LUVs treated with Aβ incubated for one day (red), LUVs treated with Fe^{3+}-Aβ (1:1) for seven days (blue), and LUVs treated with Fe^{3+}-Aβ: AMSP (1:1:10)_for seven days (green). AMSP were added after three days to the preformed fibril of Fe^{3+}-$Aβ_{1-40}$. Error bars represent standard deviations of at least three independent measurements.

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