Fight fire with fire: remodeling Aβ aggregation by *H*-aggregates of a europium(III) complex

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1. Experimental Section

1.1. Reagents and Materials

Common reagents used in the experiments were all of analytical grade and purchased from commercial suppliers unless otherwise stated. Human Aβ40 was purchased from GL Biochem Ltd. (Shanghai, China). Stock solution of Aβ was prepared according to the literature method.[1] The concentration of Aβ was immediately measured with a BCA Protein Assay Kit (Pierce). EuL3 was synthesized by previously reported method.[2] Stock solution of EuL3 was obtained by dissolving the compound in DMSO and filtered using a 0.22 μm filter (organic system). All solutions and buffers were obtained using Milli-Q water, and filtered through a 0.22 μM filter (Millipore) before use.

1.2. Methods

UV-Vis spectra were measured on a Shimadzu UV-3600 UV-VIS-NIR spectrophotometer. Time-resolved luminescence and steady-state fluorescence spectra were obtained on PerkinElmer FL6500 fluorescence spectrometer. The morphology analysis of samples were carried out on a Talos L120C transmission electron microscope. Circular dichrosim (CD) measurements were performed on a JASCO J-810 automatic recording spectropolarimeter. MTT and protein assays were quantified by using a PerkinElmer Fusion Reader.

1.3. UV-Vis measurements

The samples were prepared by diluting EuL3 stock solution (4 mM, DMSO) with a mix solution of DMSO and Tris buffer (20 mM Tris-HCl, 150 mM NaCl, pH 7.4).

The final water fraction (f_w, v/v) of the samples were 20%, 30%, 40%, 50%, 60%, 70%, 80% and 90% respectively.

1.4. Binding assays

ThT competition assay: Aβ40 (20 μM) was preincubated in buffer at 37 °C for 4 h. ThT solution (ThT final concentration 20 μM) was added to Aβ40 buffers and incubated in the dark at 37 °C for 5 min. Aliquots of EuL3 or L3-3H (1.25 μL, 4 mM) were then added to the ThT–Aβ40 system at 25 °C and the fluorescence emission spectra of ThT (λ_{ex} = 440 nm) were measured in the range of 460 – 660 nm. The inhibition constant (K_i) can be determined by the Cheng-Prusoff equation.[3]

$$K_i = IC_{50}/(1 + [ThT]/K_d)$$

where IC_{50} is the final concentration of EuL3 or L3-3H at which a 50% reduction of the fluorescence has occurred, [ThT] is the concentration of ThT, and K_d of 0.75 μM is used for the binding of ThT to Aβ fibrils.[4]

8-anilinonaphthalene-1-sulfonate (ANS) fluorescence competition assay. ANS (final concentration 20 μM) was added to the pre-incubated solution of Aβ40 (20 μM, 20 mM Tris–HCl, 150 mM NaCl, pH 7.4) and co-incubated in the dark at 37 ℃ for 5 min, respectively. Different concentrations of EuL3 were added to the ANS–Aβ40 systems at 25 ℃ and the fluorescence emission of ANS in the range of 400 – 630 nm were measured with excitation wavelengths at 380 nm.

1.5. Western blotting

The Aβ40 (20 μM) solutions were incubated with or without Cu^{2+} (20 μM) in Tris buffer (20 mM Tris-HCl, 150 mM NaCl, 1% v/v DMSO, pH 7.4) in the absence or presence of EuL3 (40 μM) at 37 ℃ for different period. After incubation, the samples were dissolved in loading buffer containing a small amount of DL-dithiothreitol. Each sample was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked for 1 h at room temperature with non-fat milk powder (5%) and incubated at 4 ℃ overnight with monoclonal anti-Aβ antibody 6E10 (1:1000 dilution in PBS buffer with 0.1% Tween 20 (PBST)). The membranes were then washed (×3) with PBST buffer and incubated with the HRP-conjugated goat anti-mouse

anibody (1: 1000 dilution in PBST) for 1 h at room temperature. Membranes were washed (×3) with PBST buffer and bands were visualized using Clinx ChemiScope 6000EXP chemiluminescence imaging system.

1.6. Dot blotting

The sample solutions were prepared as described in the Western blot assay. After incubation, the supernatant of each sample was obtained by centrifugation (12000rpm, 30 min). Then, 4 μL aliquots of the supernatant was spotted onto PVDF membranes and dried at room temperature. The membranes were blocked for 1 h at room temperature with non-fat milk powder (5%) and incubated at 4 ℃ overnight with monoclonal anti-Aβ antibody 6E10 (1: 1000 dilution in PBST). The membranes were then washed (×3) with PBST buffer and incubated with the HRP-conjugated goat anti-mouse IgG (1: 1000 dilution in PBST) for 1 h at room temperature. Membranes were washed (×3) with PBST buffer and were visualized using Clinx ChemiScope 6000EXP chemiluminescence imaging system. The images of dot blot was quantitatively analyzed by ImageJ software. The insoluble aggregates of each sample were collected through centrifugation, which was then dissolved by DMSO (5 μL) and diluted to a total volume of 100 μL using Tris buffer (20 mM Tris-HCl, 150 mM NaCl, pH 7.4). Then, the solutions were analyzed by Dot blot assay under the same conditions with that for soluble Aβ.

1.7 BCA assay

The Aβ40 (20 μM) solutions were incubated with or without Cu^{2+} (20 μM) in Tris buffer (20 mM Tris-HCl, 150 mM NaCl, 1% v/v DMSO, pH 7.4) in the absence or presence of EuL3 (40 μM) at 37 ℃ for different period. After incubation, the supernatant of each sample was obtained by centrifugation (12000 rpm, 30 min). The amount of soluble Aβ in the supernatant was determined using commercially available BCA assay by following manufacture's protocol.

1.8. Transmission electron microscopy (TEM) analysis

The sample solutions were prepared in Tris buffer (20 mM Tris-HCl, 150 mM NaCl, 1% v/v DMSO, pH 7.4). Each group contains Aβ40 (20 μM) or Aβ40 (20 μM) with EuL3 (40 μM). The samples were incubated at 37 ℃ for 12 h. After incubation,

an aliquot of each solution (10 μL) was spotted on the 300-mesh carbon-coated copper grids for 2 min at room temperature and excess sample was removed. Each grid was stained with phosphotungstic acid (3%, w/v) for 2 min. The excess phosphotungstic acid was removed and water (10 μL) was spotted on each grid to wash the sample and then was removed. Finally, each grid was examined on a Talos L120C transmission electron microscope.

1.9. Circular dichrosim measurements

The CD samples were prepared by following the same procuderes as described in TEM analysis. Briefly, samples of Aβ40 (20 μM), EuL3 (40 μM) or Aβ40 (20 μM) with EuL3 (40 μM) were incubated at 37 ℃ for 0 h or 12 h. After incubation, the CD characteristic spectra of the samples were scanned from 200 to 250 nm with 1 nm bandwidth by a JASCO J-810 automatic recording spectropolarimeter.

1.10. Time-resolved luminescence measurement

The sample of Aβ40 (20 μM) with EuL3 (20 μM) were prepared in Tris buffer (20 mM Tris-HCl, 150 mM NaCl, 1% v/v DMSO, pH 7.4) and incubated at 37 ℃ for 12 h. The precipitates were isolated by centrifugation (3000 rpm, 5 min), then were dissolved by DMSO after washing with Tris buffer. The resulting solution was mixed with $Na_2S_2O_4$ (final concentration 5 mM) and incubated at 37 ℃ for 1 h to activate the luminescence of EuL3. The time-resolved luminescence spectrum of the reaction mixture (λ_{ex} = 256 nm) was measured at 25 ℃ with the following settings: delay time = 50 μs; gate time = 2 ms; cycle time = 20 ms.

1.11. Cytotoxicity assay

The cytotoxicity was performed by MTT assay with PC12 cell line *via* the cleavage of MTT to purple formazan crystals by cell mitochondrial dehydrogenases. PC12 cells were differentiated by 1 ng/mL nerve growth factor to obtain the neuronal differentiated cells that were then were seeded in a 96-well flat bottomed microplate at a density of 2000 cells per well and incubated overnight at 37 ℃ in a humidified atmosphere with 5% CO_2. To determine the toxicity of EuL3, the cells were treated with different concentration of EuL3 (0, 15, 20, 25, 30, 35, 40, and 45 μM, final DMSO concentration 2%) for 24 h at 37 ℃ in a humidified atmosphere with 5% CO_2. To

determine Aβ-induced toxicity, Aβ (20 μM) was treated with or without EuL3 (0, 10, 20, 30, 40 μM, final DMSO concentration 2%) for 4 h. The resulting solution was added to cells in each well and incubated at 37 ℃ for 24 h. The cell viability was tested as described below. Specifically, 5% MTT (5 mg mL^{-1}, PBS) was added to each well and incubated for 4 h at 37 ℃, 5% CO_2. Then the supernatants were removed, and the formazan crystals were dissolved in 200 μL of DMSO. The absorbance at 490 nm was determined using a microplate reader. The data were normalized and calculated as a percentage of untreated cells only containing 2% DMSO as control. The cell viability was assessed using MTT assay. All experiments were conducted five times to ensure reproducibility of the results.

2. Supplementary Figures

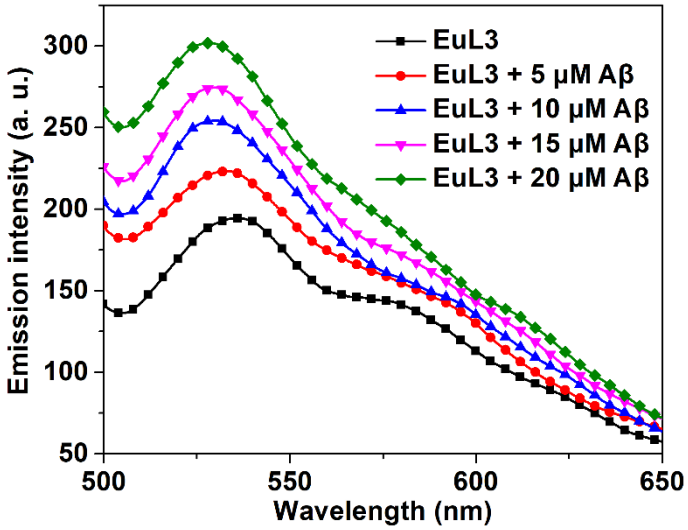

Fig. S1 The steady-state fluorescence spectra of EuL3 (40 μM, λ_{ex} = 455 nm) in the absence and presence of Aβ40 in Tris buffer (20 mM Tris-HCl, 150 mM NaCl, 1% v/v DMSO, pH 7.4).

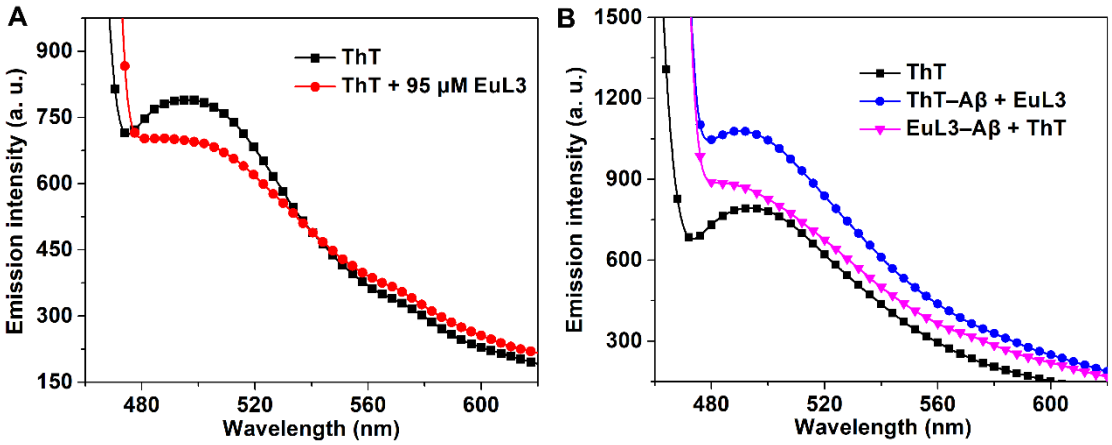

Fig. S2 (A) The emission spectra of ThT (20 μM, λ_{ex} = 440 nm) in the presence of EuL3 (95 μM) in Tris buffer (20 mM Tris-HCl, 150 mM NaCl, 1% v/v DMSO, pH 7.4). (B) The emission spectra of ThT (20 μM, λ_{ex} = 440 nm) upon addition of EuL3 (95 μM) to ThT−Aβ or addition of ThT (20 μM) to EuL3−Aβ.

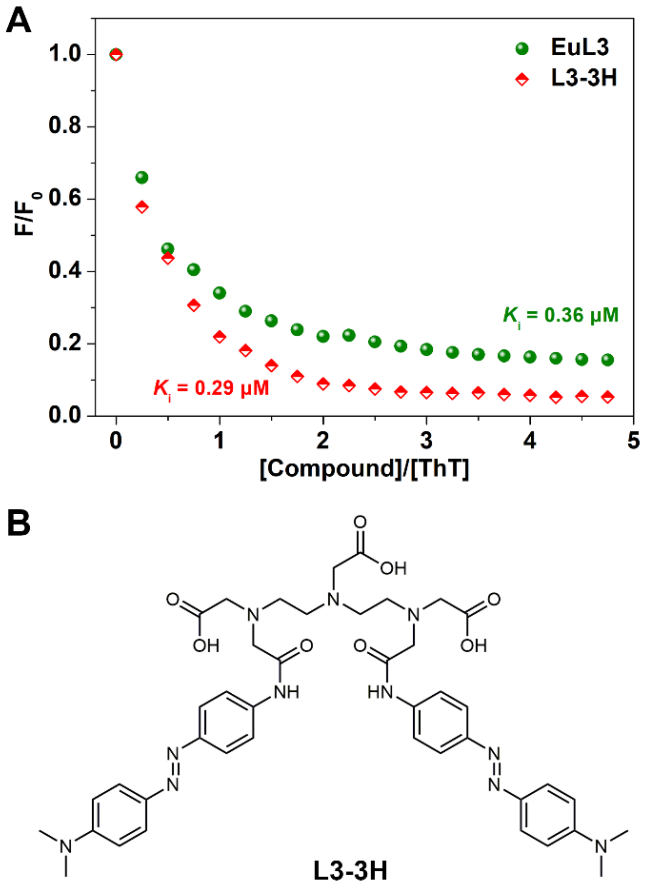

Fig. S3 (A) The emission intensity ratio (*F/F0*) of ThT (20 μM) at 484 nm *versus* the concentration ratio of EuL3 or L3-3H to ThT. Fluorescence measurements were performed in Tris buffer (20 mM Tris-HCl, 150 mM NaCl, 1% v/v DMSO, pH 7.4). (B) The structure of ligand L3-3H.

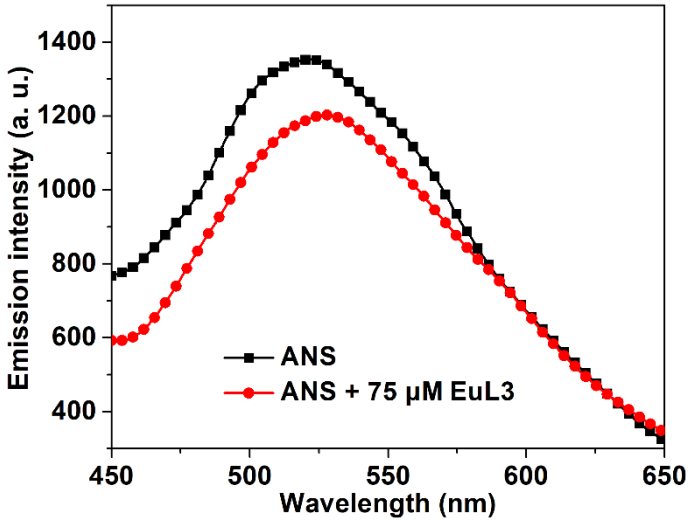

Fig. S4 The emission spectra of ANS (20 μM, λ_{ex} = 380 nm) in the presence of EuL3 (75 μM) in Tris buffer (20 mM Tris-HCl, 150 mM NaCl, 1% v/v DMSO, pH 7.4).

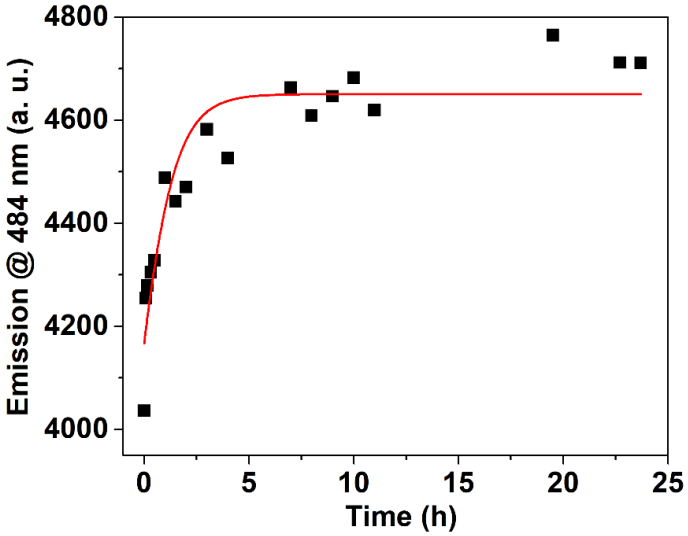

Fig. S5 Kinetics of Aβ40 (20 μM) self-aggregation monitored by ThT fluorescence assay (λ_{ex} = 440 nm) in Tris-HCl buffer (20 mM Tris-HCl, 150 mM NaCl, 1% v/v DMSO, pH 7.4) at 37 °C.

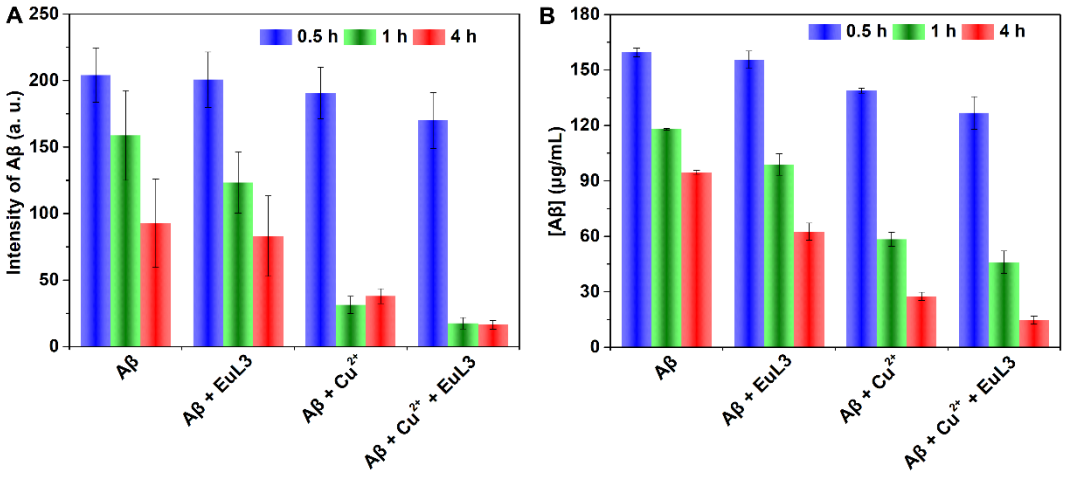

Fig. S6 (A) Quantitative analysis of soluble Aβ from the imges of Dot blot assays (Fig. 2A and B) using the image analysis programme, ImageJ. (B) The concentrations of soluble Aβ in the supernatants of Aβ solutions in the absence and presence of Cu^{2+} or EuL3, determined by BCA assay. Error bars indicate ± s.d. (n = 3 independent experiments).

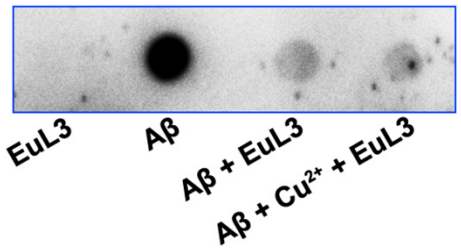

Fig. S7 Dot blot analysis of the EuL3-induced insoluble Aβ40 aggregates in the absence or presence of Cu^{2+} after incubation at 37 °C for 12 h using 6E10 antibody. The same concentration of EuL3 and Aβ40 without incubation were also measured as controls.

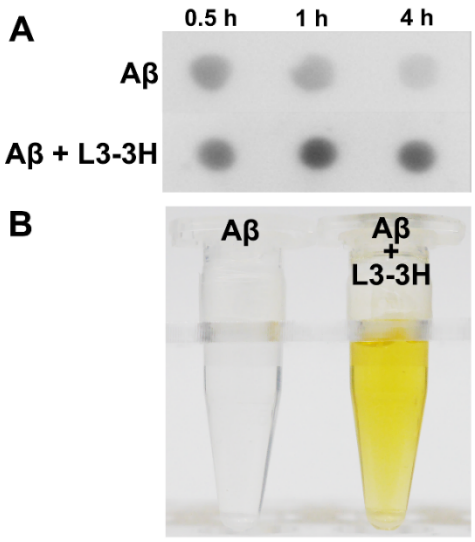

Fig. S8 (A) Dot blot analysis of Aβ40 (20 μM) in the absence or presence of L3-3H (40 μM) using 6E10 antibody. (B) Photograph of Aβ40 (20 μM) in the absence or presence of L3-3H (40 μM) after incubation at 37 ℃ for 12 h.

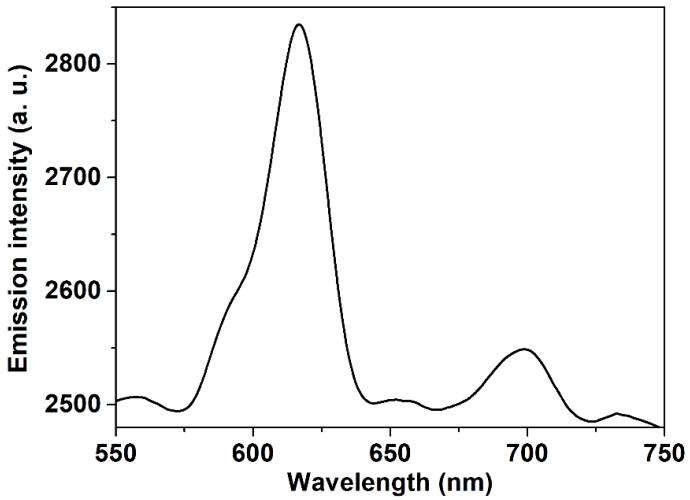

Fig. S9 The time-resolved luminescence spectra of EuL3-induced insoluble Aβ40 aggregates activated by $Na_2S_2O_4$ in Tris-HCl buffer (20 mM Tris-HCl, 150 mM NaCl, 10% v/v DMSO, pH 7.4) at 37 ℃

3. References

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2. C. Qian, Y. Ma, Y. Zhang, L. Yuan, D. Zhang, L. Zhao, J. Luo and X. Wang, *Inorg. Chem. Front.*, 2022, **9**, 2668-2675.
3. C. Yung-Chi and W. H. Prusoff, *Biochem. Pharmacol.*, 1973, **22**, 3099-3108.
4. A. Lockhart, L. Ye, D. B. Judd, A. T. Merritt, P. N. Lowe, J. L. Morgenstern, G. Hong, A. D. Gee and J. Brown, *J. Biol. Chem.*, 2005, **280**, 7677-7684.