Electronic Supplementary Information

Specific self-monitoring of metal-associated amyloid-β peptide disaggregation by a fluorescent chelator

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1Experimental Section

1.1 Reagents and Materials. Reagents used in this study were all of analytical grade, purchased from commercial suppliers and used as received unless otherwise stated. Anhydrous CCl_4 and CH_3CN were used followed by distillation and purification. 1,4,7,10-tetraazacyclododecane (cyclen) was purchased from Strem Chemicals, Inc. Human Aβ40 was purchased from GL Biochem Ltd. (Shanghai, China). Zinc chloride, copper chloride, and thioflavin T (ThT) were purchased from Sigma-Aldrich. Stock solution of Aβ40 was prepared according to the literature method.[1] TBT and cyclen stock solution (5 mM) were obtained by dissolving each chelator in dimethyl sulfoxide (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 used. C57BL/6J mice and APPswe/PSEN1 transgenic mice were purchased from Model Animal Research Center of Nanjing University (MARC). All animal experiments were in accord with institutional animal use and care regulations approved by MARC.

1.2 Methods. ^{1}H and ^{13}C NMR spectra were performed on a Bruker DRX-400 spectrometer. Electrospray ionization mass spectra (ESI-MS) were obtained by using LCQ Fleet electrospray mass spectrometer. Elemental analysis was recorded on a CHNO-Rapid analyzer (Heraeus, Germany). Circular dichroism (CD) spectra were determined by using a JASCO J-810 automatic recording spectropolarimeter (Tokyo, Japan). UV-vis spectra were acquired on a Shimadzu UV-3600 UV-VIS-NIR spectrophotometer. Fluorescence spectra were recorded on a HORIBA Fluoromax-4P fluorescence spectrometer. Turbidity, ThT, and BCA assays were determined on a Varioskan Flash microplate reader (Thermo Scientific). Transmission electron microscopy (TEM) images were obtained on a transmission electron microscope (JEOL, JEM-2100 LaB6).

1.3 Synthesis of TBT. *Synthesis of 2-(p-Tolyl)benzothiazole (**1**)*: It was synthesized according to the literature approach with some modifications.[2] Briefly, a mixture of 2-amino-benzenethiol (0.1 mol, 1.258 g) and 4-methyl benzaldehyde (0.1 mol, 1.206 g) in glycerol (30 mL) was stirred at room temperature for 10 min, followed by heating the reaction mixture to reflux for 3 h, then cooled to room temperature. The reaction mixture was washed with water (2 × 50 mL) and filtered. The crude product **1** was thus obtained and purified by silica gel column chromatography using ethyl acetate/petroleum ether (1 : 10) to yield a pure yellow solid (1.845 g, yield: 82%). ^{1}H NMR ($CDCl_3$, 400 MHz, δ, ppm): 8.06 (d, 1H, benzothiazole), 7.99 (d, 2H,

benzothiazole), 7.89 (d, 1H, benzothiazole), 7.49 (t, 1H, Ph), 7.38 (t, 1H, Ph), 7.32 (d, 2H, Ph), 2.43 (s, 3H, -CH_3). ^{13}C NMR ($CDCl_3$, 100 MHz, δ, ppm): 168.23, 154.11, 141.41, 134.90, 130.90, 129.69, 127.46, 126.22, 124.98, 123.01, 121.54, 21.50. IR (ν_{max}, cm^{-1}): 3400, 3130, 1908, 1453, 1406, 1225, 960. ESI-MS found (calcd) for $C_{14}H_{11}NS$ (m/z): 226.33 (226.06) $[M + H]^+$. Elemental analysis found (calcd) for $C_{14}H_{11}NS$ (%): C, 74.92 (74.63); H, 5.01 (4.92); N, 5.94 (6.22).

*Synthesis of 2-(4-(Bromomethyl)phenyl)benzothiazole (**2**)*: Compound **2** was synthesized based on the modified literature method.[3] Compound **1** (0.90 g, 4 mmol), NBS (0.712 g, 4 mmol) and AIBN (0.198 g, 1.2 mmol) were dissolved in 20 mL of anhydrous CCl_4. The solution was refluxed for 4 h and filtered. The solvent in filtrate was evaporated under reduced pressure. The crude product was crystallized with ethanol. White crystalline powder was obtained and dried in a vacuum (1.090 g, yield: 90%). Compound **2**: ^{1}H NMR ($CDCl_3$, 400 MHz, δ, ppm): 8.06 (t, 3H, benzothiazole), 7.87 (d, 1H, benzothiazole), 7.50 (d, 3H, Ph), 7.38 (t, 1H, Ph), 4.51 (s, 2H, -CH_2-). ^{13}C NMR ($CDCl_3$, 100 MHz, δ, ppm): 167.16, 153.98, 140.56, 135.00, 133.49, 129.68, 127.91, 126.43, 125.37, 123.27, 121.64, 32.60. IR (ν_{max}, cm^{-1}): 3400, 3134, 1903, 1473, 1406, 1222, 960, 590. ESI-MS found (calcd) for $C_{14}H_{10}BrNS$ (m/z): 303.12 (303.97) $[M + H]^+$. Elemental analysis found (calcd) for $C_{14}H_{10}BrNS$ (%): C, 55.98 (55.28); H, 3.49 (3.41); N, 4.28 (4.60).

Synthesis of 2-(4-((1,4,7,10-Tetraazacyclododecan-1-yl)methyl)phenyl)benzothiazole (TBT): 1,4,7-tris-tert-butoxycarbonyl (Boc)-1,4,7,10-tetraazacyclododecane (cyclen) (**3**) was synthesized according to the literature method.[4] The anhydrous CH_3CN solution (30 mL) of **3** (0.786 g, 1.65 mmol) and sodium carbonate (0.311 g, 3.0 mmol) was heated to reflux under N_2 for 10 min. Then CH_3CN solution of **2** (0.5 g, 1.65 mmol) was added and stirred at 80 °C for 6 h. The precipitate was filtered and the filtrate was concentrated under reduced pressure. The residue was purified by silica gel column chromatography to afford tri-Boc-protected TBT. To a cold (0 °C) solution of tri-Boc-protected TBT (0.710 g, 1.0 mmol) in methanol (15 mL) was added concentrated HCl (6 mL) dropwise. The solution was warmed to room temperature and stirred overnight. The reaction was concentrated under reduced pressure, and the crude powder was obtained followed by crystallizing from a mixture of methanol and 6 M HCl aq (2 : 1) to get TBT as a hydrochloride salt. The final product was obtained by adjusting pH value (9 ~ 10), then filtered and dried (0.325 g, yield: 89%). ^{1}H NMR ($CDCl_3$, 400 MHz, δ, ppm): 8.07 – 8.04 (t, 3H, benzothiazole), 7.89 – 7.91 (d, 1H, benzothiazole), 7.44 – 7.46 (d, 3H, Ph), 7.36 – 7.40 (t, 1H, Ph), 3.69 (s, 2H, -CH_2-Ph), 2.85 (brs, 4H, -CH_2-), 2.72 (brs, 4H, -CH_2-), 2.65 (brs, 8H, -CH_2-). ^{13}C NMR ($CDCl_3$, 100 MHz, δ, ppm): 167.91, 154.08, 142.44, 134.96, 132.46, 129.56, 127.62, 126.25, 125.09, 123.08, 121.59, 59.16, 51.39, 47.21, 46.12, 45.22. IR (ν_{max}, cm^{-1}): 3400, 3288, 3136, 2923, 2812, 1664, 1404, 1035, 754. ESI-MS found (calcd) for $C_{22}H_{29}N_5S$ (m/z): 396.58 (396.21) $[M + H]^+$, 827.75 (827.44) $[2M + 2H_2O + H]^+$. Elemental analysis found (calcd) for $C_{22}H_{29}N_5S$ (%): C, 66.58 (66.80); H, 7.04 (7.39); N, 17.11 (17.70).

1.4 Metal Binding Experiments. The fluorescence titrations of TBT (λ_{ex} = 305 nm) were determined by adding aliquots of 2.4 μL of $ZnCl_2$ or $CuCl_2$ aqueous solution (5 mM) to 3 mL of TBT solution (40 μM, 20 mM Tris-HCl, 150 mM NaCl, 8‰ v/v DMSO, pH 7.4) in a cuvette.

The stability constant (K_a) of TBT with Zn^{2+} was determined using the nonlinear least-squares analysis based on a 1:1 complex expression with equation 1:[5]

$$\frac{F}{F_0} = 1 + \left(\frac{F_{max}}{2F_0} - \frac{1}{2}\right)\left[1 + \frac{C_M}{C_L} + \frac{1}{K_a C_L} - \sqrt{\left(1 + \frac{C_M}{C_L} + \frac{1}{K_a C_L}\right)^2 - 4\frac{C_M}{C_L}}\right] \quad (1)$$

Where F and F_0 were the fluorescence intensities of TBT at 375 nm in the presence and absence of Zn^{2+}, C_M and C_L were the concentrations of Zn^{2+} and TBT, and K_a was the stability constant.

The association constant (K_a) for the binding of TBT with Cu^{2+} was calculated by nonlinear fitting with equation 2:[6]

$$\frac{F - F_{\min}}{F_{\max} - F} = K_a C_M^n \quad (2)$$

Where F was the fluorescence intensity of TBT at 375 nm, F_{min} was the fluorescence intensity without Cu^{2+}, F_{max} was the maximum intensity in the presence of Cu^{2+}, C_M was the concentration of Cu^{2+}, n was the binding stoichiometry, and K_a was the association constant.

The fluorescence responses of TBT (40 μM, λ_{ex} = 305 nm) to different metal ions (40 μM) were collected in buffer (20 mM Tris-HCl, 150 mM NaCl, 4‰ v/v DMSO, pH 7.4) at 25 °C.

1.5 Aβ Binding Experiments. *ThT competition assay:* Aβ40 (20 μM) was preincubated in buffer at 37 °C with or without Zn^{2+} (20 μM) for 7 days or 24 h, respectively. 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 TBT (0.8 μL, 5 mM) were then added to the ThT–Aβ40 system at 25 °C and the fluorescence emission spectra of ThT (λ_{ex} = 415 nm) were measured in the range of 445 – 650 nm. The inhibition constant (K_i) can be determined by the Cheng-Prusoff equation 3:[7]

$$K_i = IC_{50} / (1 + ([ThT] / K_d)) \quad (3)$$

Where IC_{50} was the final concentration of TBT at which a 50% reduction of the fluorescence has occurred, [ThT] was the concentration of ThT, K_d of 0.75 μM was used for the binding of ThT to Aβ fibrils.[8]

Docking of TBT with Aβ40: The molecular docking program Autodock 4.2.3 and Autodock tools 1.5.4 were employed to dock TBT to Aβ40.[9] The geometry of TBT was optimized using density functional theory (DFT) at the level of B3LYP/6-311G by the program Gaussian 09.[10] The molecular structures of Aβ40 (PDB: 1BA4) was obtained from Protein Data Bank.[11] All water molecules and ligands were removed from the structure of Aβ40, and essential hrdrogen atoms, Kollman united atom type charges, and salvation parameters were also added in the protein model. The Gasteiger partial charges were added to atoms of TBT, and rotatable bonds were defined. In addition, the grid box sizes of TBT–Aβ40 were 126 Å × 126 Å × 126 Å, with grid spacing of 0.375 Å, while the GA population size was set to 150, the maximum number of energy evaluation was set to 25 × 10^6, the number of GA runs was 100, and others used were default parameters. The lowest energy conformation was selected and figures of TBT docked with Aβ40 were depicted using Pymol.

1.6 Disassembly of Metal-Induced Aβ Aggregation Experiments. For all experiments described herein, the same reaction concentration (20 μM) of Aβ40, metal ions, and chelators was adopted and the final concentration of DMSO was 4‰. Each sample solution (199.2 μL, 20 mM Tris-HCl/150 mM NaCl, pH 7.4) consisting of Aβ40, Zn^{2+} or Cu^{2+} was incubated at 37 ºC for 24 h. Then, cyclen or TBT (0.8 μL, 5 mM) solution was added followed by incubation at 37 ºC for further 24 h. The degree of Aβ aggregation was studied by turbidimetry, microBCA protein assay, TEM, and CD spectroscopy. CD spectroscopy were operated as follows: the sample solutions were prepared by the same way as described above and incubated at 37 ºC for 12 h. CD spectra of the samples were recorded on a JASCO J-810 automatic recording spectropolarimeter (Tokyo, Japan) in the range of 190 – 300 nm. The other assays were carried out as described in the literature.[12]

1.7 Self-monitoring of Disassembly of Metal-Induced Aβ Aggregates. Aβ40 (20 μM) was incubated with Zn^{2+} or Cu^{2+} (20 μM) in buffer (20 mM Tris-HCl, 150 mM NaCl, pH 7.4) at 37 °C for 24 h. TBT (final concentration 20 μM, 4‰ v/v DMSO) was added to the metal-induced Aβ aggregates solutions, followed by further incubation at 37 °C for 2, 4, 6, 8, and 10 h, respectively. Each sample was conducted by turbidimetry assay and fluorescence spectroscopy of TBT (λ_{ex} = 305 nm, λ_{em} = 375 nm).

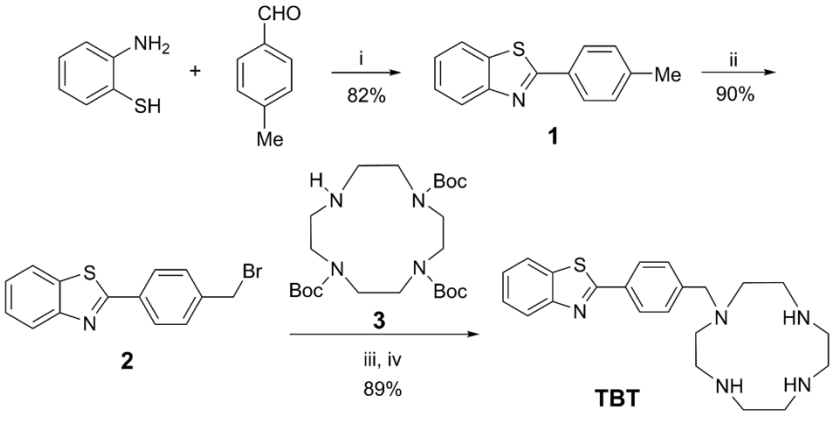
1.8 Western Blot Analysis of Brain Homogenates. Extracting of APPswe/PSEN1 transgenic mice brain (6-month-old, male) was prepared according to the literature reported.[13] TBT stock solution (0.8 μL, 5 mM),

or DMSO (0.8 μL, final concentration 4‰) was added to the supernatant (200 μL), followed by incubation at 37 °C for 24 h. The samples were dissolved in loading buffer and boiled at 95 °C for 15 min. Each sample was separated by SDS polyacrylamide gel electrophoresis (PAGE), followed by transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were taken out and blocked for 1 h at room temperature with 5% non-fat milk powder (Tris buffered saline containing 0.1% Tween-20). Then, the membranes were incubated overnight with monoclonal anti-Aβ antibody 6E10 (diluted by TBS-T containing 5% BSA, 1 : 1000, Covance Inc) at 4 °C. Then, the membranes were taken out and rainsed with TBS-T buffer for 3 × 5 min and incubated with the HRP-conjugated goat anti-mouse anibody (1: 10000) for 1 h at room temperature. Bands were visualized with SuperSignal (Thermo Scientific Inc.). The membranes were then reprobed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was used as internal reference to ensure equal protein loading.

1.9 Cytotoxicity Assay. The cytotoxicity was performed by MTT assay with PC12 cell, *via* the cleavage of MTT to purple formazan cystals by cell mitochondrial dehydrogenases. Briefly, PC12 cell were seeded in a 96-well flat bottomed microplate at 6000 cells/well and incubated at 37 °C in a humidified atmosphere with 5% CO_2. Then, Aβ40 (10 μM), Zn^{2+}, or Cu^{2+} (20 μM) was added into the tissue at 37 °C for 15 min, followed by added with chelators (8 μM, final DMSO concentration 1%). After incubation at 37 °C for 24 h, 50 μL MTT reagent (5 mg/mL) was added to each sample solution. The samples were re-incubated for 4 h at 37 °C in a humidified atmosphere with 5% CO_2. Then removed the supernatants, the formazan crystals were dissolved in 300 μL DMSO. The absorbance at 490 nm was determined using a Varioskan Flash microplate reader.

1.10 Brain Uptake Assay. Both control and treatment groups contained three mice (C57BL/6J, female, 8 − 10 weeks old). Briefly, each mouse was given TBT (4 mg/kg, dissolved in a 10 : 90 DMSO/H_2O mixture, 200 μL) *via* the tail vein injection. Then mice were sacrificed after 2, 10, 30, 60 min, and brain tissue was collected and rinsed with cold PBS. Brain tissue was diluted with water (600 μL) and homogenized. The homogenate was mixed with MeOH (1400 μL) and centrifuged. The supernatants were filtered (0.22 μm) and concentrated to 70 μL. A 20 μL sample was subjected to HPLC analysis using a C18 reverse-phase column (250 mm × 4.6 mm) (Agilent). The mobile phase was a MeOH/H_2O mixture (70 : 30, v/v). The flow rate was 1 mL/min. The absorption wavelength was 305 nm. For control experiment: C57BL/6J mice were sacrificed and the brains were removed, 200 μL of the inject solution of TBT was directly injected into the brains (n = 3) and incubated for 1 h at 37 °C, and then treated by the previous procedures. The standard was obtained by analyzing the solution of TBT (200 μL diluted to a mixture of MeOH and water).

2 Supplementary Scheme, Figures, and Tables.



Scheme S1 Synthetic route to TBT: (i) glycerol, 130 °C, 3 h; (ii) NBS, AIBN, CCl_4, 76 °C, 4 h; (iii) **3**, Na_2CO_3, CH_3CN, 80 °C, 6 h; (iv) conc. HCl, MeOH, 0 °C, then 0 °C to rt for 12 h, then NaOH.

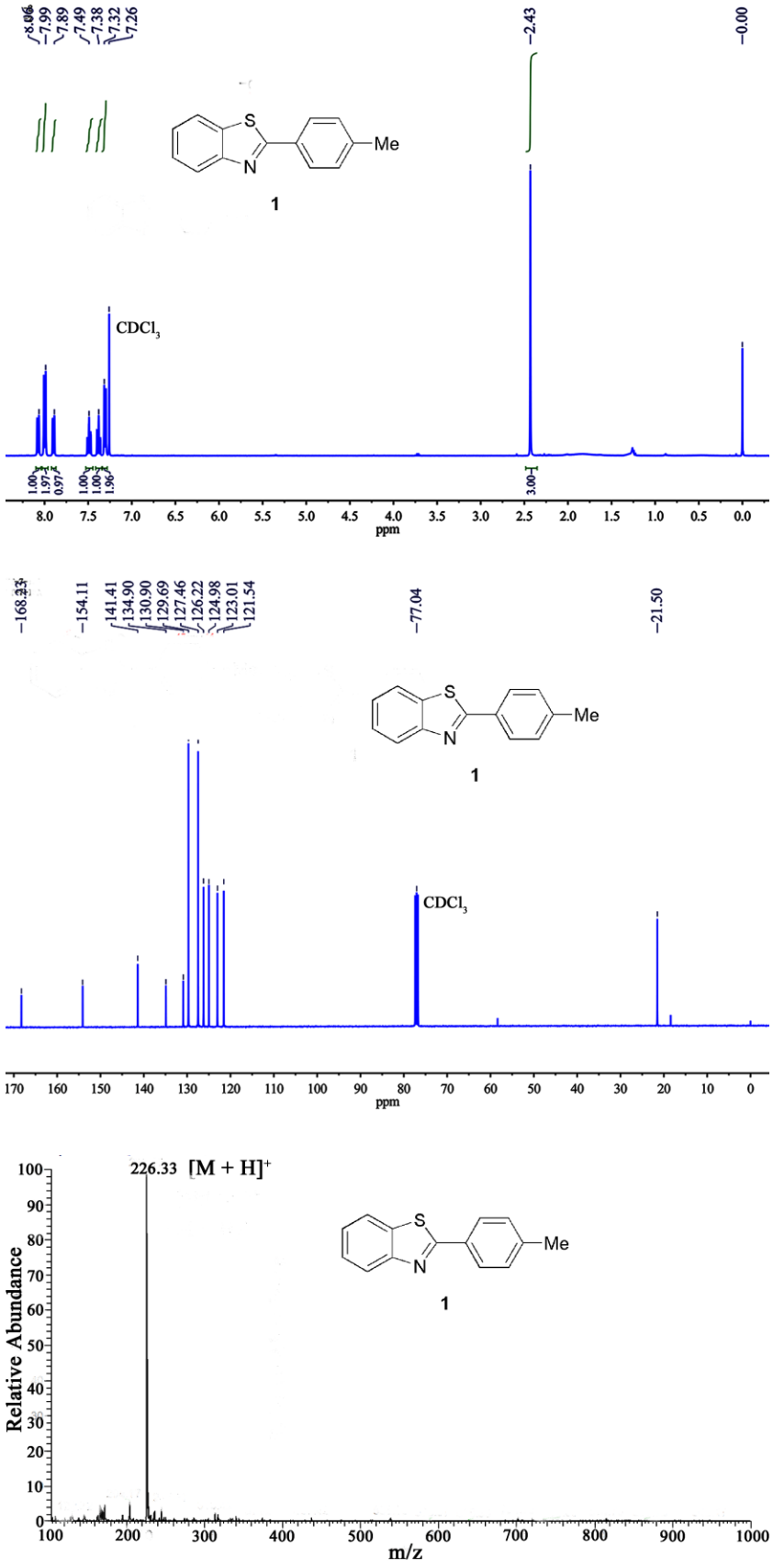


Fig. S1 ^{1}H NMR ($CDCl_3$), ^{13}C NMR ($CDCl_3$), and ESI-MS spectra for **1**.

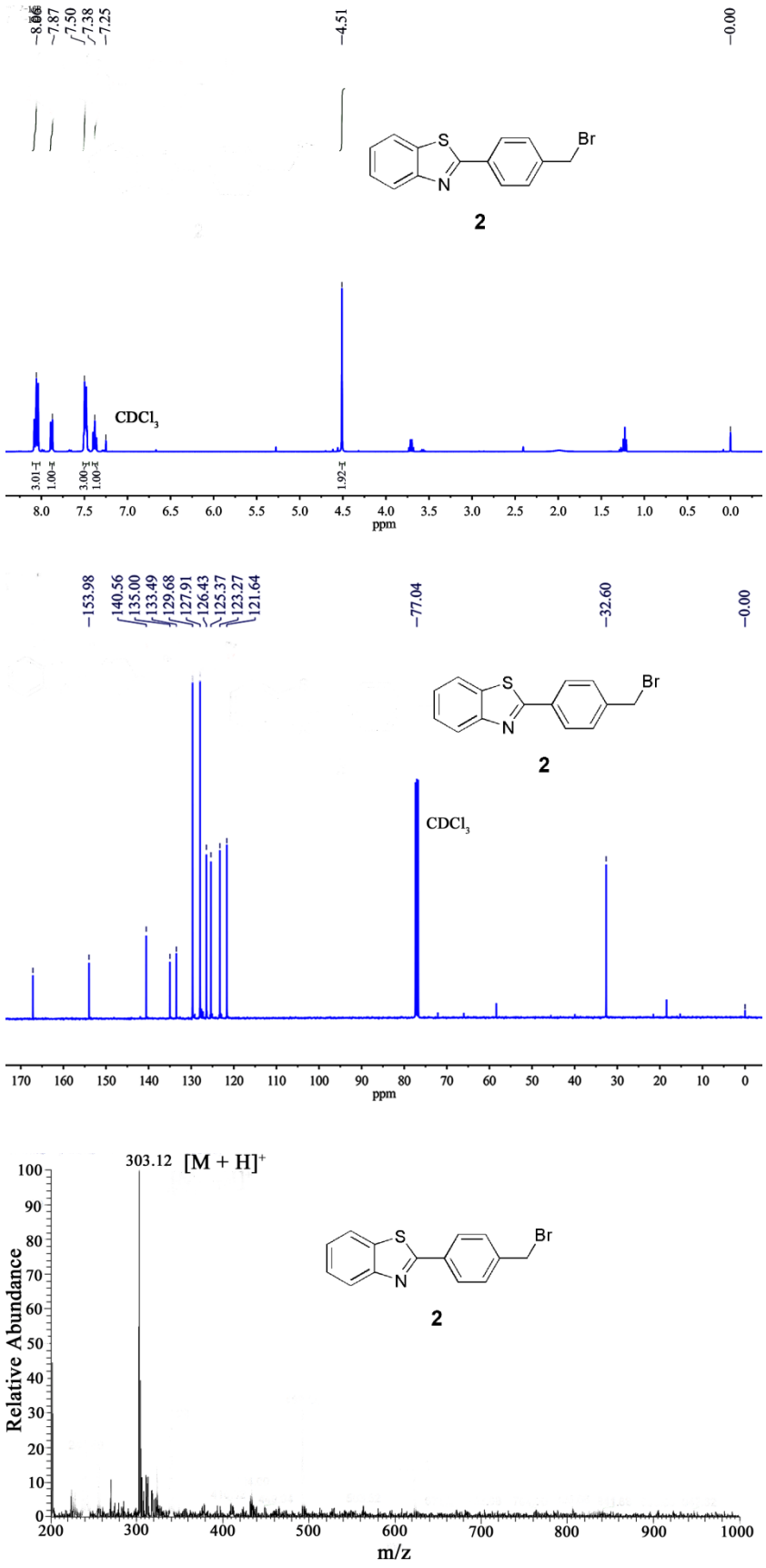


Fig. S2 ^{1}H NMR ($CDCl_3$), ^{13}C NMR ($CDCl_3$), and ESI-MS spectra for **2**.

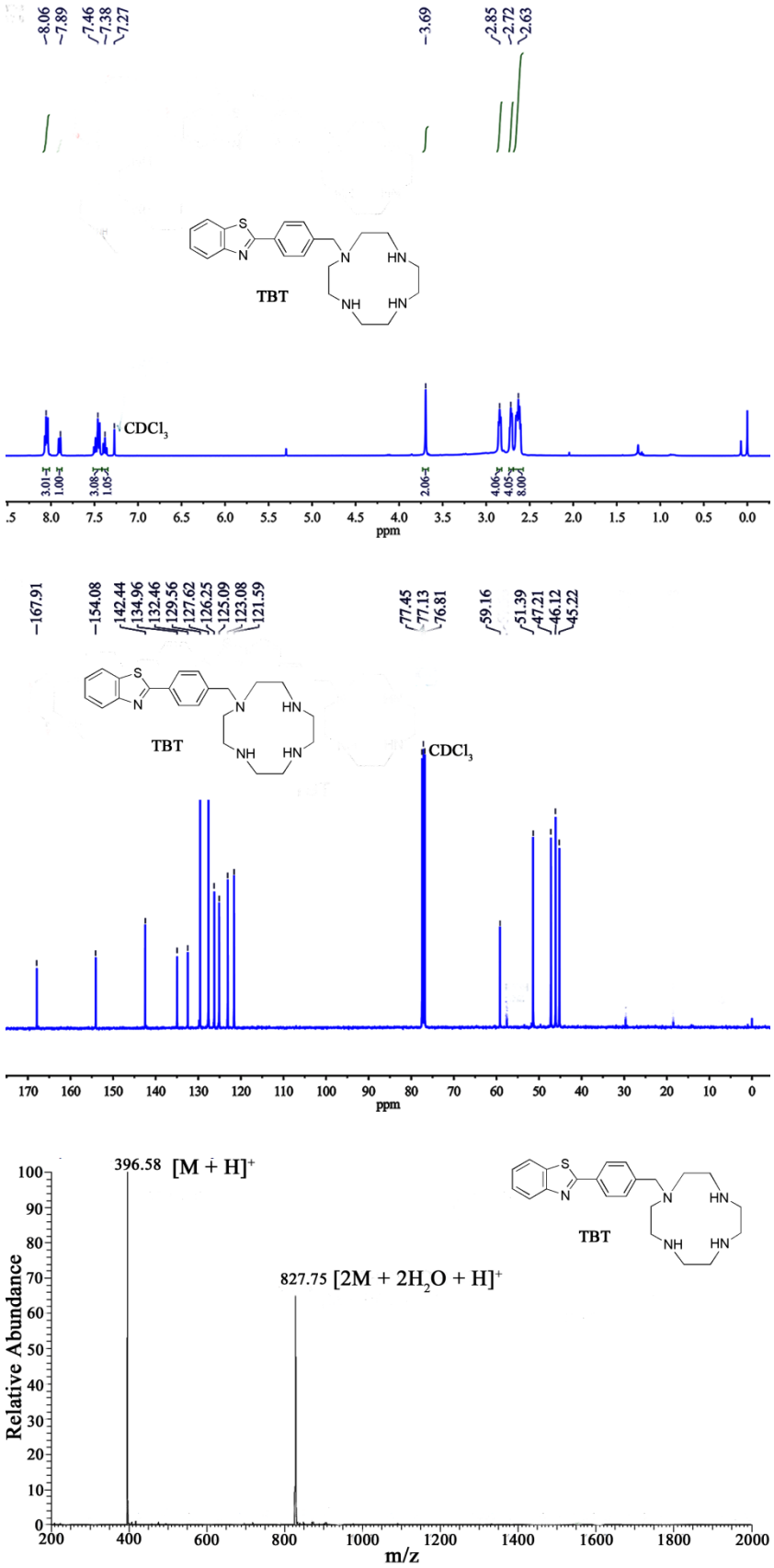


Fig. S3 ^{1}H NMR ($CDCl_3$), ^{13}C NMR ($CDCl_3$), and ESI-MS spectra for **TBT**.

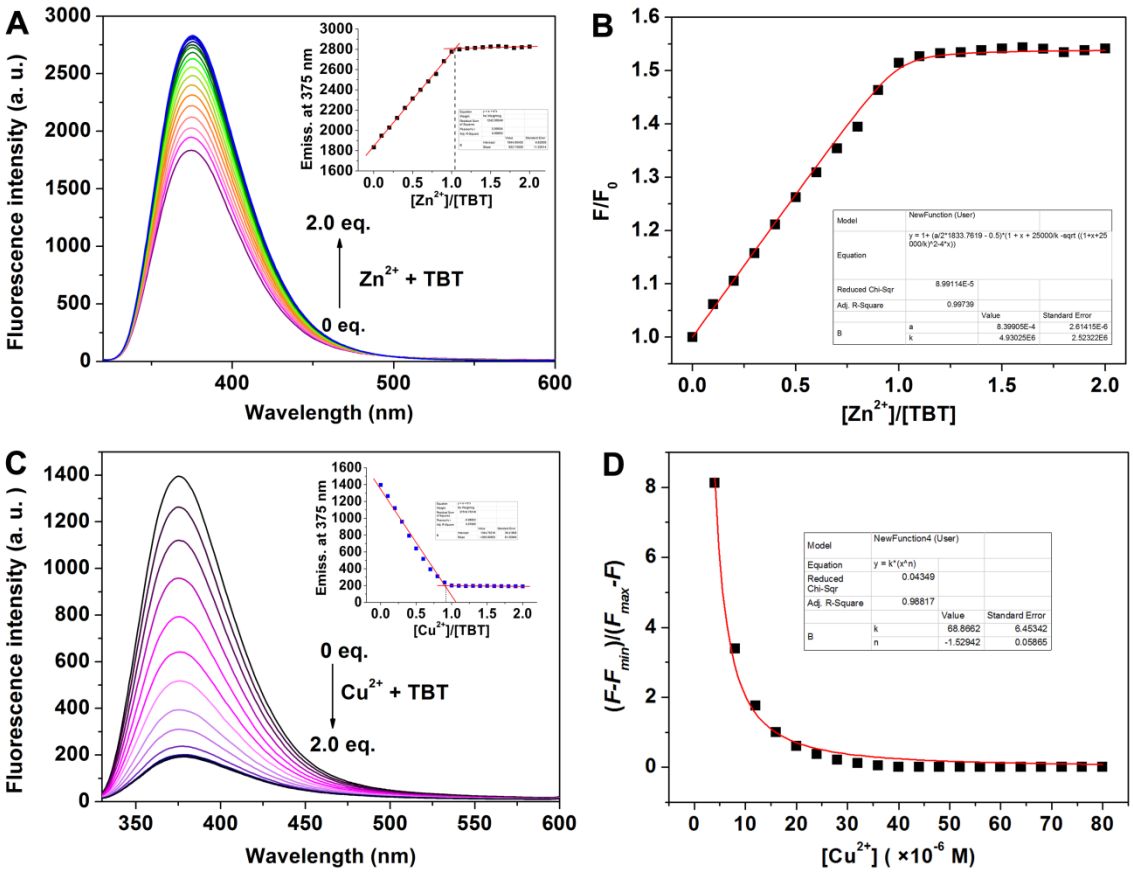


Fig. S4 (A) The fluorescence spectra of TBT (40 μM, λ_{ex} = 305 nm) upon addition of increasing concentration of Zn^{2+} in buffer (20 mM Tris-HCl, 150 mM NaCl, 8‰ v/v DMSO, pH 7.4). Inset shows the emission intensity at 375 nm of TBT versus different [Zn^{2+}]/[TBT] ratio. (B) A nonlinear least-squares curve fitness with respect to the emission intensity ratio (F/F_0) of TBT at 375 nm as a function of [Zn^{2+}]/[TBT] ratio. (C) The fluorescence spectra of TBT (40 μM, λ_{ex} = 305 nm) upon addition of increasing concentration of Cu^{2+} in buffer. Inset shows the emission intensity at 375 nm of TBT versus different [Cu^{2+}]/[TBT] ratio. (D) A nonlinear least-squares curve fitness with respect to the emission intensity ratio $(F\text{-}F_{min})/(F_{max}\text{-}F)$ of TBT at 375 nm as a function of [Cu^{2+}].

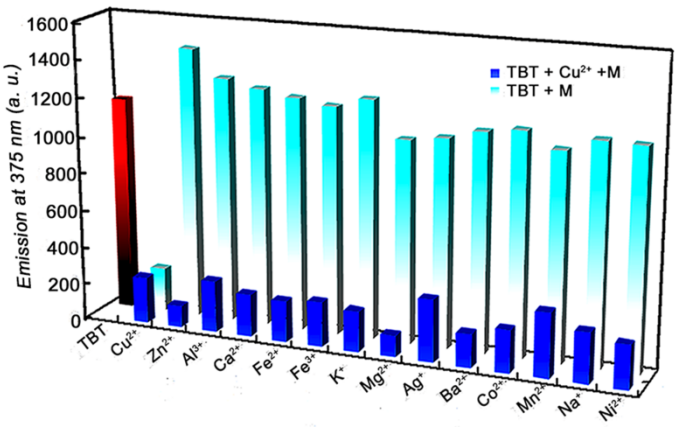


Fig. S5 Fluorescence intensity of TBT (40 μM, λ_{ex} = 305 nm) at 375 nm in response to different metal ions (40 μM) in buffer (20 mM Tris-HCl, 150 mM NaCl, 8‰ v/v DMSO, pH 7.4). Cyan bars represent the addition of different metal ions to the solution of TBT. Blue bars represent subsequent addition of Cu^{2+} to the solution.

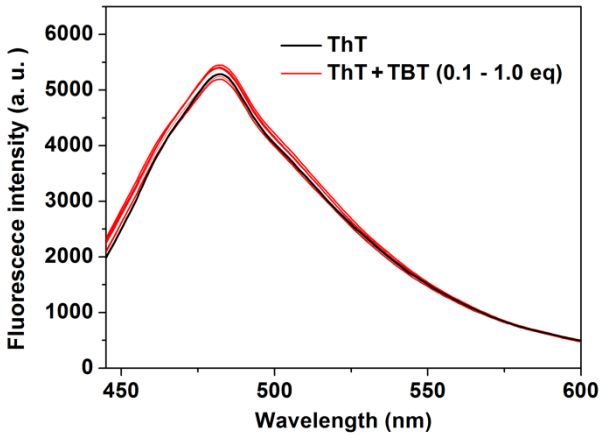


Fig. S6 The fluorescence spectra of ThT (20 μM, λ_{ex} = 415 nm) in the absence and presence of different amount of TBT.

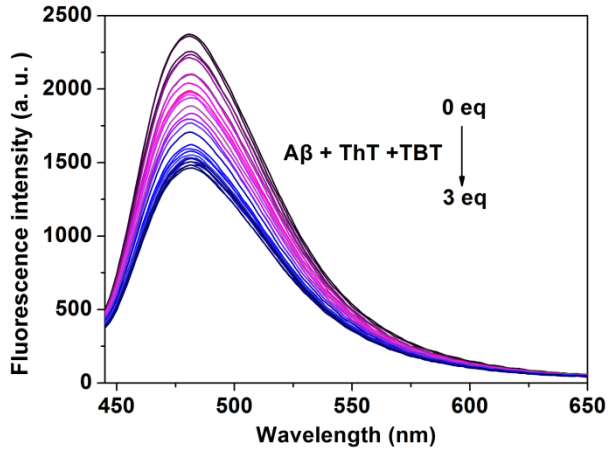


Fig. S7 The fluorescence spectra of ThT (20 μM, λ_{ex} = 415 nm) upon addition of increasing amounts of TBT in the presence of metal-free Aβ40 fibrils.

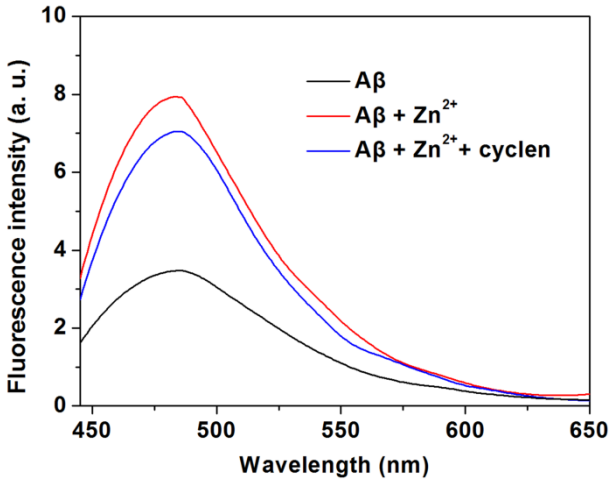


Fig. S8 The fluorescence spectra of ThT (20 μM, λ_{ex} = 415 nm) upon addition of cyclen in the presence of Zn^{2+}-induced Aβ40 aggregates.

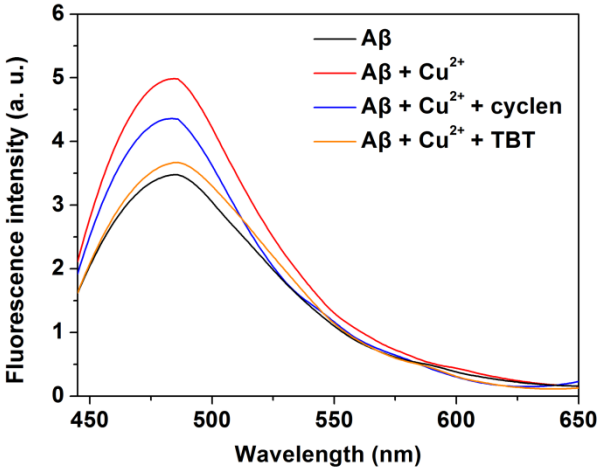


Fig. S9 The fluorescence spectra of ThT (20 μM, λ_{ex} = 415 nm) upon addition of TBT or cyclen in the presence of Cu^{2+}-induced Aβ40 aggregates.

Table S1 Interaction energies between TBT and responsive amino acid residues in molecular docking.[a]

amino acid residues	interaction energy (KJ mol^{-1})
Ala30	–2.2070
Asn27	–15.8513
Asp1	–7.4419
Asp23	–21.3580
Glu3	–7.6005
Glu11	–0.9198
Glu22	–4.2060
Gly29	–0.7791
Lys16	–1.8773
Lys28	–0.3445
Phe19	–11.2808
Phe20	–0.7012
Ser26	–15.8008

aThe MolDock score and ReRank score are –93.582 and –78.613, respectively.

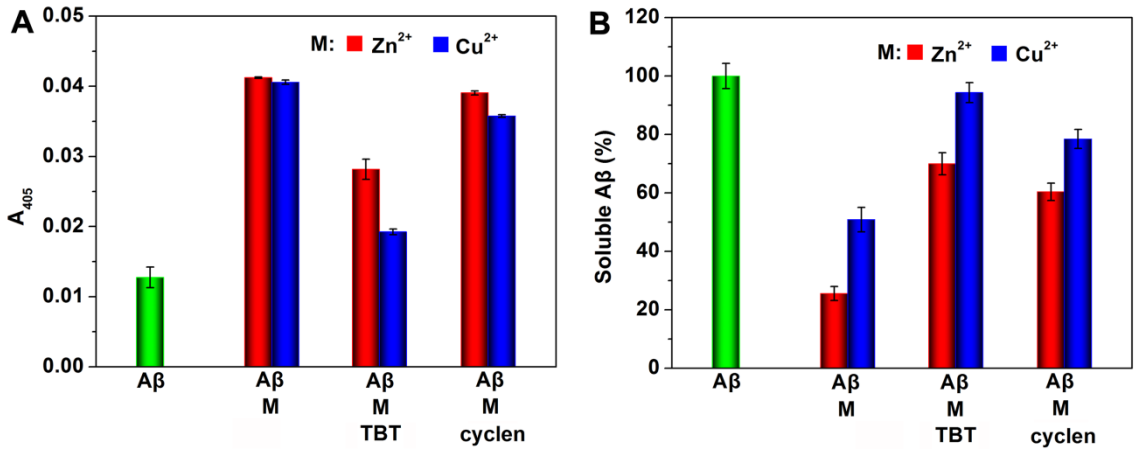


Fig. S10 The disaggregation of Zn^{2+}- or Cu^{2+}-induced Aβ40 aggregates by TBT using turbidimetry (A) and microBCA assay (B) (n = 3).

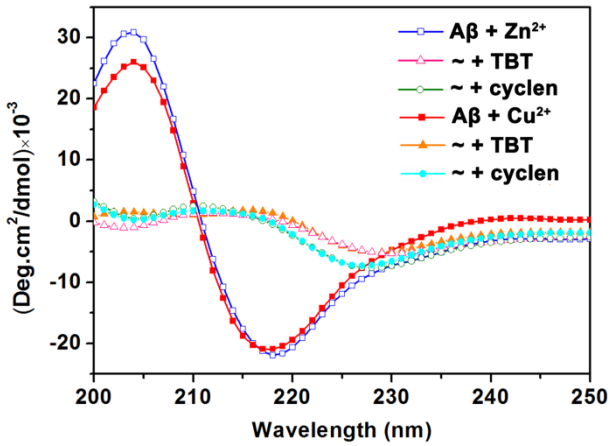


Fig. S11 The reversal effect of TBT and cyclen on Zn^{2+}- or Cu^{2+}-induced Aβ40 aggregation by CD measurement.

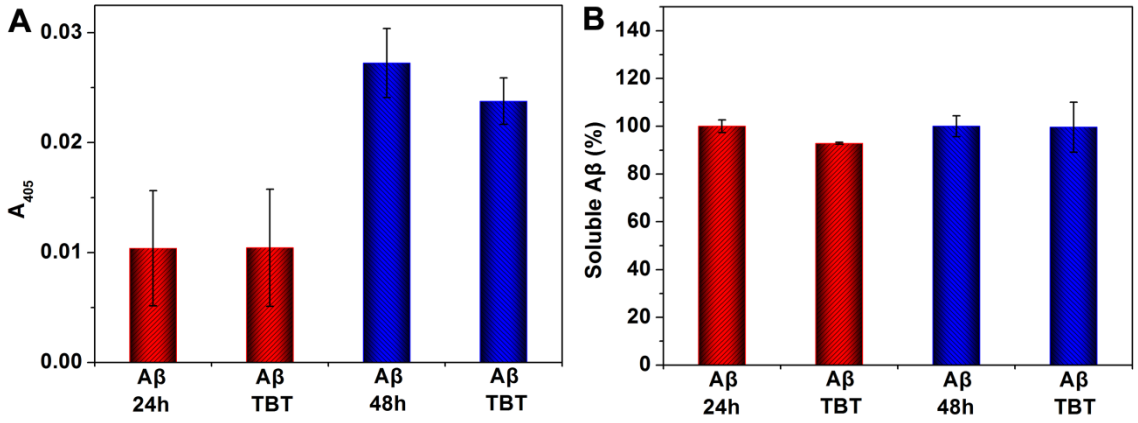


Fig. S12 The effect of TBT on metal-free Aβ40 aggregates by turbidimetry (A) and microBCA assay (B) (n = 3).

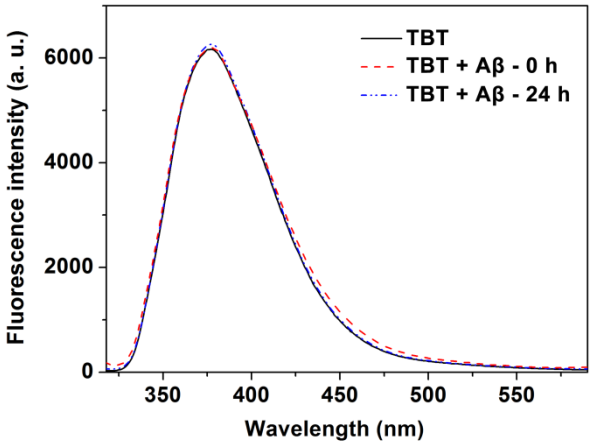


Fig. S13 The fluorescence spectra of TBT (20 μM, λ_{ex} = 305 nm) in the absence and presence of metal-free Aβ species (20 μM) with preincubation at 37 ºC for 0 h and 24 h, respectively.

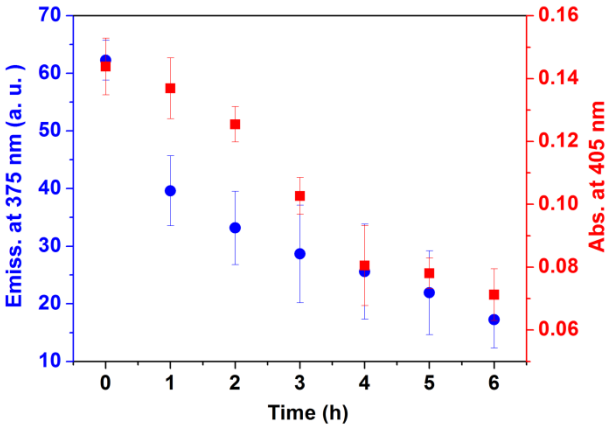


Fig. S14 Fluorescence emission intensity at 375 nm of TBT (20 μM, λ_{ex} = 305 nm) and turbidity (A_{405}) of performed Cu^{2+}–Aβ40 aggregates solution in the presence of TBT after incubation for different periods of time (n = 3).

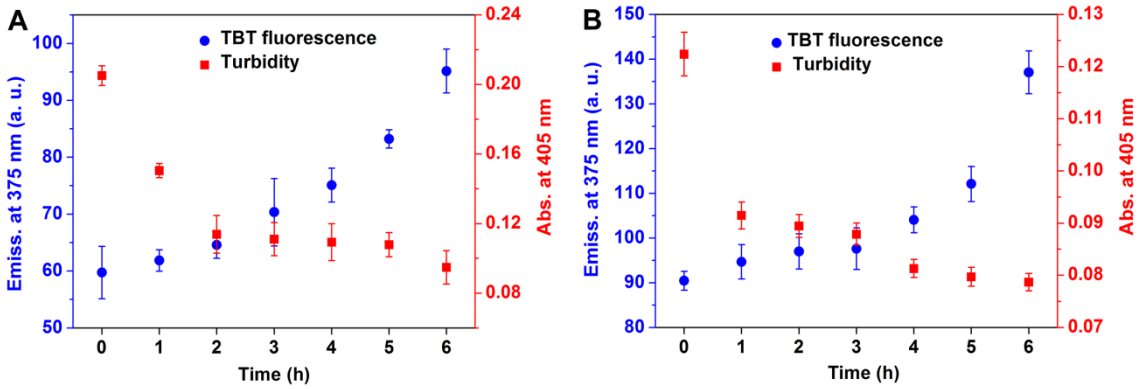


Fig. S15 Fluorescence emission intensity at 375 nm of TBT (20 μM, λ_{ex} = 305 nm) and turbidity (A_{405}) of performed Zn^{2+}–Aβ40 aggregates solution in the presence of TBT after incubation for different periods of time (n = 3) in PBS buffer (20 mM phosphate, 150 mM NaCl, pH 7.4) (A) and HEPES buffer (20 mM HEPES, 150 mM NaCl, pH 7.4) (B).

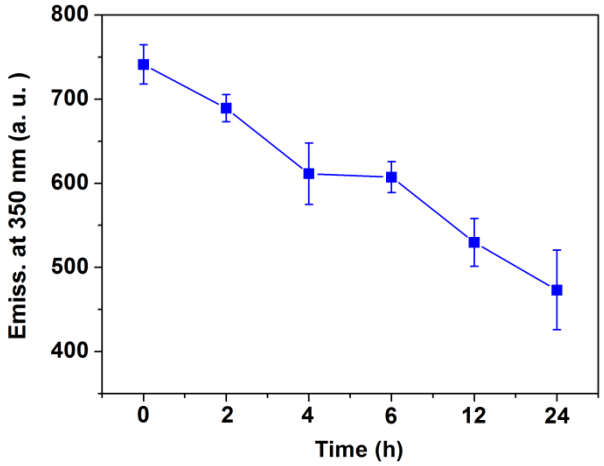


Fig. S16 Fluorescence emission intensity of TBT (20 μM, λ_{ex} = 305 nm) after incubation with brain homogenates of AD mice for different periods of time (n = 3).

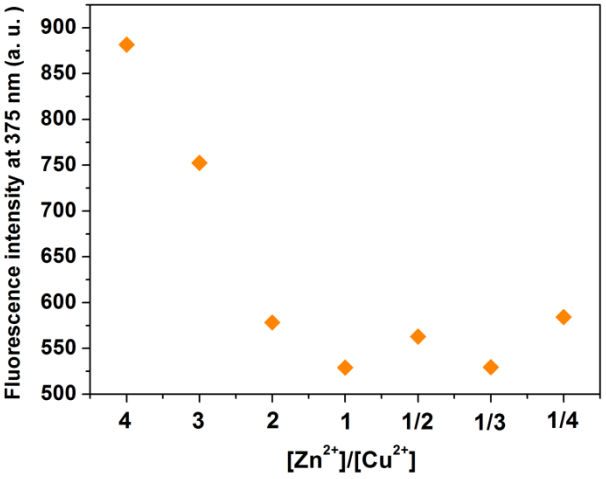


Fig. S17 The emission intensity at 375 nm of TBT *versus* different $[Zn^{2+}]/[Cu^{2+}]$ ratios.

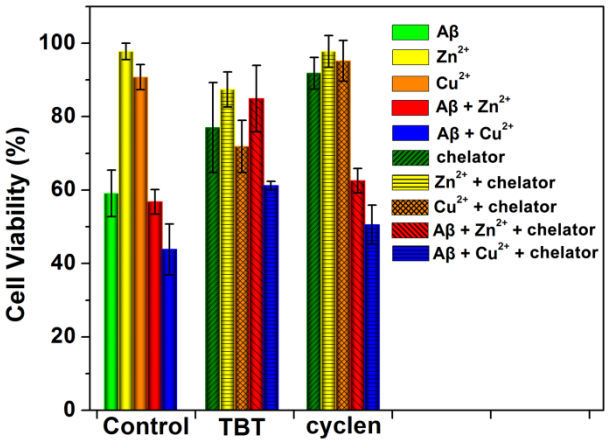


Fig. S18 Effect of TBT or cyclen on toxicity of metal–Aβ species in PC12 cells by using an MTT assay (n = 3).

Table S2 MW, ClogP, HBA, HBD, PSA, and logBB values of TBT and cyclen.[a]

Chelators	MW	ClogP	HBA	HBD	PSA	logBB[b]
TBT	395.57	2.427	3	3	51.69	–0.257
cyclen	172.27	–1.59	0	4	51.24	–0.861

[a]ClogP and PSA of TBT and cyclen were predicted by Discovery Studio 2.5 Software (Accelrys). [b]logBB = −0.0148 × PSA + 0.152 × ClogP + 0.139; compounds with logBB > 0.3 are able to cross the BBB readily, with logBB < −1.0 are only poorly distributed to the brain.

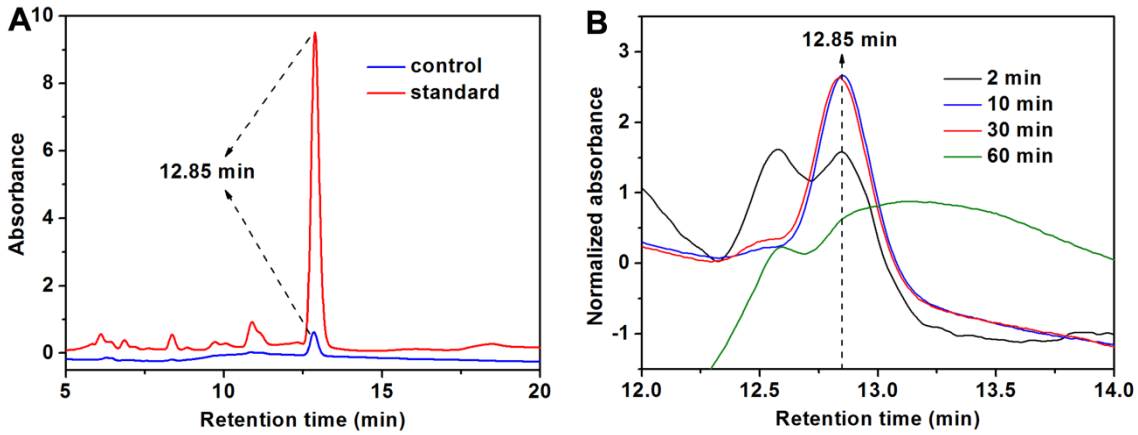


Fig. S19 (A) HPLC spectra of TBT from control and standard samples. (B) Normalized HPLC spectra of TBT from the brain extractions of C57BL/6J mice at different time (2, 10, 30, and 60 min) after i.v. injection.

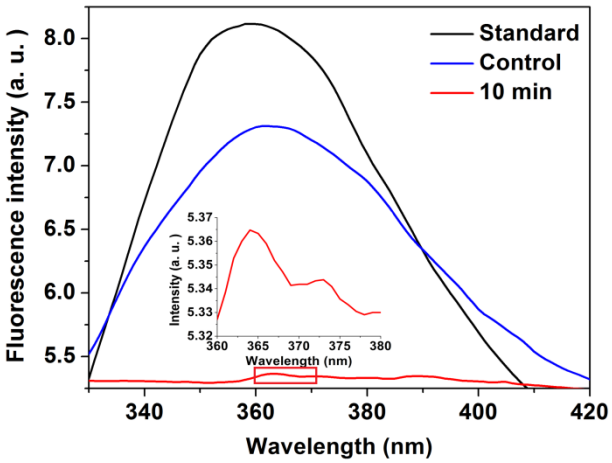


Fig. S20 Fluorescence spectra of TBT from the brain extractions of C57BL/6J mice at 10 min postinjection, and standard, and control samples.

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