Supporting information

The chelation targeting metal-Aβ40 aggregates may lead to formation of Aβ40 oligomers

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1. Materials and methods

All reagents were purchased from commercial companies and directly used unless stated otherwise. Solvents were purified by the most used methods. All solutions and buffers were prepared with using distilled water that had been passed through a Millipore-Q ultrapurification system.

The IR spectra were measured on a Perkin-Elmer Spectrum BX FT-IR instrument in tablets with potassium bromide. UV-Vis spectra were recorded on an analytik jena Specord 210 spectrophotometer. Elementary analysis was carried out on a Vario EL III elementary analysis instrument. \(^{1}\)H and \(^{13}\)C NMR spectra were recorded on a Varian Mercury 400 spectrometer at 400 and 100 MHz, respectively. Electrospray ionization mass spectra (ESI-MS) were acquired on an Applied Biosystems API 2000 LC/MS/MS system.

1.1 Preparation of A\(\beta\)40 samples

A\(\beta\)40 was purchased from GL Biochem Ltd. The sample was purified by high-performance liquid chromatography and characterized by electrospray ionization mass spectrometry (ESI-MS). A\(\beta\)40 was dissolved in dimethyl sulfoxide (DMSO) as a stock solution (1.0 mM) and stored at -30 °C before utilization.\(^1\) A\(\beta\)40 solutions with various concentration were prepared by diluting the stock solution with distilled water in an ice-water bath. Preparation of A\(\beta\)40 samples for both disaggregation and inhibition experiments was performed according to the previously reported methods with a little modification, and the samples used for morphological analysis of A\(\beta\)40 aggregates were observed by transmission electron microscopy (TEM).\(^2,3\) For the disaggregation experiments, A\(\beta\)40 samples (50 \(\mu\)M) were incubated with 50 \(\mu\)M Cu\(^{2+}\) or Zn\(^{2+}\) for 48 h with agitation, then FC-1 (50 \(\mu\)M) was added and incubated for 24 h with agitation. For the inhibition experiments, A\(\beta\)40 samples were stirred with 50 \(\mu\)M CuCl\(_2\) or Zn(OAc)\(_2\) for 5 min at room temperature, then FC-1 (50 \(\mu\)M) was added and incubated for 24 h at 37 °C with agitation. All A\(\beta\)40 samples containing Cu\(^{2+}\) or Zn\(^{2+}\) were at pH 6.6 or 7.4 in buffer (20 mM Tris-HCl/150 mM NaCl). Metal-free A\(\beta\)40 sample was taken as a control.

1.2 Determination of quantum yield

The quantum yield (\(\Phi_F\)) for fluorescence emission was determined by comparison with the integrated and corrected emission spectrum of quinine sulfate (a standard), whose quantum yield in 0.05 M H\(_2\)SO\(_4\) was assumed to be 0.55 (excitation at 366 nm). 10 \(\mu\)M FC-1 in DMSO solution was prepared. The concentration of the reference was adjusted to match the absorbance of the test sample at the wavelength of excitation. Emission for FC-1 was integrated from 375 to 550 nm with excitation at 360 nm.

1.3 X-ray structure determination

Crystals of FC-1 and its complexes 1 and 2 suitable for X-ray diffraction were sealed in thin-walled quartz Capillaries and mounted on a Bruker Smart Apex CCD Diffractometer equipped with graphite-monochromated Mo-Ka radiation (\(\lambda = 0.71073\))
Å) at 293K. Preliminary unit cell constants were determined with a set of 45 narrow frame (0.3° in ω) scans. Data sets consisted of 1286 frames of intensity data collected with a frame width of 0.3° in ω, a counting time of 10 s/frame, and a crystal-to-detector distance of 5.0 cm. Their structures were resolved by direct methods and multi-scan absorption corrections were applied using the SAINT+ program. The final refinement was performed with SHELXL-97 by full-matrix least-squares methods on $F^2$ with anisotropic thermal parameters for non-hydrogen atoms. The hydrogen atoms were generated theoretically onto atoms to which they are attached and refined isotropically with fixed thermal factors ($U_{iso}(H) = 1.2 \times U_{eq}$ (aromatic, methylene C and imine N atoms), $U_{iso}(H) = U_{eq}$ (methyl C)). Crystallographic data of FC-1 and its complexes 1 and 2 were listed in Table S2. CCDC reference numbers 759320 (FC-1), 759319 (1) and 759321(2) contained the supplementary crystallographic data. These data can be obtained free of charge at www.ccdc.cam.ac.uk/conts/retrieving.html or from CCDC, 12 union Road, Cambridge CB2 1EZ, UK; Fax: (internat.) + 44-1223-336-033; E-mail: deposit@ccdc.cam.ac.uk.

1.4 Fluorescence Assay

In order to determine the binding of FC-1 to and FC-1-induced dissociation of Aβ40 aggregates, fluorescence assays were performed by recording the strongest emission at 430 nm (excitation at 360 nm) on a FluoroMax-P spectrofluorimeter. Aβ40 aggregates were provided by incubating 100 μM Aβ40 solutions with or without 100 μM CuCl₂ or Zn(OAc)₂ in the buffer (20 mM Tris-HCl, 150 mM NaCl, pH 7.4) for 48 h at 37 °C with agitation. Upon addition of 100 μM FC-1, the freshly prepared Aβ40 aggregates were incubated for 10 min, 1 h, 1 d, 2 d, respectively, in the buffer at 37 °C with agitation. The final concentrations of tested Aβ40 samples were 10 μM in the buffer ([Aβ40]/[metal ion]/[FC-1] = 1:1:1). The Aβ40 aggregate without FC-1 was used as controls. At least, three parallel tests were performed for all measurements.

1.5 Turbidity Measurements

Turbidity assays were performed according to a previously described procedure. Each sample was tested by measuring the absorbance at 405 nm. The controls were the solutions containing FC-1, metal salts, or mixtures of FC-1 and metal salts.

1.6 BCA Protein Analysis

The solutions containing Aβ40 aggregates were prepared as above. FC-1 was added into the solutions followed by incubation at 37 °C for 24 h with agitation. The final Aβ40 concentrations in the tested samples were 45 μM in the buffer (20 mM Tris-HCl, 150 mM NaCl, pH 7.4 ([Aβ40]/[metal ion]/[FC-1] = 1:1:1). The absorbance was measured at 562 nm for all the samples. The freshly prepared Aβ40 was used as a control. After centrifugation for 20 min at 14,000 rpm, the supernatants of the samples were analyzed by a BCA Protein Assay Kit using bovine serum albumin as a standard.

1.7 CD spectra
Samples for circular dichroism (CD) were prepared by incubation of Aβ40 (25 µM) and CuSO₄ or Zn(OAc)₂ (25 µM) at 37 °C for 24 h in 10 mM sodium phosphate buffer (pH 7.4). Then FC-1 (25 µM) was added to the solutions and incubated at 37°C for another 24 h with agitation. CD spectra in the range of 200–250 nm were acquired on a Chirascan™ spectrometer. The spectra were shown as an average of 5 baseline-corrected from which the buffer plus metal spectra were subtracted. All measurements were taken using a 1 mm cuvette at 25 °C.

1.8 Chelation of Cu²⁺ from Cu-Aβ40 aggregates

Fresh Aβ40 (100 µM) was incubated with 100 µM Cu²⁺ for 24 h with agitation, then the solution was centrifuged (15000 rpm, 30 min). The precipitate was washed with water and centrifuged again (15000 rpm, 30 min). The resulted precipitate was resuspended in water (100 µL). FC-1 (5µL, 1 mM) was added to the solution, and the final concentration of FC-1 was diluted to 10 µM with a buffer (20 mM Tris-HCl/150 mM NaCl, pH 7.4). The mixture of FC-1 and Cu-Aβ40 aggregates was agitated at 37 °C. Their UV-visible absorption spectra were detected in the different time points.

1.9 Transmission electron microscopy (TEM)

Samples were prepared according to the previously reported methods. Aβ40 (100 µM, 5 µL) aggregates were added to glow-discharged, formar/carbon 300-mesh copper grids (Electron Microscopy Sciences, Hatfield, PA) and remained for 1.5 min at room temperature. Excess sample was removed with filter paper and each grid was washed twice with ddH₂O. Uranylacetate (1%, 3 µL) was added to each grid and incubated for 1 min. Excess sample was removed and grids were then dried for 15 min. Samples were visualized on a Tecnai G² transmission electron microscope at 200 kV and 75,000–250,000 magnification.

1.10 Fluorescence microscopy (FM)

Samples were put onto clear glass slide before observation under fluorescence. Images were obtained with an excitation filter ranging from 360 to 370 nm, using a Leica DMI 3000B inverted fluorescence microscopy under a 40× objective lens.

1.11 Cell imaging

Freshly prepared Aβ40 (80 µM) was incubated for 24 h, then FC-1 (80 µM) was added and incubated for 3 h at 37 °C with agitation. HeLa cells were cultured in Dulbecco's modified eagle medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Invitrogen). One day before imaging, cells were seeded in 24-well plates. The next day, the HeLa cells were incubated with 3.2 µM FC-1 or 3.2 µM FC-1-bound Aβ40 aggregates for 3 h at 37 °C with 5% CO₂, cells were washed with PBS three times. The fluorescent imaging of intracellular FC-1 was carried out under inverted fluorescence microscopy with a 40× objective lens (excited with green light). For all images, the microscopy settings, such as brightness and contrast were held constant to compare the relative intensity of
intracellular FC-1 fluorescence.

1.12 Cytotoxicity assay

The cytotoxicity of Aβ40 and Cu-Aβ40 aggregates with and without FC-1 was assessed by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide, Promega) assay. Briefly, the HeLa cells supplemented with 10% FBS, 100 U/mL of penicillin, and 100 U/mL of streptomycin were seeded at about 1000-10000 cells/well (200 μL) in 96-well plates, and incubated at 37 °C in a 5% CO₂ humidified air atmosphere. The FBS-containing medium was replaced with a FBS-free medium when growth reached 80% confluence as a monolayer. After 4 h of incubation in the medium, the wells were divided into seven experimental groups and one control group. The cells were monitored by fluorescent microscopy after further incubation of 18 h at 37 °C in a 5% CO₂ humidified air atmosphere. Then, after removing the medium, 20 μL of MTT (5 mg/mL) in PBS was added to wells along with 200 μL of the culture medium. After incubation of 4 h, the MTT-containing medium was replaced by DMSO of 150 μL. Finally, the 96-well plates were oscillated for 10 min to fully dissolve the formazan crystal formed by living cells in the wells. The relative viability of the cells in each well was produced by absorbance of at 490 nm each well determined with the Biotek Synergy™ 2 Multi-detection Microplate Reader.9,10

2. Preparation of FC-1 and its complexes 1 and 2

![Scheme S1. Synthesis of FC-1 and its complexes 1 and 2.](image)

2.1 4-benzothiazol-2-yl-benzenamine, 3

4-Aminobenzoic acid (1.37 g, 10 mmol) and 2-aminothiophenol (1.25 g, 10 mmol)
were mixed with polyphosphoric acid (5 g) and heated to 220 °C for 4 h under an Ar atmosphere according to the previously reported procedure. The reaction mixture was cooled to room temperature and poured into 10% K₂CO₃ (aq) solution. The precipitate was filtered under reduced pressure and re-crystallized from ethyl acetate to obtain pure product (2.03 g, 90%). M.P. 156−157 °C. 

1H NMR (400 MHz, CDCl₃, ppm): δ 4.02 (s, 2H), 6.72 (d, 2H, J 7.2 Hz), 7.33 (t, 1H, J 7.6 Hz), 7.45 (t, 1H, J 7.6 Hz), 7.86 (d, 2H, J 8.8 Hz), 7.91 (d, 1H, J 7.2 Hz), 8.00 (d, 2H, J 8.8 Hz). 

13C NMR (100 MHz, DMSO-d₆, ppm): δ 168.2, 153.9, 152.2, 133.6, 128.8, 126.3, 124.8, 121.9, 121.8, 120.1, 113.7. 

Anal. Calcd for C₁₃H₁₀N₂S: C, 69.03; H, 4.42; N, 12.39. Found: C, 69.00; H, 4.46; N, 12.39. ESI-MS: m/z 225 (M-H⁺); calculated, 225.

IR (KBr, cm⁻¹): 3458 (m), 3294 (m), 3179 (m), 1635 (s), 1603 (s), 1476 (s), 1432 (s), 1312 (s), 1227 (m), 1179 (s), 963 (m), 826 (s), 759 (s), 729 (m), 624 (w).

UV-Vis spectra: MeOH solution, λmax, 342 nm; ε, 41300 M⁻¹cm⁻¹.

2.2 FC-1

2-picolyl chloride hydrochloride (6.56 g, 40 mmol) in 5 mL water was neutralized by addition of 5 M NaOH solution. A mixture of 4-benzothiazol-2-yl-benzenamine (4.52 g, 20 mmol) and KI (0.68 g, 4 mmol) in 150 mL EtOH was added into this solution. The reaction mixture was allowed to stir at 80 °C for 2 days. During the reaction course, pH of the mixture was maintained between 7−9 by periodic addition of 5 M NaOH. After cooling to room temperature, the solvent was removed under reduced pressure. The residue was washed with H₂O and extracted into CH₂Cl₂ (3×100 mL), and then the organic fraction was combined and dried with MgSO₄. After evaporation of CH₂Cl₂, the product was obtained as a yellow solid (1.22 g, 15%) via column chromatography (silica, CH₂Cl₂/acetone, 1/4, v/v). Yellow crystals suitable for X-ray diffraction were got by slow evaporation of a dichloromethane solution of FC-1 at room temperature. M.P. 201–202 °C. 

1H NMR (400 MHz, CDCl₃, ppm): δ 4.90 (s, 4H), 6.79 (d, 2H, J 8.8 Hz), 7.22 (d, 2H, J 6.0), 7.30 (q, 2H, J 8.4 Hz), 7.43 (t, 2H, J 8.0 Hz), 7.68 (q, 2H, J 7.6 Hz), 7.83 (d, 1H, J 8.4 Hz), 7.89 (d, 2H, J 8.4 Hz), 7.96 (d, 1H, J 7.2 Hz), 8.63 (d, 2H, J 3.6 Hz). 

13C NMR (100 MHz, CDCl₃, ppm): δ 168.2, 157.7, 154.2, 150.2, 149.8, 136.9, 134.5, 128.9, 125.9, 124.3, 122.6, 122.3, 121.3, 120.7, 112.3. Anal. Calced for C₂₅H₂₀N₄SCl₂Cu·1/6AcOEt,·4/3H₂O: C, 73.55; H, 4.88; N, 13.10. ESI-MS: m/z 409 (M+H⁺); calculated, 409. IR(KBr, cm⁻¹): 3350 (w), 3049 (w), 2920 (m), 2850 (m), 1672 (w), 1608 (vs), 1569 (m), 1530 (m), 1484 (vs), 1432 (vs), 1385 (s), 1353 (s), 1278 (m), 1194 (s), 1148 (m), 1046 (m), 965 (m), 813 (s), 755 (s), 727 (s), 694 (w), 616 (w). UV-Vis spectra: MeOH solution, λmax, 350 nm; ε, 46600 M⁻¹cm⁻¹.

2.3 C₂₅H₂₀N₄SCl₂Cu·1/6CH₃CN·4/3H₂O, 1

CuCl₂·2H₂O (0.17 g, 1 mmol) was added to MeOH solution (30 mL) of FC-1 (0.41 g, 1 mmol), the precipitate was immediately formed when stirred at 60 °C. The resulted solution was filtered after 6 h, and the precipitate was dissolved in 40 mL MeCN. Green crystals suitable for X-ray diffraction were obtained at room temperature after nine days (0.34 g, 60%). Anal. Calced for C₂₅H₂₀N₄SCl₂Cu·1/6
CH₃CN·4/3H₂O: C, 55.23; H, 3.68; N, 10.31. Found: C, 55.26; H, 3.72; N, 10.97. UV-Vis spectra: \( \lambda_{\text{max}} \) (MeOH)/nm 310 and 710 (ε/ M⁻¹ cm⁻¹ 43800 and 465).

2.4 C₂₅H₂₀N₄SCl₂Zn·1/2CH₃OH, 2

ZnCl₂ (0.14 g, 1 mmol) was added to MeOH solution (30 mL) of FC-1 (0.41 g, 1 mmol), the precipitate was formed immediately when stirred at 60 °C. The resulted solution was filtered after 6 h, and the precipitate was dissolved in 5 mL DMF. Yellow crystals suitable for X-ray diffraction were obtained by vapor diffusion with CH₃OH after a month(0.25 g, 45%). Anal. Caled for C₂₅H₂₀N₄SCl₂Zn·1/2CH₃OH: C, 55.15; H, 3.68; N, 10.29. Found: C, 55.18; H, 3.72; N, 10.93. UV-Vis spectra: \( \lambda_{\text{max}} \) (MeOH)/nm 350 (ε/ M⁻¹ cm⁻¹ 43800).

3. The tables for data of crystal structures and others

Table S1. Lipinski's rules and logBB Values of FC-1 and CQ.

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<th>FC-1</th>
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<td>MW</td>
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<tr>
<td>clogP</td>
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<tr>
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<tr>
<td>HBD</td>
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<tr>
<td>TPSA</td>
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<tr>
<td>logBB</td>
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<td>0.21</td>
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</table>

Here, MW: molecular weight; clogP: calculated logarithm of the octanol-water partition coefficient; HBD: hydrogen-bond donor atoms; HBA: hydrogen-bond acceptor atoms; TPSA: polar surface area.

Lipinski’s rules: MW ≤ 450, HBD ≤ 5, HBA ≤ 10, TPSA ≤ 90, clogP ≤ 5. logBB = −0.0148 TPSA + 0.152clogP + 0.130 (logBB > 0.3, readily cross the BBB; logBB < −1.0, only poorly distributed to the brain).¹²,¹³

Table S2. Crystal data and structure refinement for FC-1, 1 and 2.

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<td>P-1</td>
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<td>Monoclinic</td>
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<tr>
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<tr>
<td>b/Å</td>
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4. X-ray structure, absorption and fluorescence spectra

**Figure S1.** An ORTEP view of the structures of FC-1. The hydrogen atoms have been omitted for clarity.

**Figure S2.** UV-visible absorption spectra of FC-1 and its complexes 1 and 2 in MeOH at 37 °C. The concentrations of FC-1, 1 and 2 all were 20 μM.
Figure S3. UV-visible absorption spectra of the different proportion of FC-1 and Cu$^{2+}$ in DMSO solution at 37 °C. The concentration of FC-1 was kept at 10 μM, while that of Cu$^{2+}$ ranged from 0 to 50 μM.

Figure S4. Job’s plot of the coordination of Cu$^{2+}$ (a) or Zn$^{2+}$ (b) to FC-1 in Tris-HCl (100 mM, pH 7.4, H$_2$O:DMSO = 9:1, v/v) at 37 °C. The concentrations of FC-1 and metal ion were kept constant at 10 μM.
Figure S5. Fluorescence spectra (excitation at 360 nm) of FC-1 and its complexes 1 and 2 in MeOH at 37 °C. The concentrations of FC-1, 1 and 2 were all 5 μM.

Figure S6. The fluorescence property of FC-1 (10 μM) over the pH range with an excitation at 360 nm.
**Figure S7.** The fluorescence property of FC-1 (10 μM) over the incubation time at 37 °C in 20 mM Tris-HCl buffer (pH 7.4, H₂O:DMSO = 9:1, v/v).

**Figure S8.** The effect of metal ions (10 μM) on the fluorescent property of FC-1 (10 μM) in 100 mM Tris-HCl buffer (pH 7.4) (H₂O:DMSO = 9:1, v/v) with an excitation at 360 nm. The samples were incubated for 30 min at room temperature prior to measurements.
**Figure S9.** The emission intensity of FC-1 (10 μM) changes with increasing concentrations of CuCl₂ in 100 mM Tris-HCl buffer (pH 7.4) (H₂O:DMSO = 9:1, v/v) at 37 °C. The insets show the relationship between the emission intensity and concentrations of Cu²⁺ (0, 20, 40, 60, 80, 100, 120, 140, 160, 180 and 200 μM).

**Figure S10.** The comparison of fluorescent property between FC-1 (excitation at 360 nm, 1 nm slit width) and ThT (excitation at 440 nm, 4 nm slit width). The fluorescent intensity was the strongest emission of FC-1 (at 430 nm) and ThT (at 490 nm), respectively. Aβ40 (10 μM) was incubated for 48 h at 37 °C in the absence and presence of Zn²⁺ or Cu²⁺ (10 μM), FC-1 or ThT (10 μM) was then added and incubated for 10 min at 37 °C with agitation.
**Figure S11.** FM images (magnification: 40) of FC-1-bound Aβ40 aggregates. The first row showed bright-field microscopy images to show the presence of aggregates, and the second showed FM images. Aβ40 (100 μM) was incubated for 5 days at 37 °C in the absence and presence of Zn²⁺ or Cu²⁺ (100 μM), then FC-1 (100 μM) was added and incubated for 10min at 37 °C. (a) Aβ40 + FC-1; (b) Cu²⁺-Aβ40 + FC-1; (c) Zn²⁺-Aβ40 + FC-1.

**Figure S12.** UV-visible absorption spectra of the different time course of FC-1 and Cu²⁺-Aβ40 aggregates in DMSO aqueous solution (H₂O:DMSO = 9:1, v/v) at 37 °C.
Figure S13. The influence of metal ions, Aβ40 aggregates or metal-Aβ40 aggregates on the emission (any unit) of FC-1 at 430 nm (excitation at 360 nm). The Aβ40 and metal-Aβ40 aggregates were prepared by incubating 10 μM Aβ40 for 2 days at 37 °C in the absence and presence of 10 μM Zn²⁺ or Cu²⁺. FC-1 (10 μM) was added, respectively, into the samples containing 10 μM Zn²⁺ or Cu²⁺, Aβ40 aggregates and metal-Aβ40 aggregates, then incubated for 10 min, 1 h, 1, 2, 3, 5 and 7 days at 37 °C with agitation, respectively.

Figure S14. Turbidity test of Aβ40 (10 μM) and Cu²⁺- or Zn²⁺-Aβ40 aggregates (10 μM) after incubation for 48 h at pH 7.4 and 37 °C in the absence and presence of 10 μM FC-1. The absorbance was measured at 405 nm.
Figure S15. BCA protein analysis of soluble Aβ40 in the metal-Aβ40 systems before or after addition of FC-1. The freshly prepared Aβ40 solution was taken as reference.

Figure S16. CD spectra of metal-Aβ40 systems in the presence and absence of FC-1. (a) Cu$^{2+}$-Aβ40 (black line), Cu$^{2+}$-Aβ40 +FC-1 (red line); (b) Zn$^{2+}$-Aβ40 (black line), Zn$^{2+}$-Aβ40 +FC-1 (red line).
Figure S17. TEM images of Aβ40 samples for inhibition experiment. Cu^{2+}- and Zn^{2+}-Aβ40 aggregates done by incubating the reactions containing 50 μM freshly prepared Aβ40 and 50 μM Cu^{2+} (a) or Zn^{2+} (b), followed by addition of 50 μM FC-1 and re-incubation for 1 day at 37 °C with agitation.

Figure S18. FM images of HeLa cells exposed to FC-1 or FC-1-containing Aβ40 aggregates (excited with UV light). a, Bright-field image of HeLa cells, b, FM image of HeLa cells exposed to 3.2 μM FC-1, c, FM image of HeLa cells exposed to 3.2 μM FC-1-bound Aβ40 aggregates.

5. References