Electronic Supplementary Information

Spiropyran-based fluorescent probe for specific detection of β-amyloid peptide oligomers in Alzheimer's disease

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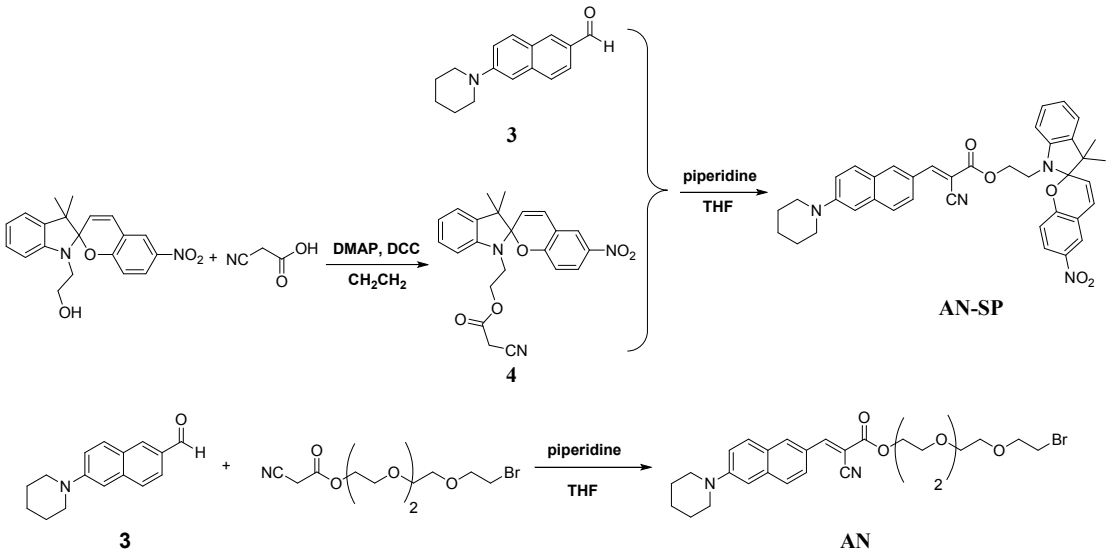
Contents

1. Materials and general methods

All of the starting materials were obtained from commercial suppliers and used as received. Moisture sensitive reactions were performed under an atmosphere of dry argon. Palladium acetate and (±)-2,2'-bis(diphenylphosphino)-1,1'-binaphthalene were provided by Acros. Methyl 6-bromo-2-naphthoate and other chemicals were supplied from J&K Scientific Ltd. Aβ (1-42) monomer was purchased from GL Biochem (Shanghai) Ltd. Column chromatography was carried out on silica gel (200–300 mesh). ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on a Mercury plus-Varian instrument. Proton chemical shifts are reported in parts per million downfield from tetramethylsilane (TMS). HR-MS was obtained on an LTQ-Orbitrap mass spectrometer (ThermoFIsher, San Jose, CA). UV-visible spectra were recorded on a Shimadzu UV-2550 spectrometer. Steady-state emission experiments at room temperature were measured on an Edinburgh instruments spectrometer (FS-920).

All the in *vivo* experiments were performed in compliance with the relevant laws and institutional guidelines, and the institutional committees of both Fudan University and Shanghai University of Medicine & Health Sciences have approved the experiments.

2. Synthesis details of AN-SP



Scheme S1

Compound **3** was synthesized according to our previous report. [1]

Synthesis of compound 4. To a mixture of 2-cyanoacetic acid (382.6 mg, 4.0 mmol) and 1-(2-Hydroxyethyl)-3,3-dimethylindolino-6'-nitrobenzopyrylospiran (1.4 g, 4.0 mmol) in CH_2Cl_2 (2.5 mL), DMAP (4.9 mg, 0.04 mmol) was added dropwise at 0 °C. Finally, DCC (206.3 mg, 4.5 mmol) was added and the reaction mixture was stirred at 0 °C for 6 hours. The reaction slurry was diluted with CH_2Cl_2 and was filtered. The filtrate was dried over MgSO4 and concentrated under reduced pressure. The residue was purified by silica gel flash chromatography (dichloromethane: acetone = 20: 1) to yield **4** as a red solid, 86% yield. ^{1}H NMR (400 MHz, CD_3CN) δ 8.08 (d, *J* = 2.4 Hz, 1H), 8.01 – 7.99 (m, 1H), 7.17 – 7.12 (m, 2H), 7.06 (d, *J* = 10.4 Hz, 1H), 6.87 (t, *J* = 7.2 Hz, 1H), 6.71 (t, *J* = 9.0 Hz, 2H), 6.02 (d, *J* = 10.4 Hz, 1H), 4.36 – 4.23 (m, 2H), 3.59 – 3.48 (m, 3H), 3.44 – 3.38 (m, 1H), 1.25 (s, 3H), 1.14 (s, 3H). ^{13}C NMR (100 MHz, CD_3CN) δ 164.88, 160.26, 147.68, 142.19, 136.98, 129.20, 128.80, 126.64, 123.76, 122.89, 120.85, 119.96, 116.31, 115.07, 107.80, 107.67, 65.22, 53.55, 42.98, 26.14, 25.46, 20.01.

Synthesis of compound AN-SP. To the mixture of compound **3** (50.2 mg, 0.21 mmol) and compound **4** (96.4 mg, 0.23 mmol) in THF, piperidine (2.0 μL, 0.02 mmol) was added and the mixture was stirred at 50 °C for 6 hours. After completion, the crude mixture was concentrated under reduced pressure and the product was obtained via flash column chromatography (EtOAc: petroleum ether = 1: 5) as a red solid (83% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.26 (s, 1H),8.20 (s, 1H), 8.10 – 8.07 (m, 1H), 8.02 – 7.99 (m, 2H), 7.79 – 7.66 (m, 2H), 7.33 (s, 1H), 7.23 (d, *J* = 7.2 Hz, 1H), 7.11 (d, *J* = 6.8 Hz, 1H), 7.00 – 6.90 (m, 2H), 6.77 – 6.72 (m, 2H), 6.10 (d, *J* = 10.0 Hz, 1H), 3.76 – 3.64 (m, 2H), 3.78 – 3.62 (m, 1H), 3.55 – 3.49 (m, 5H), 1.87 – 1.70 (m, 6H), 1.29 (s, 3H), 1.21 (s, 3H).

^{13}C NMR (100MHz, $CDCl_3$) δ 163.55, 159.50, 156.03, 146.61, 141.22, 138.00, 136.05, 134.99, 130.91, 128.52, 128.03, 127.52, 126.20, 125.99, 122.95, 122.27, 122.04, 120.18, 119.44, 118.75, 116.71, 115.65, 108.58, 106.81, 106.77, 68.10, 64.10, 52.96, 49.56, 42.57, 26.01, 25.59, 24.43, 19.97.

HRMS (ESI, m/z): calcd for $C_{39}H_{37}N_4O_5$ $[M+H]^+$, 641.2764, found 641.2759.

IR (cm^{-1}): 2925, 2854, 2217, 1723, 1578, 1483.

Synthesis of compound **AN** was depicted in our precious report.[1]

3. The measurement of K_d

The apparent binding constant (K_d) of **AN-SP** to Aβ oligomer (5 μM) was measured from the double reciprocal of the fluorescence intensity maximum (F_{max}) and the concentration of the probe. The plot is shown in Fig. S3. The K_d corresponds to the -1/(x-intercept) of the linear regression.

4. Preparation of Aβ aggregates, oligomer and monomer

Aβ aggregates and oligomer: Aβ (1-42) monomer was dissolved in PBS (pH = 7.31). This solution was magnetically stirred at 1200 rpm for three days at room temperature. Then pre- aggregated Aβ solution was obtained. The preparation of Aβ oligomer was performed according to Kayed's reported procedure [2] and confirmed by TEM (Fig. S1).

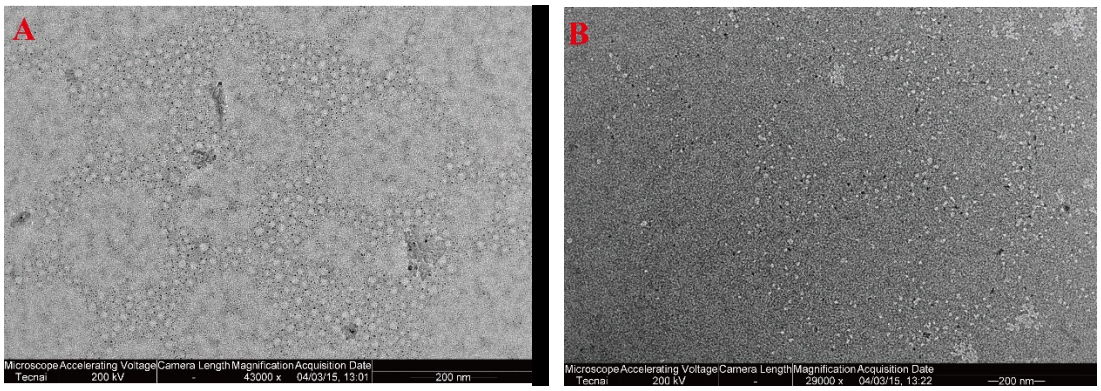


Fig. S1 TEM (negative staining with PTA) of Aβ42 aggregates (A), Aβ42 oligomers (B).

Aβ monomer: Aβ monomer was purchased from GL Biochem (Shanghai) Ltd and further purified using HPLC. Purified monomer was stored in hexafluoroisopropanol. 10 μL of Aβ monomer in HFIP were dried with argon gas and then reconstituted in 1.0 mL of distilled water.[3] This procedure is important for the following measurements.

5. AD transgenic mouse model

A triple transgenic model for Alzheimer's disease was used to examine the possible co-localization of **ANSP** probe staining and Aβ or tau immunoreactivity. The triple transgenic mice were generated by crossing 5XFAD transgenic mice with the tau P301S single transgenic mice. The 5XFAD transgenic mice [4] and P301S tau transgenic mice [5] were obtained from the Jackson Laboratory (Bar Harbor, Maine, USA). To maintain on a C57BL/6J background, the original 5XFAD mice were backcrossed to C57BL/6J mice for eight generations. These 5XFAD transgenic mice overexpress both mutant human APP (695) with the Swedish (K670N, M671L), Florida (I716V), and London (V717I) familial Alzheimer's disease (FAD) mutations and human PS1 harboring two FAD mutations, M146L and L286V. Expression of two transgenes is regulated by neural-specific elements of the mouse Thy1 promoter. Mutant tau transgenic mice express the P301S mutant form of human microtubule-associated protein tau with one N-terminal insert and four microtubule binding repeats, under control of the mouse prion promoter. Mice were genotyped by PCR as described previously Male or female mice at age of 9-10 months old were used in this study. [6] Animals were handled in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals.

6. Colocalization of probe labeling and AD pathology in the transgenic mouse brain

The brain tissue and immunofuorescent labeling were performed as previously described.[1] Mice were deeply anesthetized and transcardially perfused with PBS, followed by 8% formadehyde (Sigma-Aldrich) in PBS (pH 7.4). The brains were post-fixed and frozen-sectioned (14 μm thickness) with a microtome (Leica Microsystems, GmbH, Wetzlar, Germany) for further analyses. Briefly, free-floating sections were blocked in a blocking solution containing 10% goat serum, 1% BSA and 0.4% Triton X-100. Incubation with primary antibodies took place overnight at 4°C. Then, one of following antibodies was employed each for colocalization studies:

β-Amyloid oligomer-specific monoclonal antibody (Agirisera, Sweden), β-Amyloid monoclonal antibody (Sigma-Aldrich), Aβ40 and Aβ42 polyclonal antibodies (Cell Signaling), or conformation-dependent tau antibody MC1 (Peter Davies), at dilutions from 1:400 to 1:100. After rinsing, sections were incubated in the solution of goat anti-mouse or goat anti-rabbit secondary antibody conjugated with Alexa 488 (Molecular Probes, Carlsbad, CA, USA) for 2 h at room temperature. The images for Aβ or tau immunofuorescent reactivity followed by **AN-SP** probe (40 μM) for Aβ oligomers were taken sequentially using a microscope (BX51, Olympus, Japan) equipped with a DP72 digital camera.

7. Correlation of probe labeling and immunoblotting on blots

Frozen cortical tissues of transgenic mice were homogenized (20 strokes with Dounce tissue grinder) in 10× vol (w/v) of ice-cold extraction buffer (50 mM Tris-HCl, 150 mM NaCl, 0.3% Triton X-100, protease inhibitor cocktail, pH 7.6). Samples were then centrifuged at 14,000 rpm for 15 min at 4°C, and the supernatants were quantified for the protein concentration by bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). Protein samples were then portioned into aliquots and stored at −80°C until used. [7]

Extracted protein samples in a buffer without β-mercaptoethanol were separated on 12% SDS-PAGE gels, and transferred to nitrocellulose membranes (Bio-Rad). The membranes were firstly imaged with EC3 Imaging System (UVP BioImaging System, USA) before and after probing with **AN-SP** at 10 μM for 5 min. Then membranes were blocked for 1 h at room temperature in TBST buffer containing 5% skim milk, and subsequently probed with 6E10 monoclonal antibody (1:3,000, Covance Princeton, NJ, USA) or β-amyloid polyclonal antibodies (1:5,000, Cell Signaling, Danvers, MA, USA). The Aβ immunoreactivity was detected with horseradish peroxidase conjugated secondary antibody, and visualized with the enhanced chemiluminescence method.

8. ***In vivo* probe labeling and *ex vivo* imaging**

In vivo probe labeling was performed using both 5XFAD transgenic mice and the triple transgenic mice at ages 8-10 months. Mice were injected intraperitoneally with **AN-SP** probe (20 μmol/kg) 12 hours and/or 2 hours before brain tissues were collected. At the specific time points following SP injection, the mice were deeply anesthetized and brain tissues were processed and imaged as described above.

9. Cytotoxicity Assay MTT assay

The cytotoxicity was performed by 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT) assay with Hela and RAW cell lines. Cells growing in log phase were planted into a 96-well cell culture plate at 1×10^5/ well. The cells were incubated for 12 h at 37°C under 5% CO_2 in an incubator. A solution of **ANSP** (100.0 μL/well) at concentrations of 1.0, 2.5, 5.0, 10.0, 25.0, 50.0, 100.0 μM in nutrient with 10‰ DMSO was added to the wells of the treatment group, respectively. The cells were subsequently incubated for 6 h and 12 h at 37 °C under 5% CO_2. Thereafter, MTT (0.5 mg/mL) was added to each well and the plate was incubated for an additional 4 h at 37 °C under 5% CO_2. The optical density OD490 value (Abs.) of each well was measured by means of a Tecan Infinite M200 monochromator-based multifunction microplate reader, which was used as cell viability.

10. Computational Methods

Quantum mechanical calculations

The geometry optimization for **AN-SP** compound was performed by using density functional theory at the B3LYP/6-31G* level [8] at the gas phase using Gaussian 09 program3.

Molecular docking search

AN-SP docking search with Aβ oligomer were executed by using Autodock vina1.1.2 software package. The docking simulations were carried out with a box centered on the Aβ oligomer and employing 50 × 50 × 50 grid points. For the Aβ oligomer

structure, we served X-ray RCSB database (PDB ID: 4NTR) determined Aβ trimers derived from the β-amyloid peptide as a working model for toxic Aβ oligomer. [9]

Molecular dynamics (MD) simulations

We performed all-atom, explicit-water MD simulations using NAMD soft package[10] in Charmm force field. Cl ions were added to keep system neutral and a TIP3P periodic water box was added, 9208 water molecules were added into the system. Then a 1000 steps energy minimization was carry out at 298K, 1BAR condition. Finally MD simulation run at the same temperature and pressure. Other parameter were the default value of the software.

11. HPLC spectrum of AN-SP

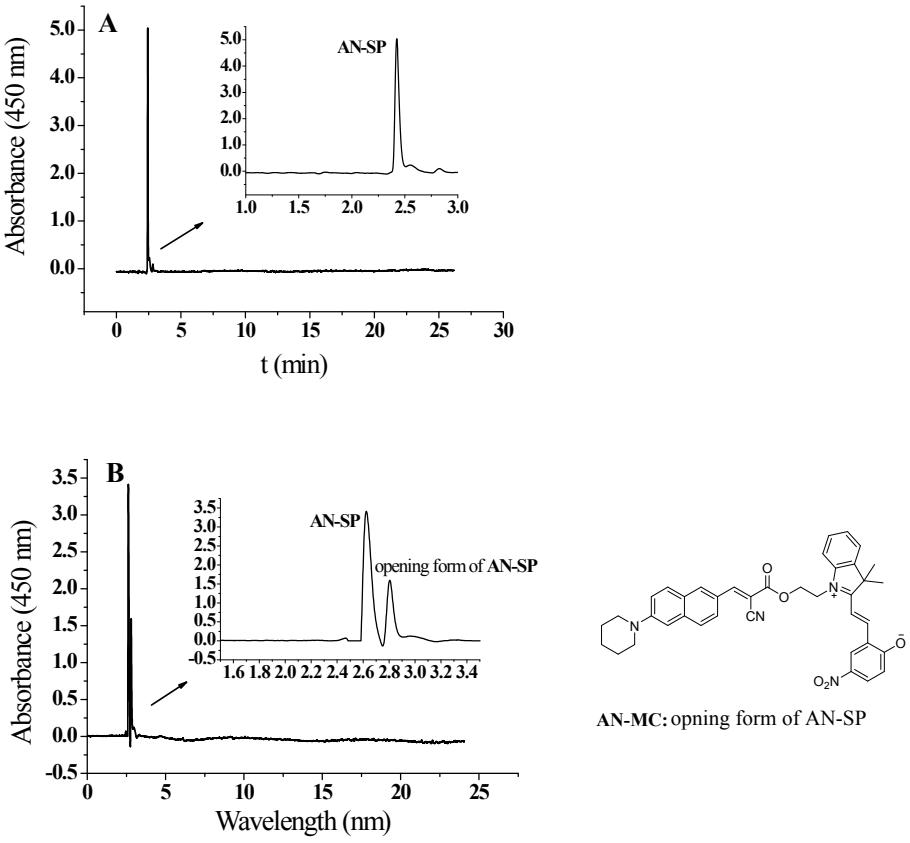


Fig. S2 (A) The HPLC spectrum of **AN-SP** (2.0×10^{-6} M) in DMSO; (B) The HPLC spectrum of **AN-SP (**2.0×10^{-6} M) in PBS.

12. **Additional absorption and fluorescent spectra**

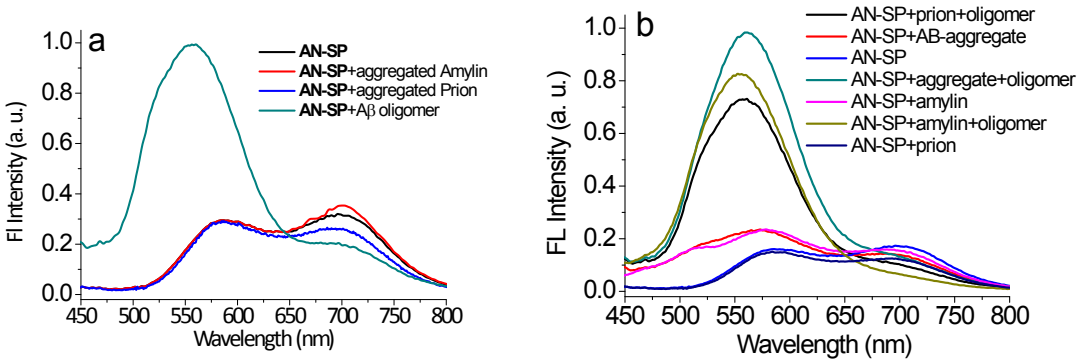


Fig. S3 (a) Fluorescence spectra of **AN-SP** (2.0×10^{-6} M, 5% DMSO in PBS) and with Aβ-oligomer (5.0×10^{-6} M) and other aggregated peptides (aggregated amylin and aggregated prion, 5.0×10^{-6} M). (b) Fluorescence spectral change of **AN-SP** (2.0×10^{-6} M, 5% DMSO in PBS) when Aβ oligomers were added to the solution of prion and amylin with **AN-SP**.

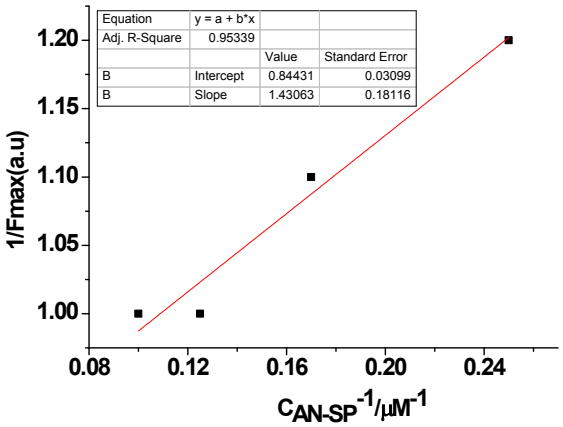


Fig. S4 Determination of the apparent binding constant (K_d) of probe (R^2=0.95) to Aβ oligomer.

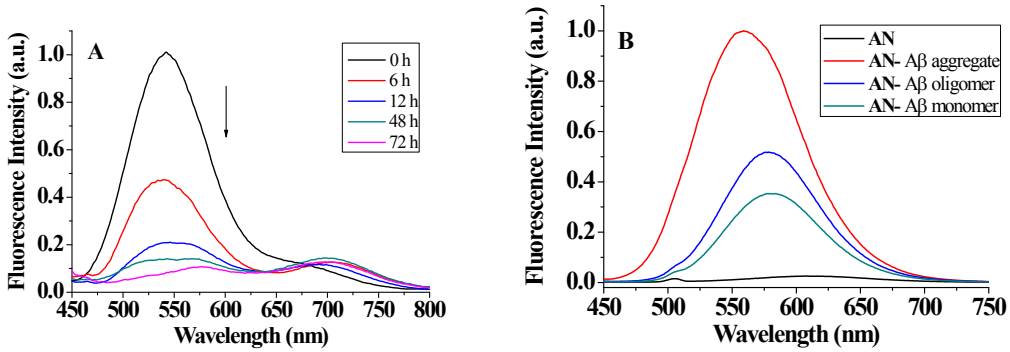


Fig. S5 Fluorescence spectral change of (A) **AN-SP** (2.0×10^{-6} M, 5% DMSO in PBS) with different incubation time of Aβ oligomer (5.0×10^{-6} M) and (B) **AN** (2.0×10^{-6} M, 5% DMSO in PBS) with Aβ aggregate, monomer, and oligomer (5.0×10^{-6} M). λ_{ex}=430 nm.

13. Calculated binding model of the probes with Aβ oligomer

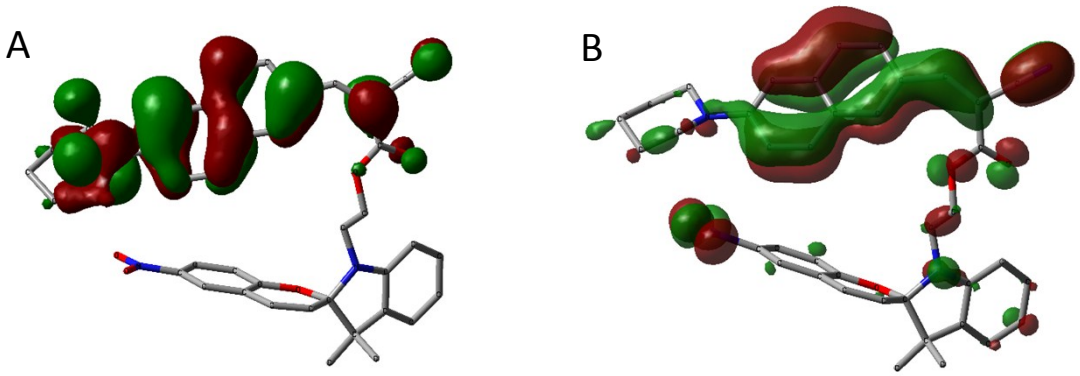


Fig. S6 The HOMO (A) and LUMO (B) level of **AN-SP**

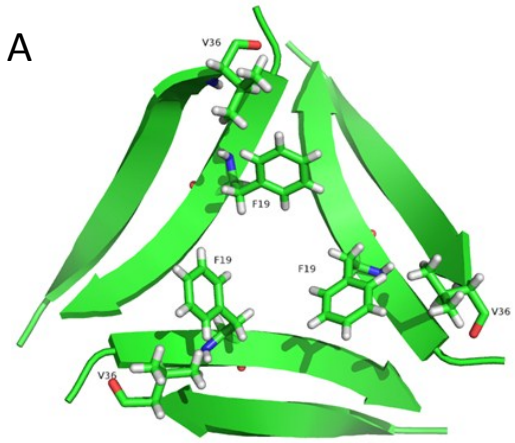


Fig. S7 Hydrophobic domain of the Aβ trimer

14. Toxicity of AN-SP and additional brain section images

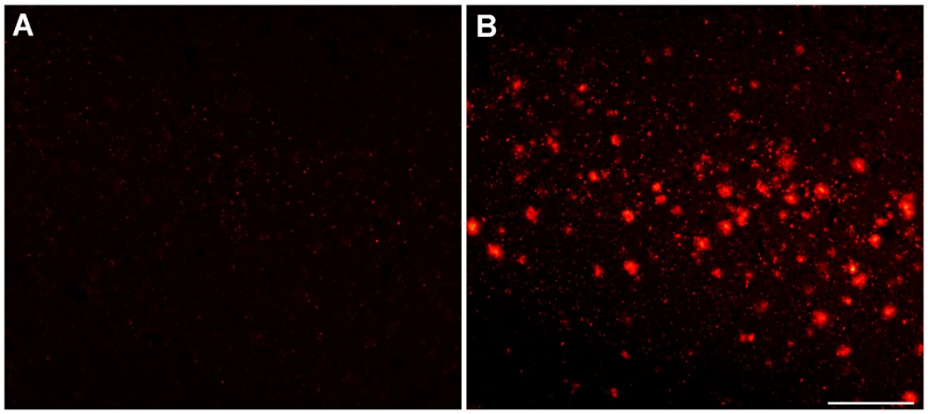


Fig. S8 The fluorescent images of **AN-SP** *in vitro* labeling on brain sections from an AD transgenic mouse model. A: negative control without **AN-SP** labeling; B: **AN-SP** (0.1 μM) *in vitro* labeling. Scale bar: 200 μm.

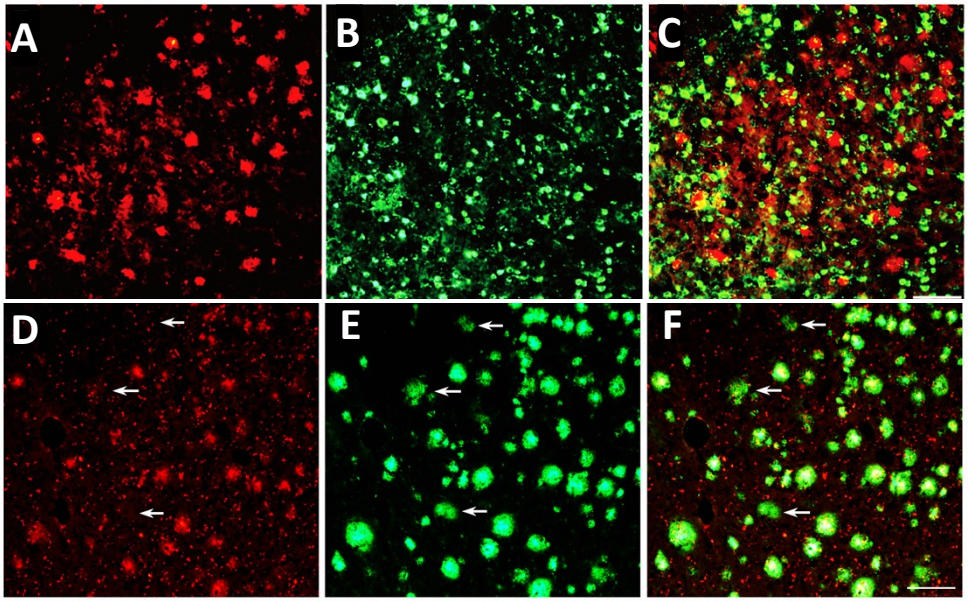


Fig. S9 The brain sections of APP/PS1/Tau triple transgenic mice staining with (A and D) 60 μM **AN-SP**, (B) conformation-dependent tau antibody MC1 and (E) β-amyloid antibody pAβ; C and F: the merged images. Scale bar: 100 μm.

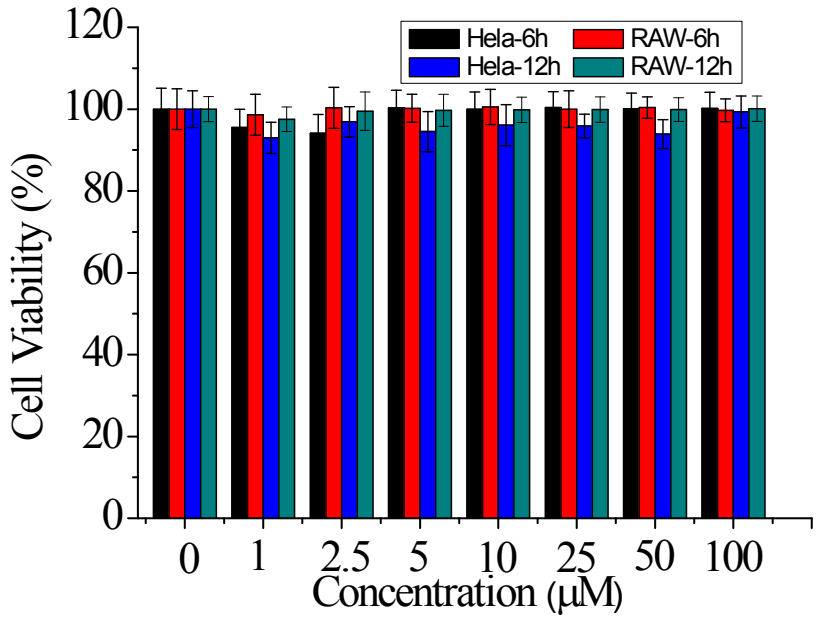


Fig. S10 Cell viability values (%) estimated by MTT assay in Hela and RAW cells, which were cultured in the presence of 0-100 μM **AN-SP** for 6 h and 12 h at 37 °C.

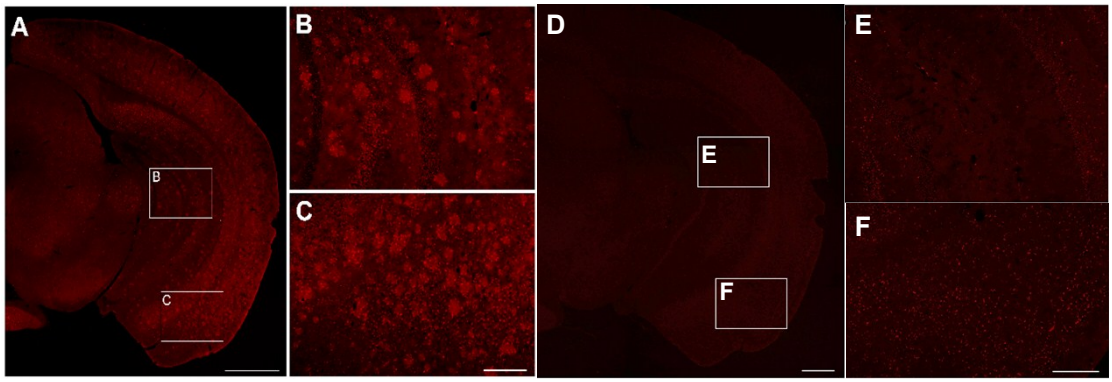


Fig. S11 *In vivo* binding and *ex vivo* imaging of **AN-SP** in the brain of AD transgenic mouse (A-C) and wild-type mice (D-F) models. In an APP/PS1 transgenic mouse at 8 months old, the labeling of **AN-SP** on plaques were detected in the mouse brain after 12 hours following **AN-SP** injection (ip). No staining signals were seen in the brain of wild-type mice following a similar procedure of **AN-SP** *in vivo* labeling. Scale bar, 200 μm..

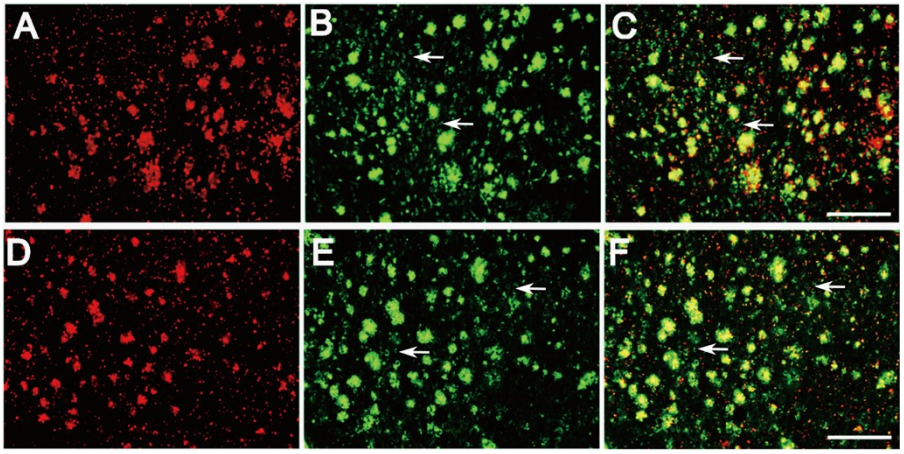


Fig. S12 Brain sections from AD transgenic mice with *in vivo* **AN-SP** labeling were immunostained with 6E10 antibody (A-C) or 4G8 antibody (D-F), 4G8 stains against Aβ monomers and 6E10 stains all forms of Aβ. As shown in B and E, the immuno-reactivity of 6E10 (B) or 4G8 (E) were found in both plaques and adjacent neurons (arrows), whereas **AN-SP** (A, D) labels plaque-like structure only, indicating that **AN-SP** and 6E10 or 4G8 antibody target different Aβ species. Scale bar: 200 μm.

15. Characteristic of the compounds

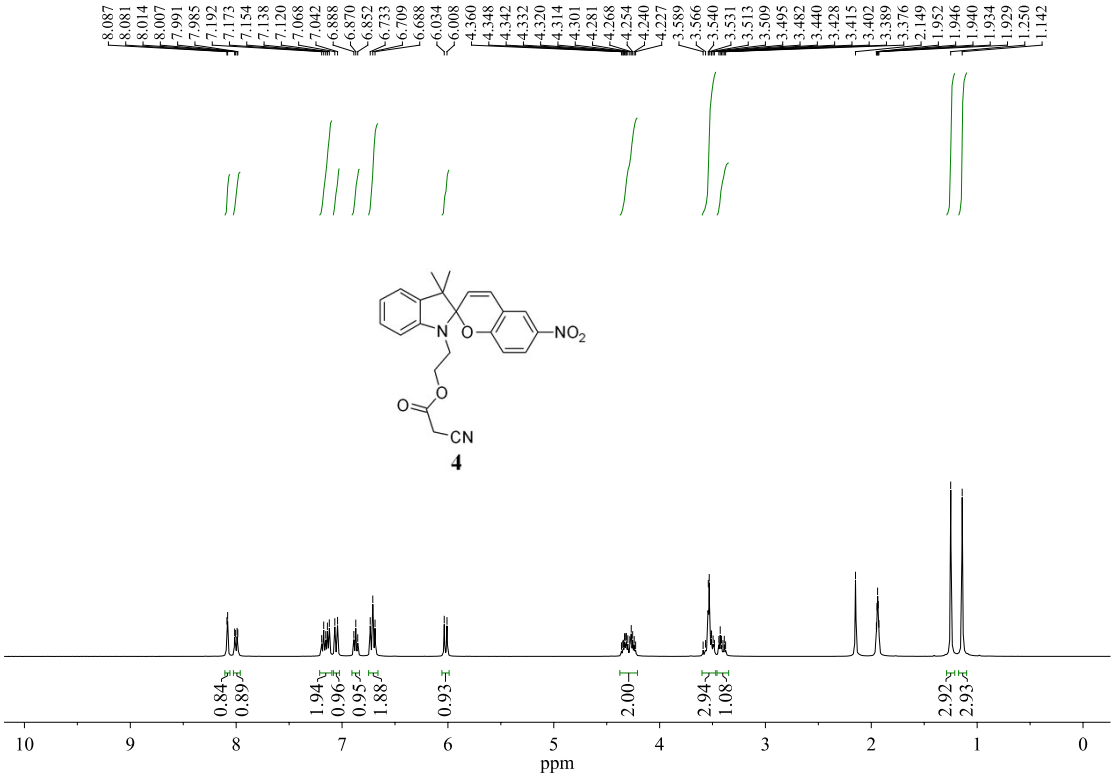


Fig. S13 The ^{1}H NMR spectrum of **4** (400 MHz, CD_3CN).

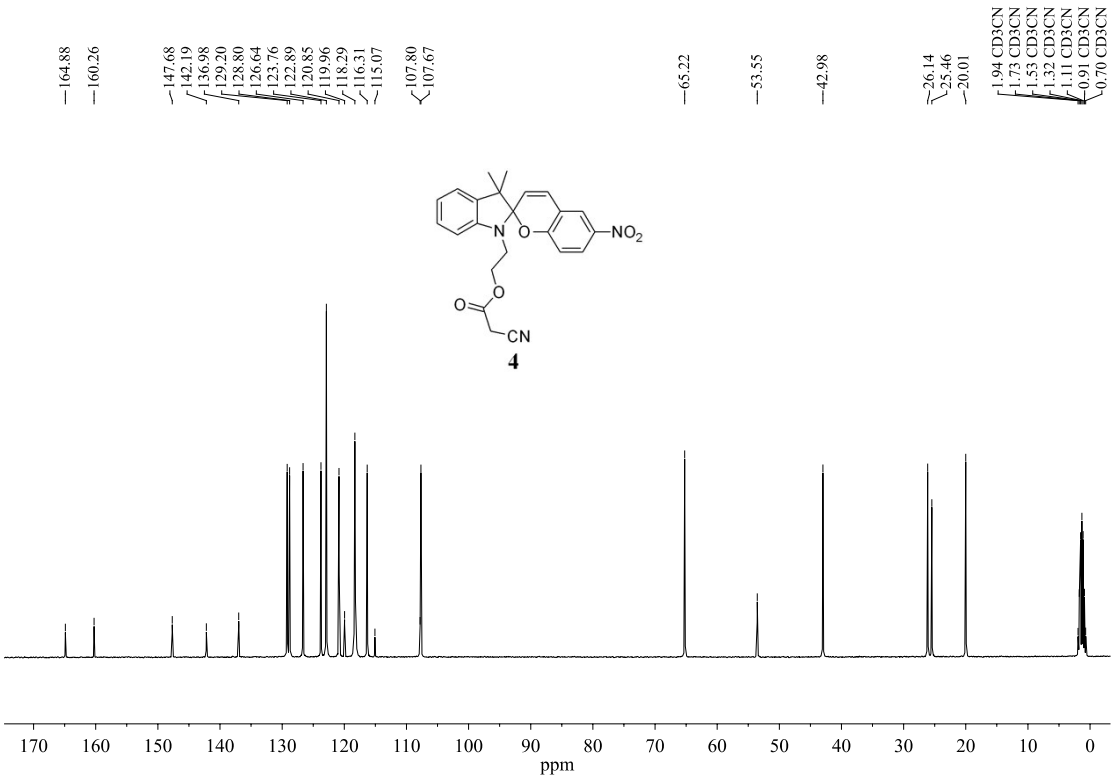


Fig. S14 The ^{13}C NMR spectrum of **4** (100 MHz, CD_3CN)

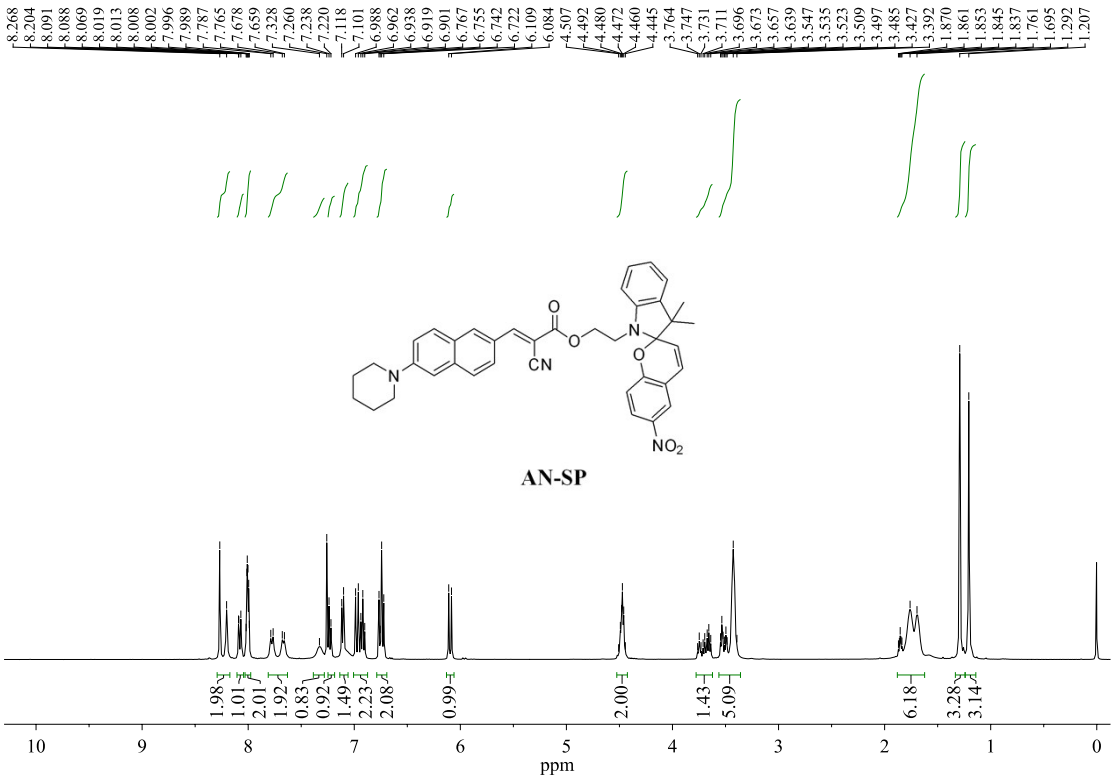


Fig. S15 The ^{1}H NMR spectrum of **AN-SP** (400 MHz, $CDCl_3$).

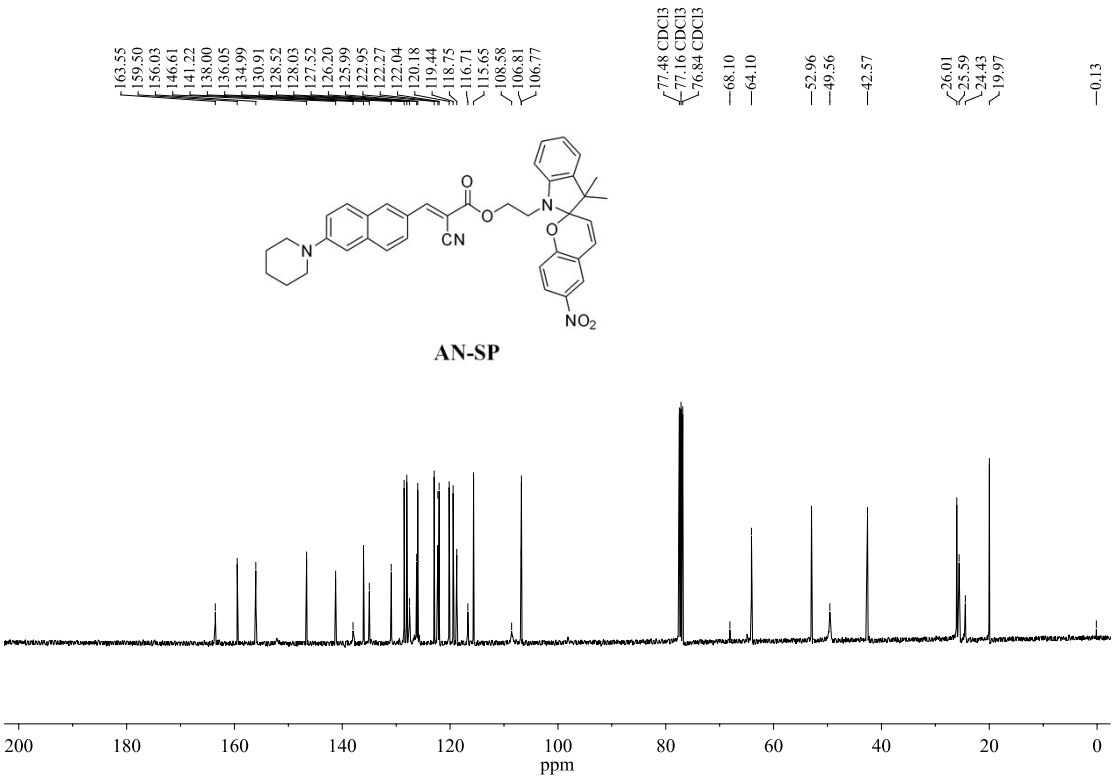


Fig. S16 The ^{13}C NMR spectrum of **AN-SP** (100 MHz, $CDCl_3$)

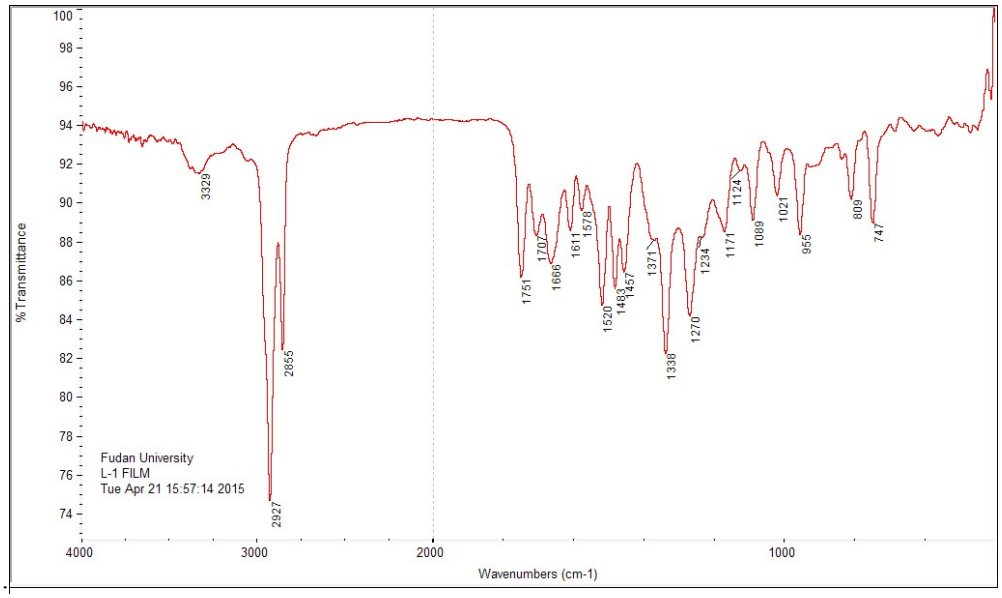


Fig. S17 The IR of **AN-SP**

Reference

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