Electronic Supplementary Information

Ratiometric fluorescence imaging of lysosomal NO in living cells and mice brains with Alzheimer's disease

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EXPERIMENTAL SECTION

Chemicals and Instruments. The diethylamine NONOate sodium salt hydrate (DEANO) was purchased from Sigma-Aldrich (Shanghai, China). Poly[(9,9-dioctylfluorenyl-2,7-diyl)-alt-(2-methoxy-5-(2-ethylhexyloxy)phenylenevinylene-1,4-diyl)] (PFPV) was purchased from Xi'an Polymer Light Technology Co. Ltd. (Xi'an, China). 4,7-Dibromo-5,6-Dinitrobenzo[c][1,2,5]thiadiazole, distearoyl phosphoethanolamine-polyethylene glycol (DSPE-PEG), polysorbate 80 (PS 80), 4-(4-(4,4,5,5-Tetramethyl-1,3,2-dioxaborolan-2-yl) phenyl) morpholine and tetrakis (triphenylphosphine) palladium (0) ($Pd(PPh_3)_4$) were obtained from Adamas (Shanghai, China). 3-(4,5-Dimethyl-2-thiazoly)-2,5-diphenyl-2H-tetrazolium bromide (MTT), 4',6-diamidino-2-phenylindole (DAPI), LysoTracker Blue, MitoTracker Green, and ER-Tracker Blue were bought from Beyotime Biological Technology Co., Ltd (Shanghai, China). Dulbecco's modified Eagle's media (DMEM) was acquired from Cellcook Biotech Co., Ltd (Guangzhou, China). All aqueous solutions were prepared with ultra-pure deionized water (≥18.25 MΩ·cm).

Zeta potential and dynamic light scattering were obtained with a Malvern Zetasizer Nano ZS90 (Malvern, UK). The fluorescence and UV absorption spectra were taken with a Hitachi F-4700 fluorescence spectrophotometer (Hitachi, Japan) and a Hitachi U-3900 spectrophotometer (Hitachi, Japan), respectively. High-resolution mass spectrometry (HRMS) was obtained with Agilent 6200 instrument and Bruker solanX 70 FT-MS. The absolute fluorescence quantum yields were obtained with an FLS1000 fluorescence spectrophotometer with an integrating sphere setup (Edinburgh, UK). Fluorescence imaging experiments were performed on a confocal laser-scanning microscopy (TCS SP8, Leica, Germany). Fusion FX imaging system (VILBER, France) was used for brain imaging.

Synthesis of 4,4'-((5,6-dinitrobenzo[c][1,2,5]thiadiazole-4,7-diyl)- bis(4,1-phenylene)) dimorpholine (Compound 1). 4,7-dibromo-5,6-dinitrobenzo[c][1,2,5]thiadiazole (576 mg, 1.5 mmol), 4-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)morpholine (1.012 g, 3.5 mmol), $Pd(PPh_3)_4$ (174 mg, 0.15 mmol) and K_2CO_3 aqueous solution (1 N, 5 mL) in toluene (20 mL) were

added into a 50 mL three-necked flash under N_2 atmosphere. The mixture was degassed with N_2 flow for 20 min, and then heated in oil bath at 115℃ for 15 h. After cooling to room temperature, the reaction mixture was diluted with water and extracted with ethyl acetate (3 × 20 mL). The organic phase was washed with saturated NaCl solution, and dried over anhydrous Na_2SO_4, and then concentrated in vacuum to obtain a red residue. The crude product was purified by silica gel column chromatography using eluent (petroleum ether / EtOAc, v/ v = 2 / 1) to afford a red solid of Compound 1 (263 mg, 32%). ^{1}H NMR (400 MHz, Chloroform-d) δ/ppm = 7.68 – 7.62 (m, 1H), 7.55 (d, J = 7.9 Hz, 1H), 4.15 (q, J = 4.1, 3.3 Hz, 2H), 3.58 – 3.41 (m, 2H).

Synthesis of 4,7-bis(4-morpholinophenyl)benzo[c][1,2,5]thiadiazole-5,6-diamine (LSBT). Compound 1 (40 mg, 0.073 mmol), iron powder (50 mg, 0.081 mmol) and AcOH (2 mL) were added into a 10 mL sealed tube. The reaction mixture was stirred at 100℃ for 3 h. After cooling to room temperature, the reaction was neutralized with saturated $NaHCO_3$ aqueous solution and then extracted with EtOAc (3 × 10 mL). The extract liquor was washed with saturated NaCl aqueous solution, dried over anhydrous Na_2SO_4, and concentrated in vacuum to give a dark-yellow residue. The residue was purified by chromatography on a silica gel column (CH_2Cl_2/MeOH, v/v= 100:1) to furnish the desired compound LSBT as a yellow solid (15 mg, 42% yield). ^{1}H NMR (400 MHz, DMSO-d6) δ 7.28 – 7.18 (m, 2H), 6.98 – 6.90 (m, 2H), 6.85 – 6.76 (m, 1H), 3.77 – 3.70 (m, 4H), 3.12 – 3.06 (m, 4H). ^{13}C NMR (101 MHz, DMSO-d6) δ/ppm=151.48, 144.63, 135.82, 117.86, 115.93, 113.96, 66.72, 66.52, 55.37, 50.67, 48.22. LR−ESI−MS (m/z): $[M + H]^+$ =489.2.

Preparation of LyNO-Pdots. The LyNO-Pdots were prepared by a modified nanoprecipitation method. Typically, PFPV polymer, LSBT and DSPE-PEG were dissolved in THF solution at a concentration of 1 mg·mL^{-1}, respectively, and the obtained solution was stored at 2-8℃ for further use. Then 12 μL of PFPV, 400 μL of LSBT and 100 μL of DSPE-PEG were added into 2 mL of THF. Under vigorous ultrasound, the mixture was rapidly injected into 10 mL of PS-80 aqueous solution (0.5 mg·mL^{-1}). After continuous sonication for 5 min, the solution was distilled under

reduced pressure to remove THF. The obtained colloidal solution was filtered through 0.2 μm membrane filter to obtain the final product of LyNO-Pdots.

Preparation of Reactive Oxygen/Nitrogen Species. In a typical procedure, the NO solution was prepared by passing gas NO into the deoxygenated water, and the NO concentration of the stock solution was measured by the Griess Method. Nitrite (NO_2^-), nitrate (NO_3^-) and hypochlorite anion (ClO^-) were prepared by dissolving sodium nitrite, sodium nitrate and NaClO in deionized water, respectively. H_2O_2 solution (1 mM) and tert-butyl hydroperoxide (TBHP) solution were obtained by diluting H_2O_2 (30%) and TBHP with ultrapure water. Hydroxyl radical (•OH) was prepared by Fenton reaction between H_2O_2 solution (6 mM) with ferric ion solution (1 mM). Peroxynitrite ($ONOO^-$) solution was generated by H_2O_2 solution (1 mM) reacting with $NaNO_2$ (1 mM). The single oxygen ($^1O_2$) was formed by reacting NaClO (1 mM) with H_2O_2 (1 mM).

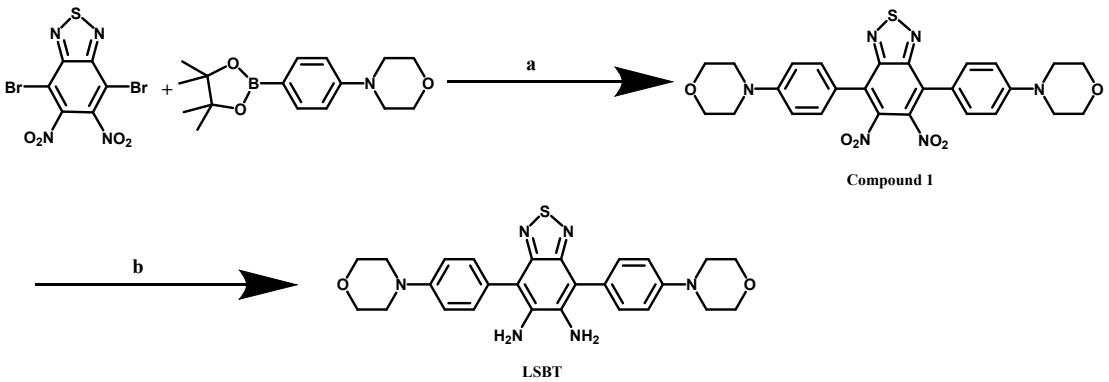
Cell Culture and Cytotoxicity Assay. Brain-derived Endothelial cells (bEnd.3) were acquired from the cell bank of Chinese Academy of Sciences (Shanghai, China). bEnd.3 cells were cultured in DMEM medium containing 1% penicillin/streptomycin and 10% fetal bovine serum (FBS) under a 5% CO_2 atmosphere at 37°C. The cell cytotoxicity of LyNO-Pdots was evaluated by MTT assay. Typically, bEnd.3 cells with 1×10^4 cells per well were seeded on 96-well plates and incubated for 24 h at 37°C under 5% CO_2. After 80% confluence, LyNO-Pdots at concentrations ranging from 0 to 105 $\mu g\cdot mL^{-1}$ were added to the wells, followed by incubation for 24 h. Afterwards, 10 μL of MTT was added to each well individually and the cells were incubated for another 4 h. Subsequently, the formazan solvent was added to dissolve the formazan crystal. Finally, the absorbance was recorded with a plate reader at 570 nm.

Fluorescence Imaging of NO in bEnd.3 Cells. Cells were seeded on 35 mm glass bottom culture dishes with 8000 cells per well for fluorescent imaging, and incubated at 37°C for 24 h. The cells were treated with 105 $\mu g\cdot mL^{-1}$ LyNO-Pdots for 8 h. After washing two times with PBS buffer, the cells stained with LyNO-Pdots were incubated with various concentrations of DEANO (NO donor) for 1 h. Finally, the cells were washed three times with phosphate-buffered solution (PBS)

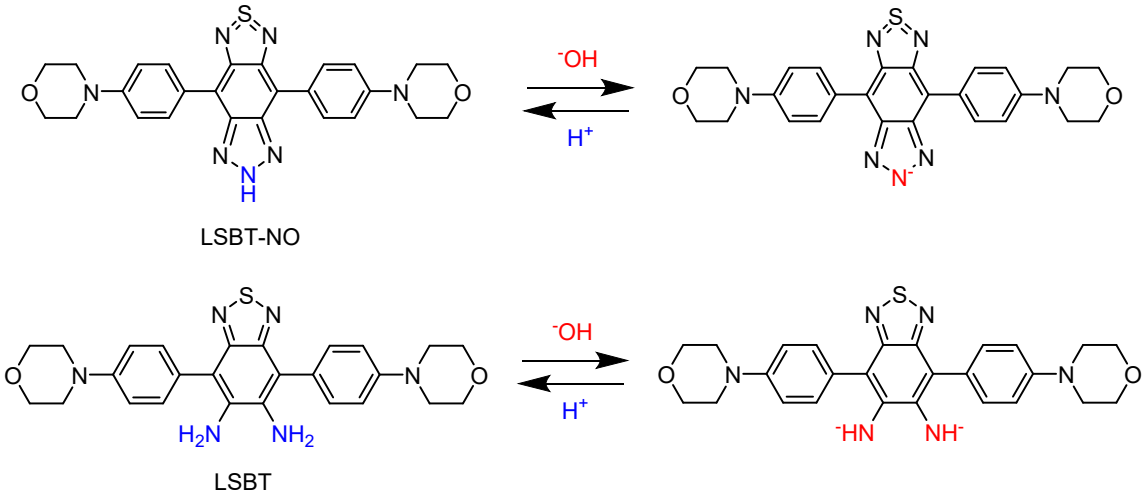
and then observed with confocal laser scanning microscopic (CLSM). For colocalization experiments, the LyNO-Pdots pretreated cells were incubated with 2 μM LysoTracker Blue for 1 hour, 20 nM MitoTracker Green for 15 min and ER-Tracker Blue for 15 min, respectively.

***In Vitro* Evaluation of BBB Permeability.** To construct an in vitro BBB model, bEnd.3 cells were seeded on a 1.1 mL transwell with a diameter of 0.4 μm culture system at a density of 1×10^6 cells/insert and grown in on insert membrane. After about 5 h of incubation, the transendothelial electrical resistance (TEER) can reach the 150-300 $\Omega\cdot cm^2$ interval, implying the formation of a continuous and dense monolayer film. Then, LyNO-Pdots were added to the upper dish and the fluorescence intensity of the probe-containing medium was recorded as F1. After incubation for 5 h, the fluorescence intensity (F2) of the lower medium was measured. The BBB penetration rate of the LyNO-Pdots was obtained by calculating the ratio of F2 to F1.

Fluorescence Brain Imaging of NO in AD Mice. Normal wide-type C57BL/6 mice (WT, 10-month old, 25±2.0 g) and double-transgenic APPSWE/PS1dE9 (APP/PS1) mice (Tg, 10 month-old, 25±1.0 g) were raised under standard conditions with sufficient food and water. For imaging, the mice were treated by injecting 100 μL of LyNO-Pdots into the tail vein of APP/PS1 mice and C57BL/6 mice at 4 $mg\cdot kg^{-1}$ based on their weight, respectively. The APP/PS1 mice were injected with normal saline via tail vein as a control group. The brain imaging of AD mice were recorded with the Fusion FX imaging system. The excitation wavelength is 480 nm and the fluorescence is collected with 535 nm and 590 nm, respectively. After 2 hours of tail vein injection, these mice were executed. Subsequently, the brain of the mice was dissected, and fluorescence imaging of the tissue sections of the hippocampus was performed with confocal laser scanning microscopy. In this study, animal experiments were conducted in accordance with the protocol approved by the Animal Ethics Committee of Anhui Normal University (Approval No. AHNU-ET2022029).



Scheme S1 Synthetic route of the dye LSBT. Reaction conditions: (a) K_2CO_3, $Pd(PPh_3)_4$, Toluene, N_2, reflux 15 h; (b) Fe, AcOH, 3 h.



Scheme S2 Mechanism of LSBT-NO acid promoting fluorescence characteristics and the influence of acid-base environment on the structural changes of LSBT.

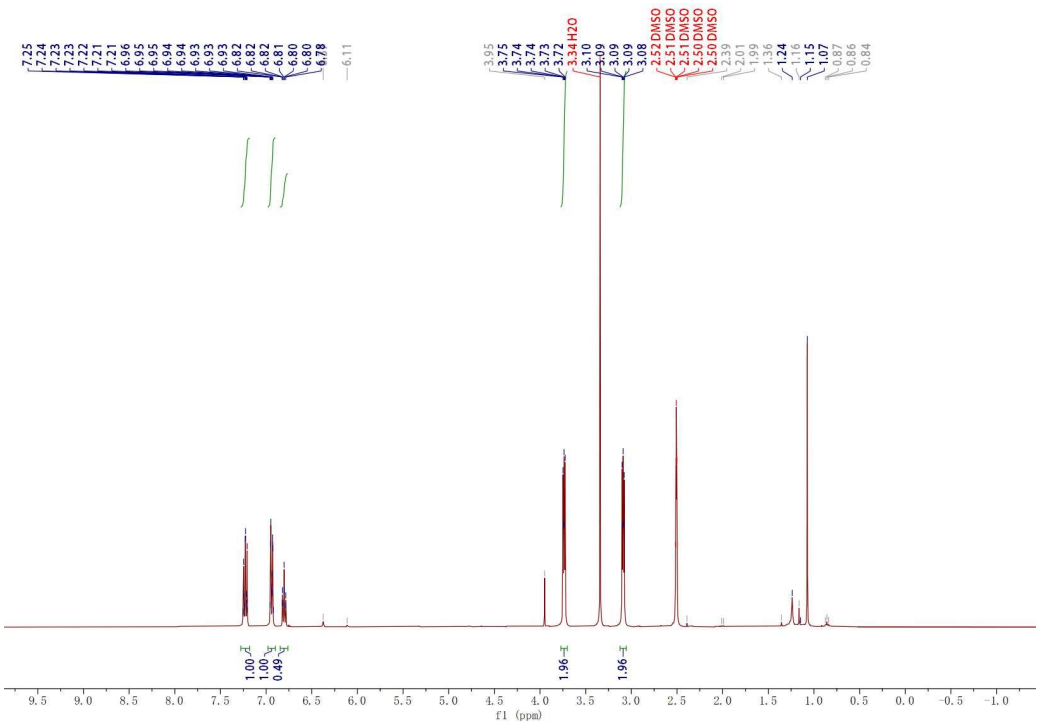


Fig. S1 ^{1}H NMR spectra of LSBT in DMSO-D6.

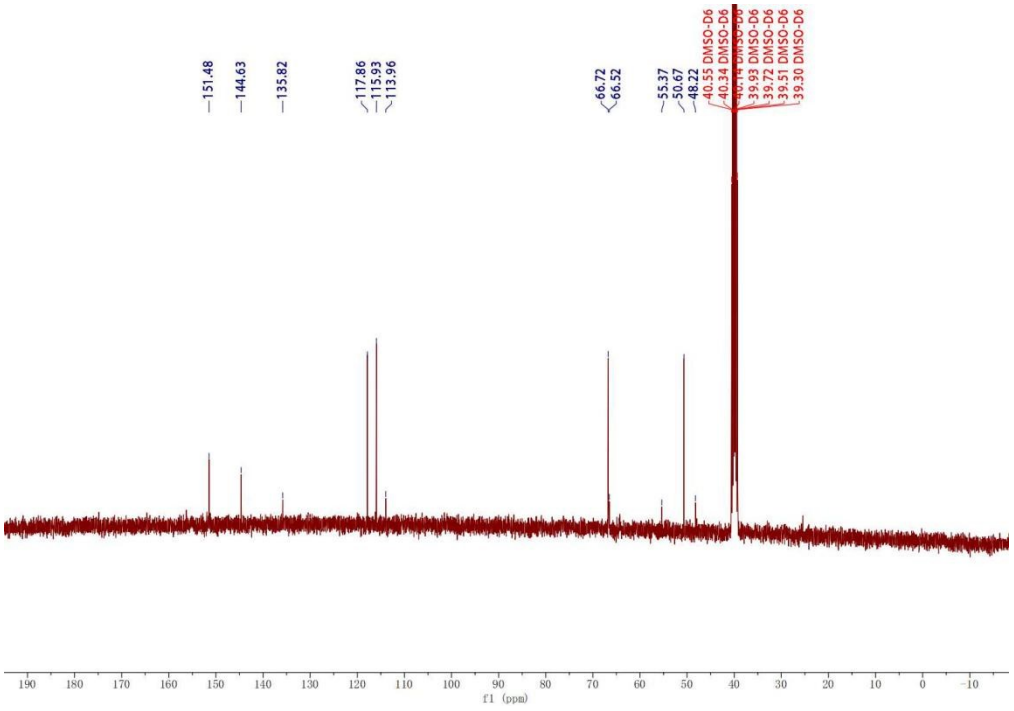


Fig. S2 ^{13}C NMR spectra of LSBT in DMSO-D6.

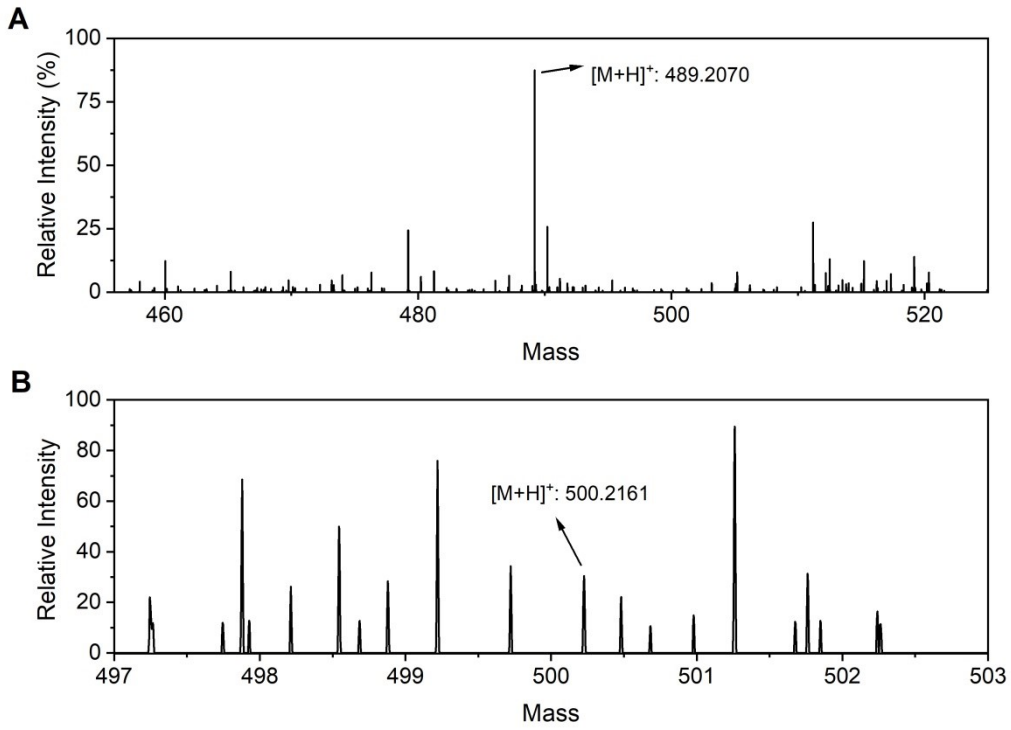


Fig. S3 Mass spectra of LSBT before (A) and after (B) reacting with NO.

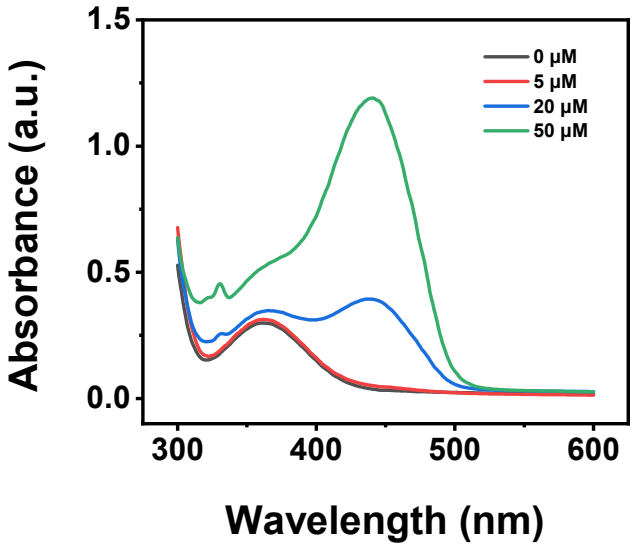


Fig. S4 UV-vis spectra of LSBT in the presence of NO with different concentrations.

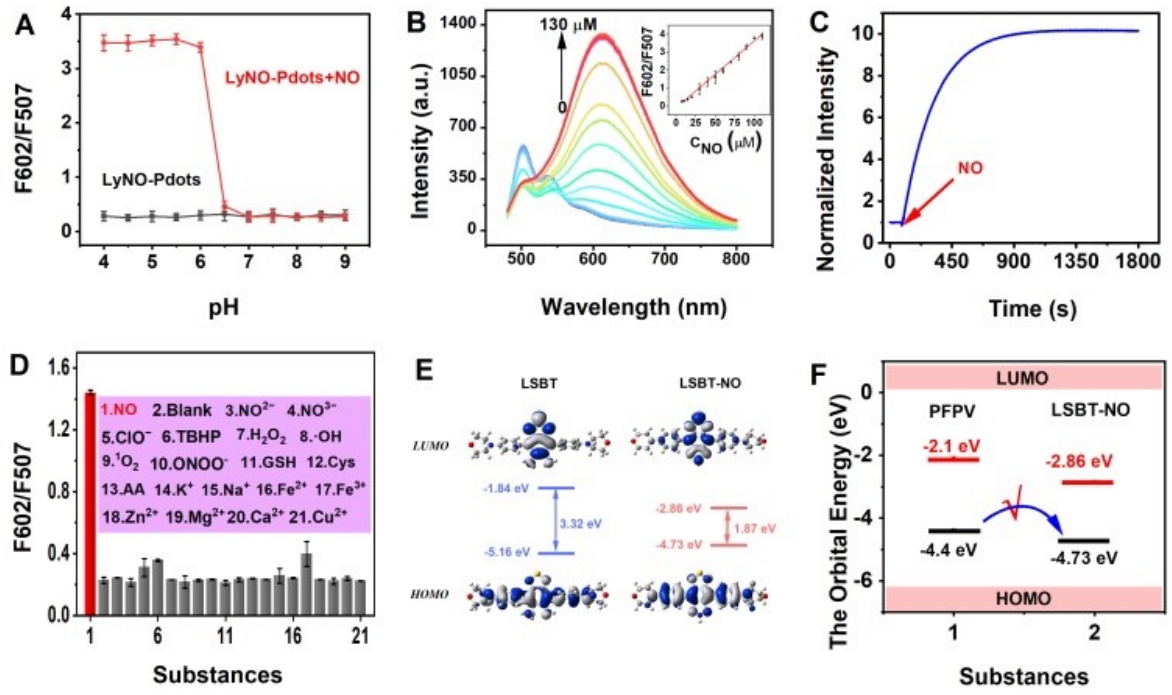


Fig. S5. (A) The intensity ratios (F602/F507) of LyNO-Pdots in the presence or absence of NO at different pH values. (B) Fluorescence spectra of LyNO-Pdots in the presence of various concentrations of NO. Inset: linear plot of intensity ratios vs. NO concentrations. (C) Time-dependent fluorescence intensity curve of LyNO-Pdots at 602 nm in the presence of 100 μM NO. (D) Fluorescence response of LyNO-Pdots incubated with NO and potential interfering substances. (E) Molecular orbitals of LSBT and LSBT-NO are visualized at the B3LYP/6-31+G (d) level in Gas phase. (F) Schematic energy level diagram of PFPV and LSBT-NO.

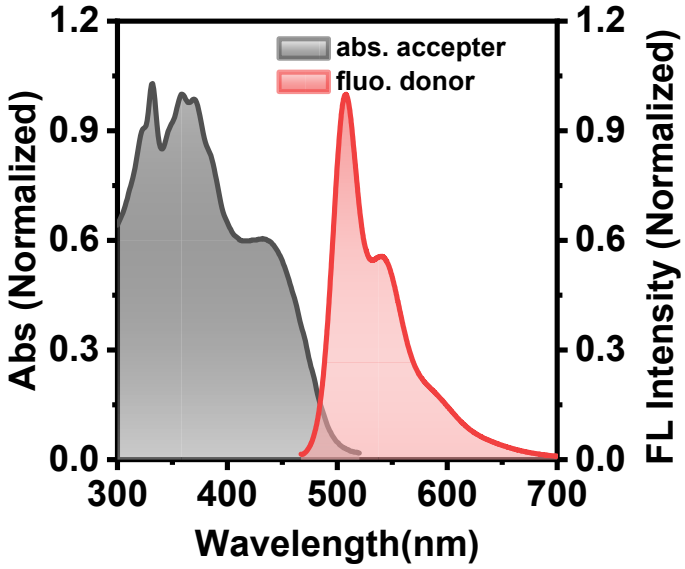


Fig. S6 The absorption spectra of LSBT-NO and emission spectra of PFPV Pdots.

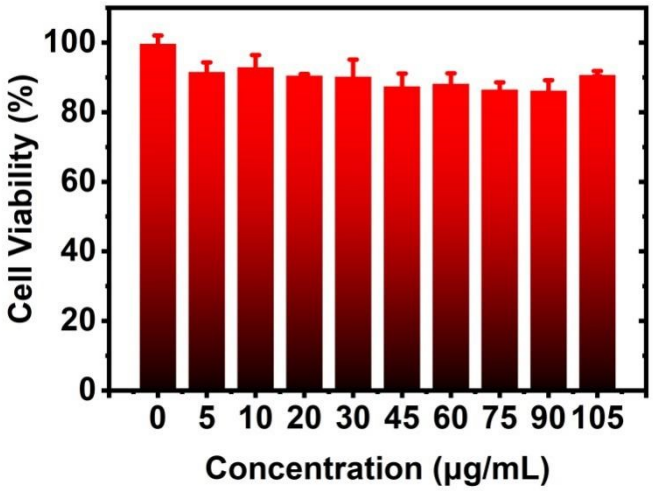


Fig. S7 MTT method was used to measure the cell viability of bEnd. 3 cells with LyNO-Pdots probes of different concentrations. The co incubation time of cells and materials is 24 hours.

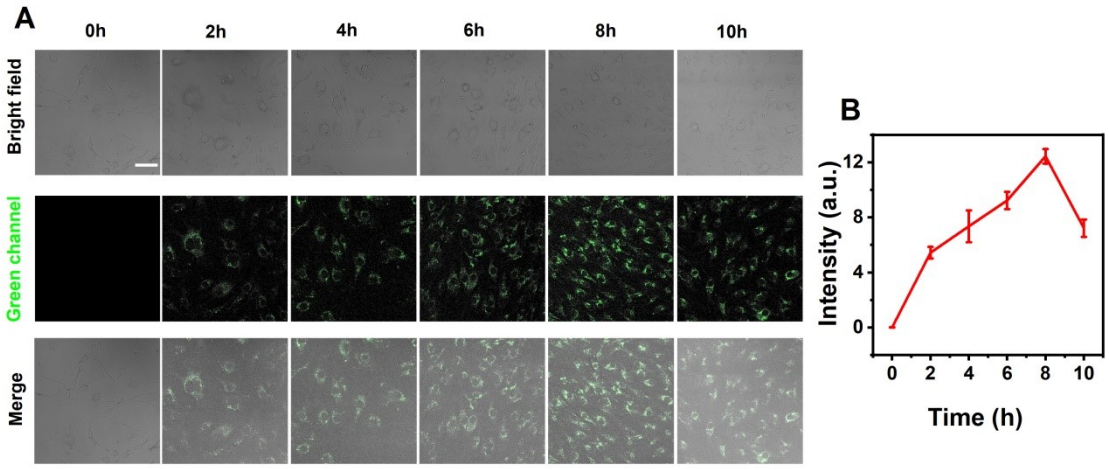


Fig. S8 The incubation time of bEnd.3 cells with LyNO-Pdots (105 μg·mL^{-1}) was optimized. (A) The change of fluorescence intensity in cells with the change of incubation time. (B) Change curve of fluorescence intensity during incubation time. λex = 488 nm, λem = 500~580 nm. Scale bar = 50 μm.

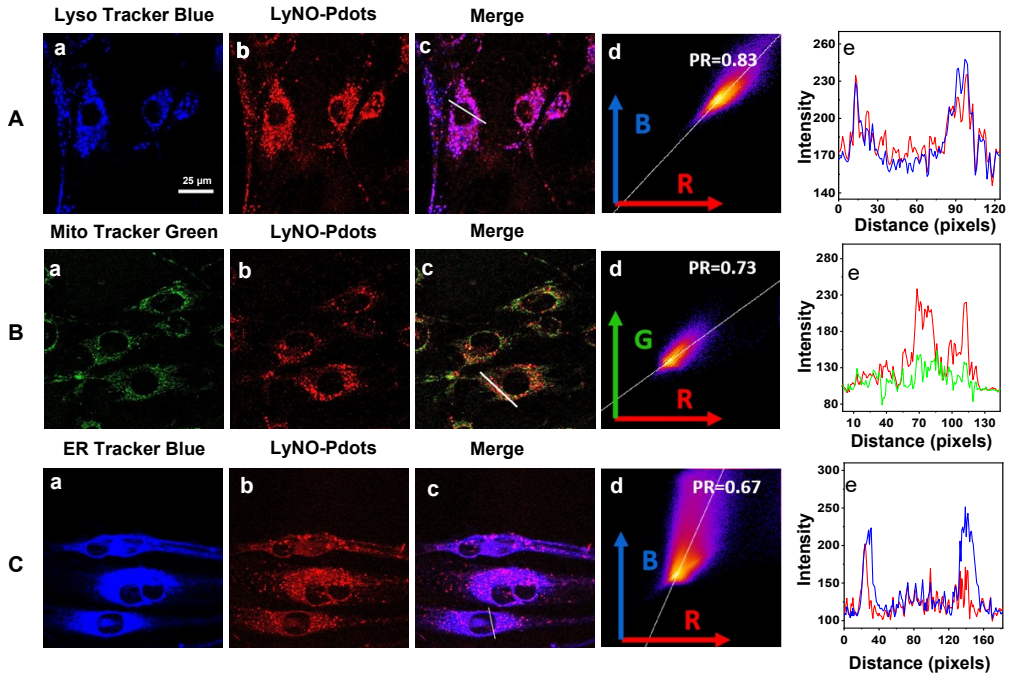


Fig. S9 Colocalization of DEANO (NO donor) activated LyNO-Pdots with lysosomes (A), mitochondria (B), and endoplasmic reticulum (C) in bEnd.3 cells, and corresponding fluorescence intensity profile (A-e, B-e, C-e). LyNO-Pdots channel: λ_{ex} = 488 nm, λ_{em} = 590-680 nm. Blue channel: λ_{ex} = 405 nm, λ_{em} = 425-435 nm. Green channel: λ_{ex} = 488 nm, λ_{em} = 570-580 nm. Scale bar =25 μm.

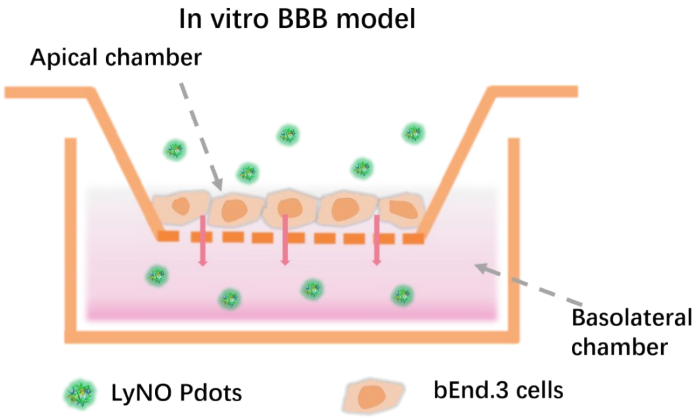


Fig. S10 Schematic diagram of an experimental model for crossing the blood-brain barrier.

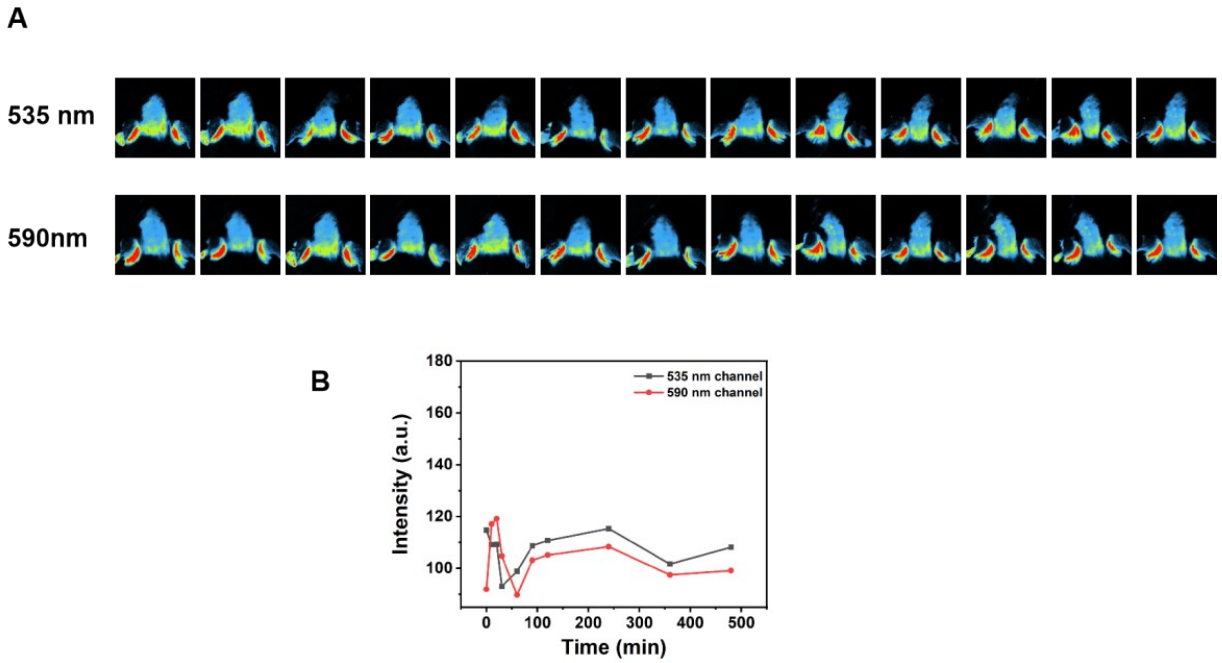


Fig. S11 The variations in the fluorescence intensities from different channels in the brain of AD mice after injecting normal saline into the tail vein. (A) Visualization of fluorescence intensity in the brain. (B) Changing trends in fluorescence intensities from different channels. λ_{ex} = 480 nm, LyNO Pdots: 1 mg·mL^{-1}, n=3.