Supplementary Information (SI) for ChemComm.

Supporting Information

ROS/Viscosity Dual-activating Nanoprobe with CD44-Targeting Ability for NIR-II Imaging of Early Atherosclerotic Plaques

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Experimental Procedures

Materials and Methods

All chemicals and reagents used in the synthesis were purchased from commercial suppliers (Aladdin, Bidepharm, Makclin Biochemical, and J&K) and used without further purification. Dulbecco's Modified Eagle Medium (DMEM), penicillin, and streptomycin were obtained from Solarbio, while fetal bovine serum (FBS) was purchased from Beyotime. The ^{1}H NMR was analyzed by an AVANCE III HD (400 MHz) spectrometer. High-resolution mass spectrometry (HRMS) was performed using waters Xevo G2 Qtofmass spectrometer. UV-visible absorption spectra were measured with a UV-2310II spectrophotometer. NIR-II fluorescence emission spectra were recorded using an Edinburgh FLS1000 spectrometer. Confocal fluorescence images of cells were obtained with a laser confocal microscope (Lecia SP5). In vivo fluorescence images of mice were recorded with an NIR-II small animal fluorescence imaging system (Beijing RayLight Technology Co., Ltd.).

Synthesis of Probes

Synthesis of Compound 1

2-Bromobenzaldehyde (185 mg, 1 mmol), indolin-2-one (212 mg, 0.6 mmol), and Cs_2CO_3 (978 mg, 3 mmol) were added to a 100 mL two-necked flask, followed by dissolution in 4 mL of DMSO. The mixture was stirred at 120 °C for 2 hours under a nitrogen atmosphere. After completion, 100 mL of water was added to the mixture, which was then extracted with ethyl acetate (EtOAc) three times (3 × 50 mL). The combined organic extracts were washed with saturated brine, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (PE/EA = 2:1) to afford Compound 1 as a product (175 mg, yield: 80%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.87 (s, 1H), 8.85 (s, 1H), 8.54 (s, 1H), 8.35 (s, 1H), 8.27 (s, 1H), 7.90 (s, 1H), 7.78 (s, 1H), 7.63 (s, 1H), 7.09 (s, 1H).

Synthesis of Compound 2

Compound 4 (219 mg, 1 mmol), NaH (60 mg, 1.5 mmol, 60% dispersion in mineral oil), and 15 mL of anhydrous DMF were mixed and stirred in an ice bath for 0.5 hours. Iodoethane (188 mg, 1.2 mmol) was then added, and the reaction mixture was stirred at 50 °C for 4 hours. After the reaction, the solvent was removed under reduced pressure, and the residue was purified by flash column chromatography (PE/EA = 20:1) to give Compound 2 as a yellow solid (180 mg, yield: 73%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.68 (s, 1H), 8.47 (s, 1H), 8.14 (s, 2H), 7.82 (s, 1H), 7.66 (s, 2H), 7.07 (s, 1H), 4.03 (s, 2H), 1.42 (s, 3H).

Synthesis of Compound 3

Under a nitrogen atmosphere, methylmagnesium chloride solution (3.0 M in THF, 1 mL, 3 mmol) was slowly added dropwise to a solution of Compound 5 (247 mg, 1 mmol) in anhydrous THF (10 mL). The mixture was stirred at 60 °C for 4 hours, then cooled, neutralized with 2 M hydrochloric acid, and stirred for an additional 0.5 hours. A 5 mL potassium iodide solution (1 M) was added, and the resulting precipitate was filtered and washed with deionized water to obtain Compound 3 as an orange solid (210 mg, yield: 56%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.17 – 8.93 (m, 2H), 8.12 (d, J = 21.3 Hz, 2H), 7.34 (d, J = 37.2 Hz, 4H), 4.77 (s, 2H), 3.29 (s, 3H), 1.59 (s, 3H).

Synthesis of Compound 4

4-Hydroxyisophthalaldehyde (75 mg, 0.5 mmol) was dissolved in anhydrous DMF (3.0 mL), and K_2CO_3 (138 mg, 1 mmol) was added. After stirring for 10 minutes, 4-(bromomethyl)phenylboronic acid pinacol ester (180 mg, 0.6 mmol) was added, and the mixture was stirred at room temperature for 12 hours. The reaction solution was dissolved in ethyl acetate (100.0 mL), washed three times with saturated ammonium chloride solution, and dried over anhydrous sodium sulfate. After concentration under reduced pressure, the crude product was purified by silica gel column chromatography to afford Compound 4 as a white solid (160 mg, 89%). ^{1}H NMR (400 MHz, Chloroform-d) δ 10.58 (s, 1H), 9.97 (s, 1H), 8.38 (s, 1H), 8.12 (s, 1H), 7.90 (s, 2H), 7.45 (s, 2H), 7.21 (s, 1H), 5.35 (s, 2H), 1.37 (s, 12H).

Synthesis of DCy-BOH

Compound 3 (93 mg, 0.25 mmol) and Compound 4 (37 mg, 0.1 mmol) were dissolved in a mixed solvent of toluene/n-butanol (5.0 mL/5.0 mL). The mixture was stirred overnight at 100 °C under nitrogen protection. After completion, the solvent was removed under reduced pressure, and the crude product was purified by silica gel column chromatography to obtain DCy-BOH as a reddishbrown solid (50 mg, 49%). ^{1}H NMR (400 MHz, Methylene Chloride-d_2) δ 10.27 (d, J = 45.1 Hz, 3H), 9.86 – 9.32 (m, 3H), 9.22 – 8.69 (m, 3H), 8.60 – 6.92 (m, 15H), 6.57 (d, J = 159.6 Hz, 3H), 5.13 (s, 2H), 4.73 (s, 2H), 4.31 (s, 2H), 1.56 (d, J = 17.6 Hz, 6H), 1.47 (t, J = 7.2 Hz, 12H). ESI HRMS: calcd. for $C_{57}H_{51}BN_2O_3^{2+}$ $[M]^{2+}$: 411.1991, found m/z: 411.0827.

Synthesis of BSA-HA

200 mg of hyaluronic acid (HA, molecular weight 40–100 kDa) was dissolved in 20 mL of deionized water, and the pH was adjusted to 5.5 with dilute hydrochloric acid. 200 mg of EDC and 100 mg of NHS were added, and the mixture was stirred in the dark for 2 hours for activation. Then 200 mg of BSA was added, and the pH was adjusted to 7.5 with PBS (pH = 9). The reaction was allowed to proceed for 12 hours. The reaction solution was dialyzed (MWCO: 5000) for 48 hours to remove EDC and NHS, and the dialysate was finally lyophilized to obtain a white cotton-like solid, which was sealed and stored at −20 °C for further use.

Preparation of DCy-BOH@BSA-HA

1 mg of DCy-BOH was dissolved in 50 µL of DMSO, and this solution was added dropwise to 950 µL of an aqueous solution containing BSA-HA (BSA-HA concentration: 10 mg/mL). The mixture was sonicated for 10 minutes to form the DCy-BOH@BSA-HA nanocomposite probe. The DCy-BOH@BSA-HA probe should be freshly prepared before use

Fluorescence spectrum test

The DCy-BOH compound was dissolved in dimethyl sulfoxide (DMSO) to prepare a 1 mM stock solution. This stock solution was then diluted in a CH_3CN/PBS (1:1, v/v) mixture to obtain a 20 µM working solution for measurement. All fluorescence emission spectra were recorded on an Edinburgh FLS1000 spectrophotometer using an excitation wavelength of 808 nm.

NIR-II Fluorescence quantum yield (QY) Test

The NIR-II fluorescence quantum yield (QY) of DCy (in CH_3CN/H_2O = 1/1, v/v) was determined following a reported method [1]. Briefly, IR-1061 with a known QY of 1.7% in dichloromethane (DCM) was used as the reference. A series of four solutions with absorbance at 808 nm below 0.1

were prepared for both the sample and the reference. The QY of DCy was then calculated using the following equation by comparing the slopes obtained from the linear regression of the integrated fluorescence intensity versus absorbance.

$$QY_{(sample)} = QY_{(ref)} \times \frac{slope_{(sample)}}{slope_{(ref)}} \times \frac{n^2_{(sample)}}{n^2_{(ref)}}$$

Herein, the parameter n denotes the solvent refractive index, and the slope is derived from the linear fit of the integrated fluorescence intensity versus absorption.

Cell Experiments

RAW 264.7 macrophages were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (double antibodies) in a humidified incubator at 37 °C with 5% CO_2. For induction of ROS-overexpressing M1 macrophages: RAW 264.7 cells were stimulated with LPS (1 μg/mL) for 24 hours to establish M1 macrophage models, while the control group was treated with NAC (50 μM) as a ROS inhibitor. Intracellular ROS overexpression in RAW 264.7 macrophages was stained using the DCFH-DA ROS detection kit, and images were acquired by laser confocal microscopy with an excitation wavelength of 488 nm and emission collection range of 500–550 nm.

Animal Experiments

BALB/c, C57BL/6, and ApoE$^{-/-}$ mice (approximately 5 weeks old) used in this study were purchased from Jiangsu Jicui Yaokang Biotechnology Co., Ltd. All procedures complied with the guidelines of the National Research Council. All animal experiments were performed under the Guidelines for the Care and Use of Tsinghua University (approval number: PA240 12302). For the LPS-induced acute inflammatory mouse model, normal saline or LPS (2 mg/mL, 25 μL) was injected into the upper hindlimbs (left and right sides) of mice to simulate normal and inflamed tissues, respectively. At 12 hours after saline/LPS treatment, DCy-BOH@BSA (50 μL, 1 mg/mL, BSA concentration: 10 mg/mL) was injected into normal and inflamed tissues, and NIR-II images were captured every 30 minutes post-injection.

ApoE$^{-/-}$ mice fed a high-fat diet for 16 weeks were used as atherosclerotic (AS) models, while healthy C57BL/6 mice fed a normal diet for 16 weeks served as controls; these mice were used for ex vivo fluorescence imaging of the aorta. Mice were divided into three groups (n = 3 per group) for tail vein injection: (1) ApoE$^{-/-}$ + DCy-BOH@BSA-HA; (2) ApoE$^{-/-}$ + DCy-BOH@BSA; (3) C57BL/6 + DCy-BOH@BSA-HA (DCy-BOH concentration: 1 mg/mL; BSA-HA and BSA concentration: 10 mg/mL; volume: 200 μL). At 8 hours post-injection, aortas and organs were dissected from AS and normal mice, followed by ex vivo NIR-II fluorescence imaging of the aorta using an NIR-II imaging system (Imaging parameters: 808 nm laser, 900 nm long-pass filter.).

Supplementary Figures

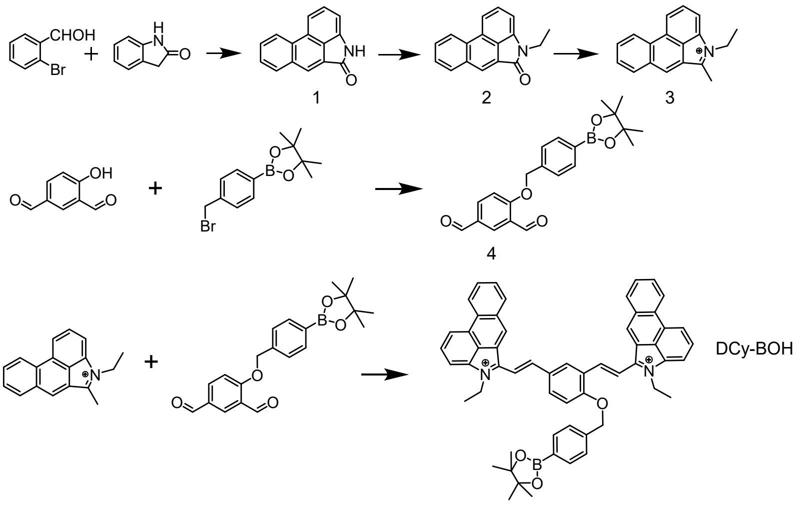

Figure S1. Synthetic Route Diagram.

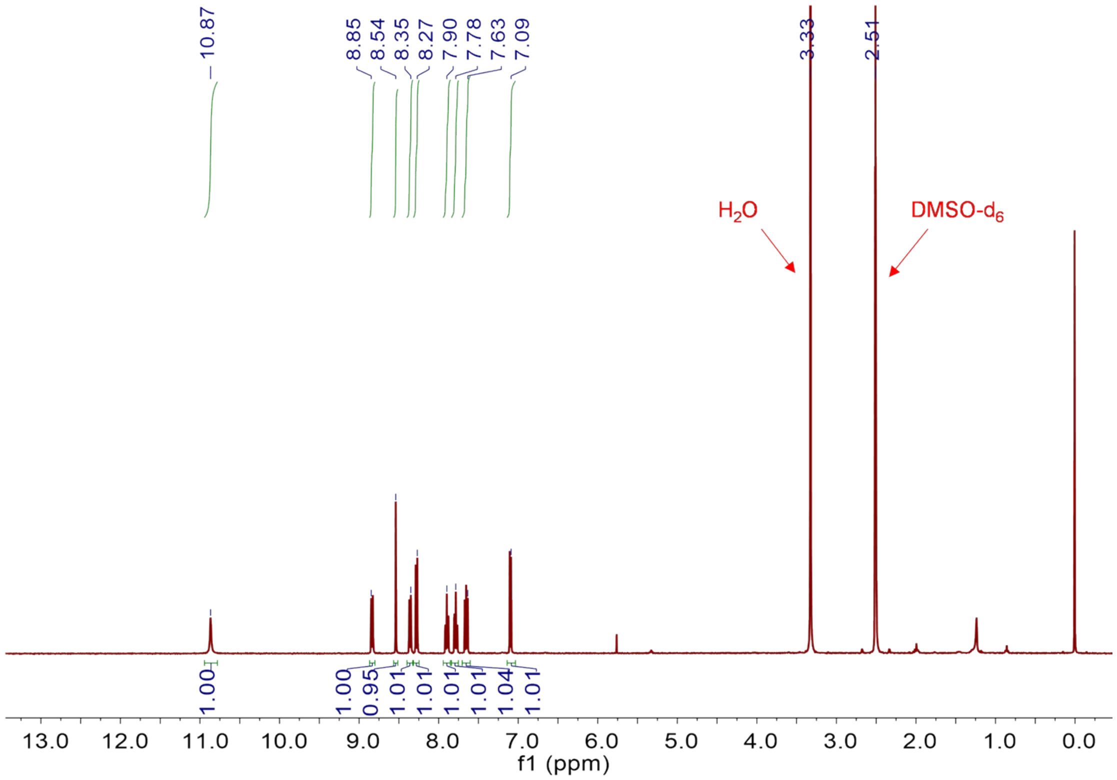

Figure S2. ^{1}H NMR spectrum of compound 1 (DMSO-d_6, 400 MHz).

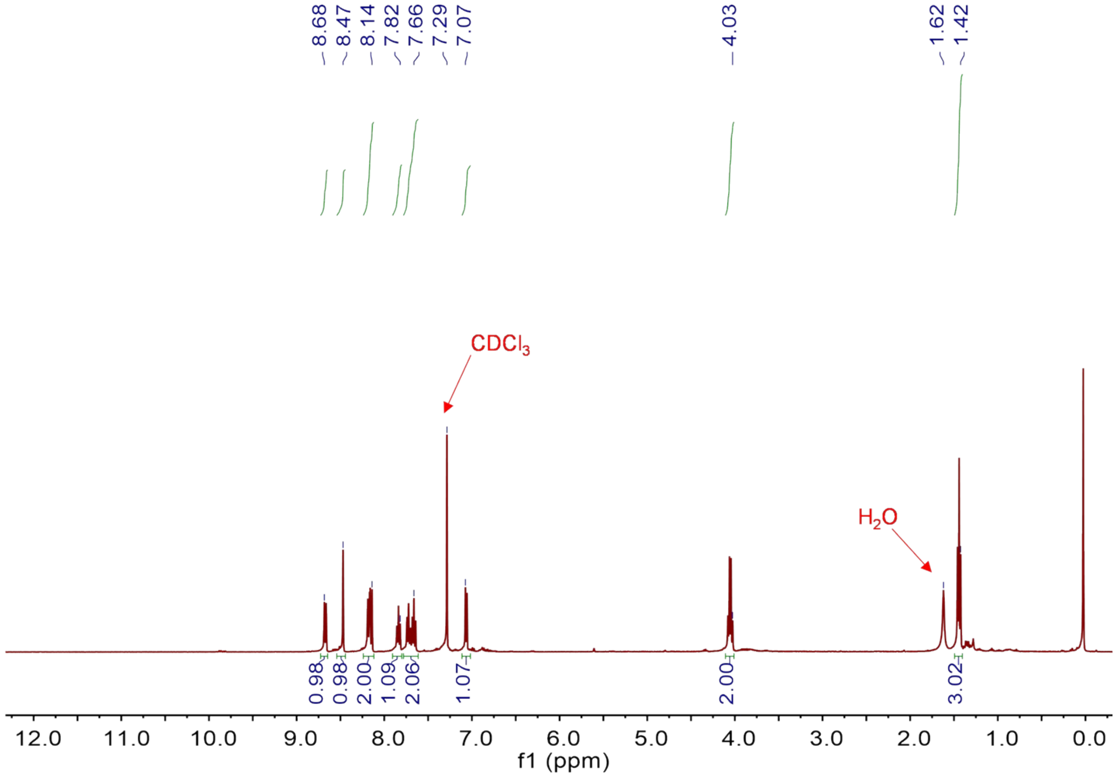

Figure S3. ^{1}H NMR spectrum of compound 2 (CDCl$_3$, 400 MHz).

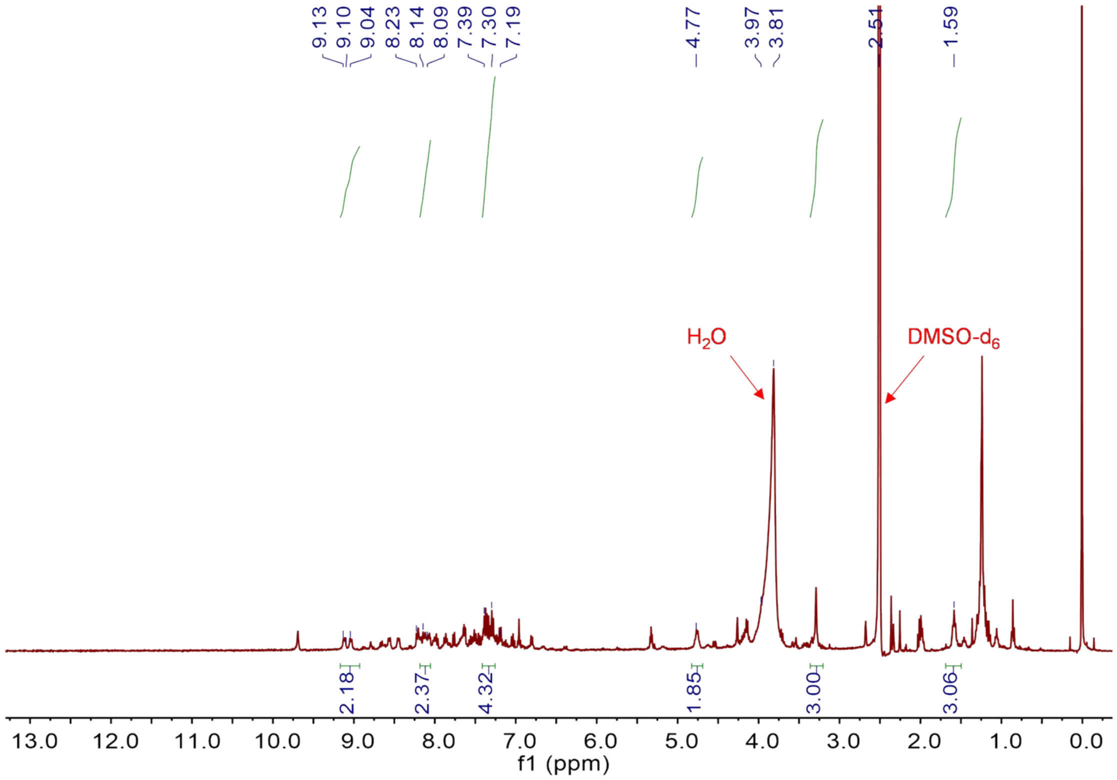

Figure S4. ^{1}H NMR spectrum of compound 3 (DMSO-d_6, 400 MHz).

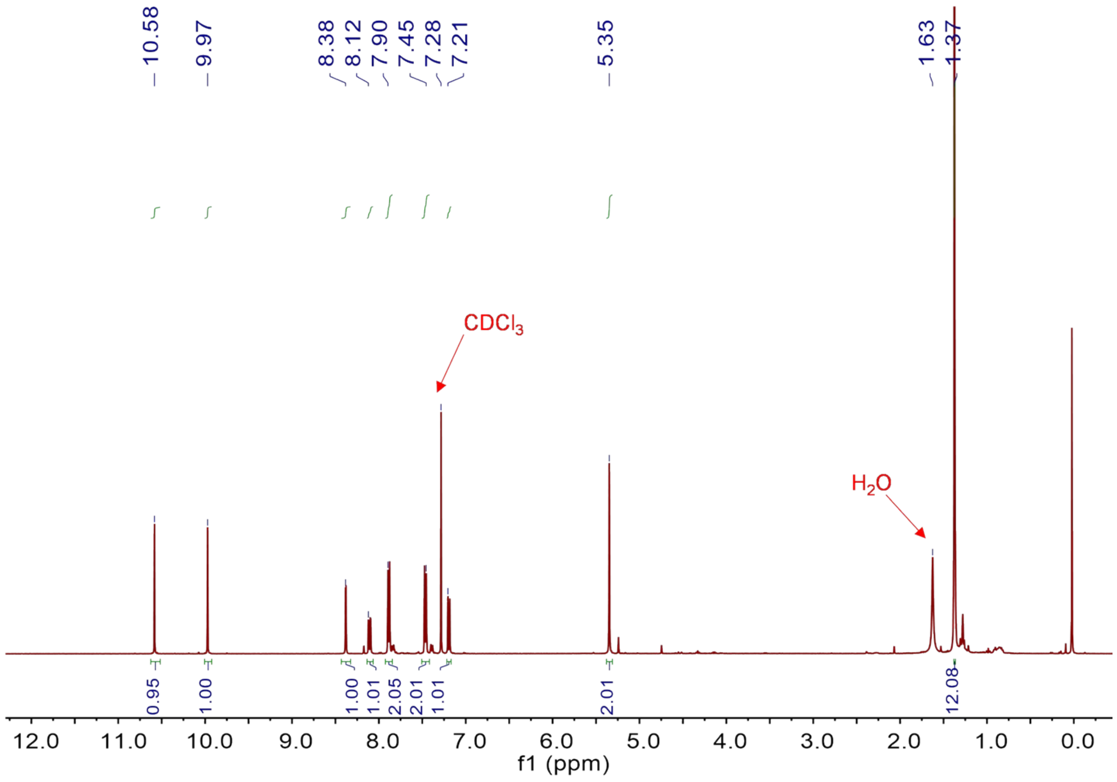

Figure S5. ^{1}H NMR spectrum of compound 4 ($CDCl_3$, 400 MHz).

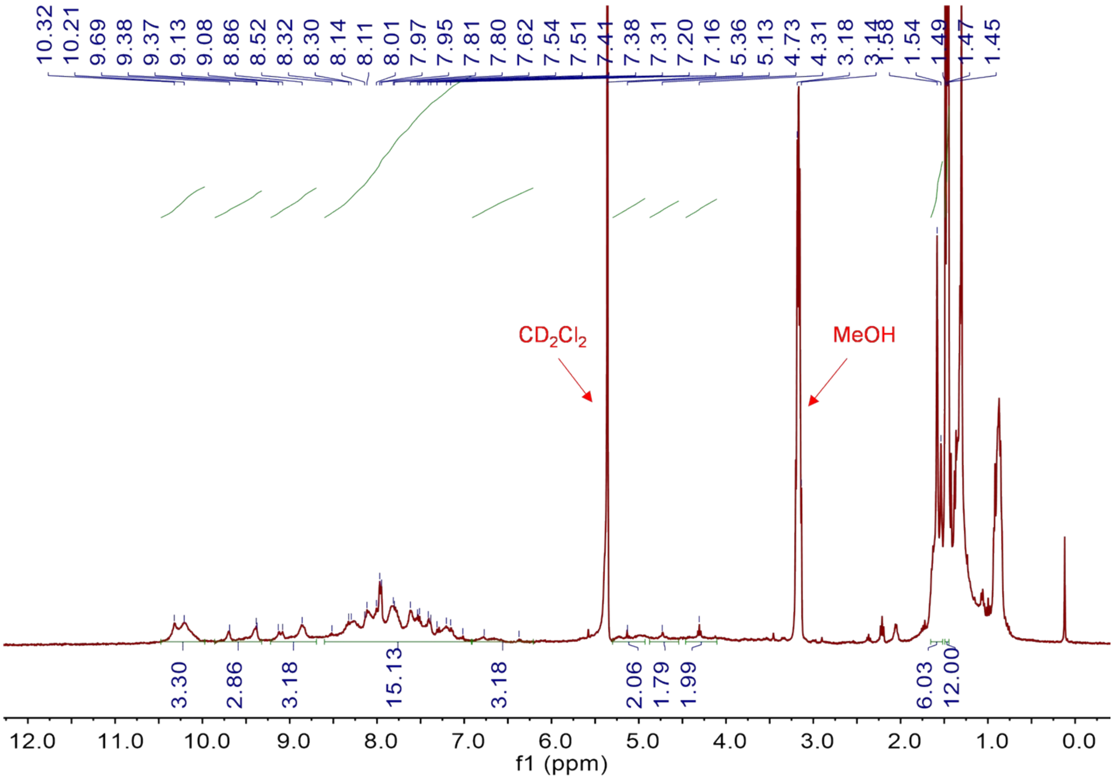

Figure S6. ^{1}H NMR spectrum of compound DCy-BOH (CD_2Cl_2, 400 MHz).

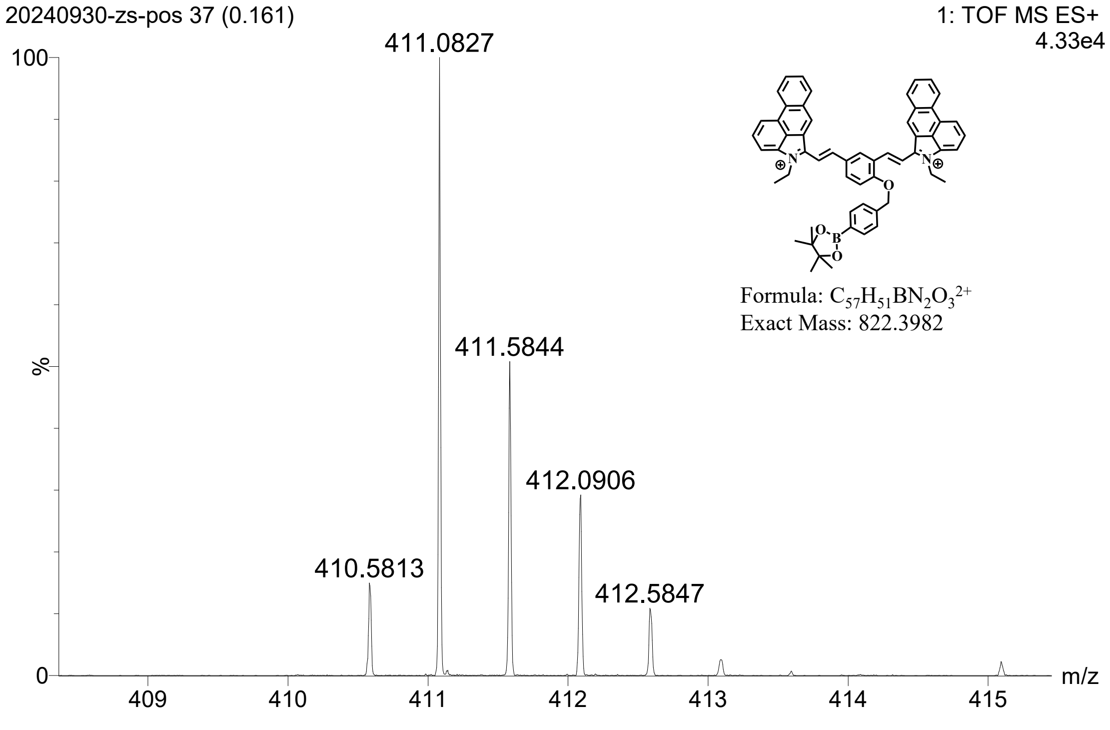

Figure S7. The high-resolution mass spectrum of DCy-BOH.

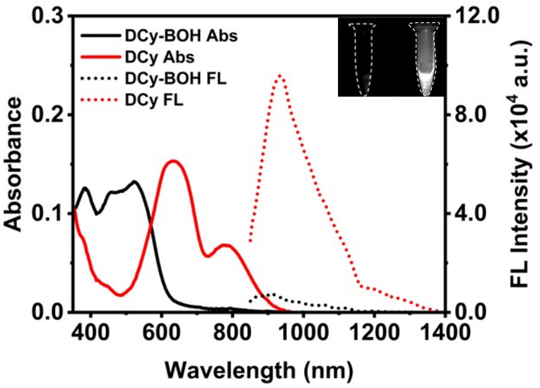

Figure S8. Absorption and emission spectra of DCy-BOH and DCy (10 μM, PBS/CH_3CN = 1/1, Inset: NIR-II FL imaging, Left: DCy-BOH, Right: DCy).

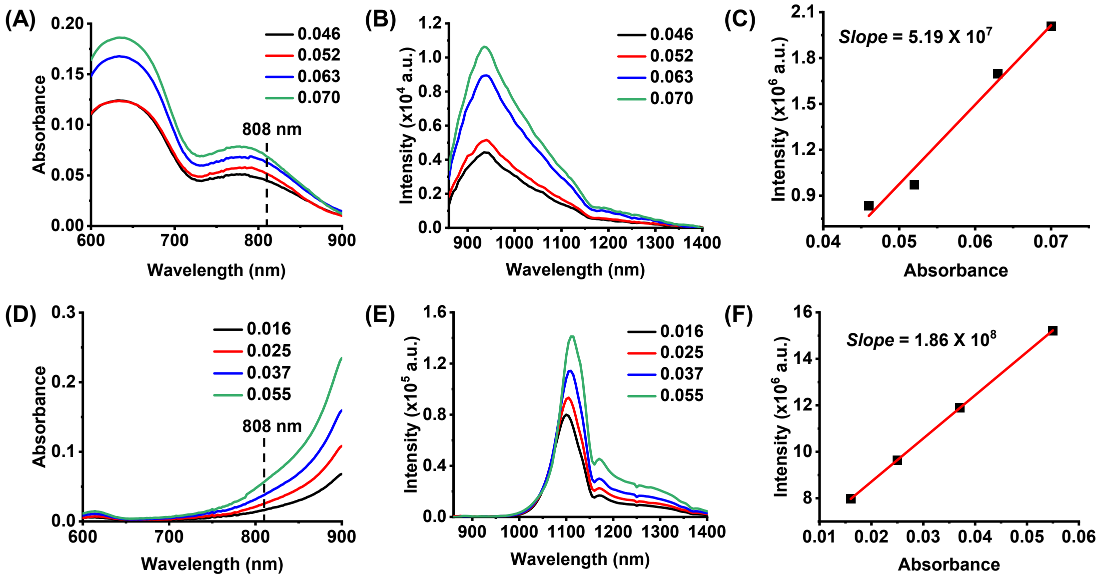

Figure S9. (A, D) UV-vis absorption spectra (B, E) NIR-II fluorescence emission spectra and (C, F) linear correlations between absorption concentrations and integrated fluorescence intensities (900-1400 nm) of DCy and IR-1061 at varying concentrations.

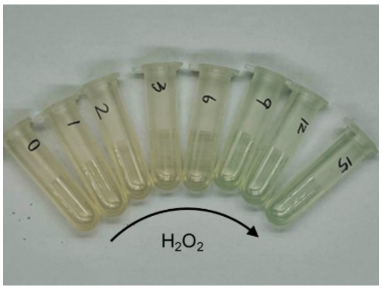

Figure S10. Color changes of DCy-BOH solution (20 μM, PBS/CH_3CN = 1/1) after adding different equivalents of H_2O_2.

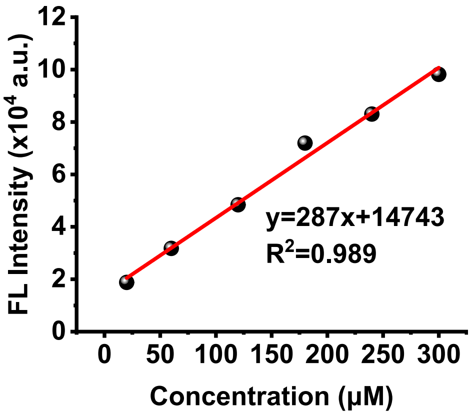

Figure S11. Color changes of DCy-BOH solution (20 μM, PBS/CH_3CN = 1/1) after adding different equivalents of H_2O_2.

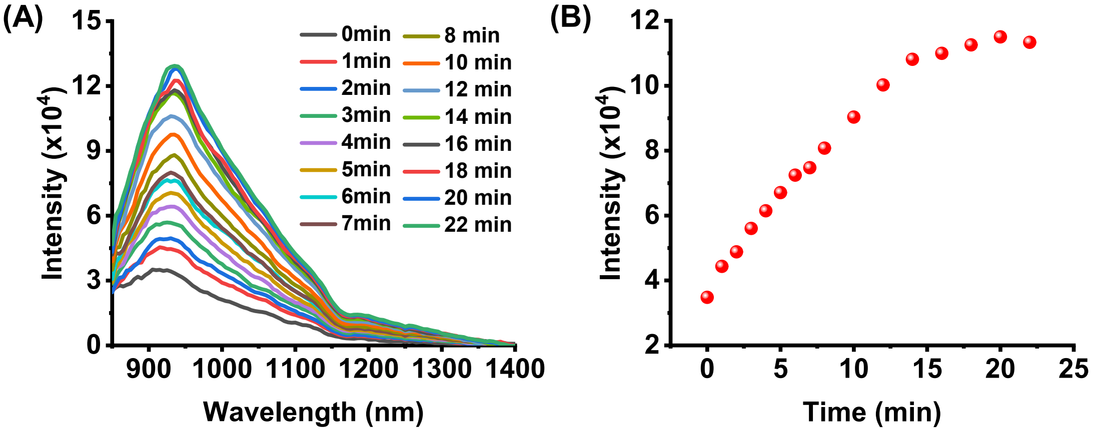

Figure S12. Time-dependent fluorescence changes of DCy-BOH (20 μM, PBS/CH_3CN = 1/1, v/v) following the addition of H_2O_2 (300 μM).

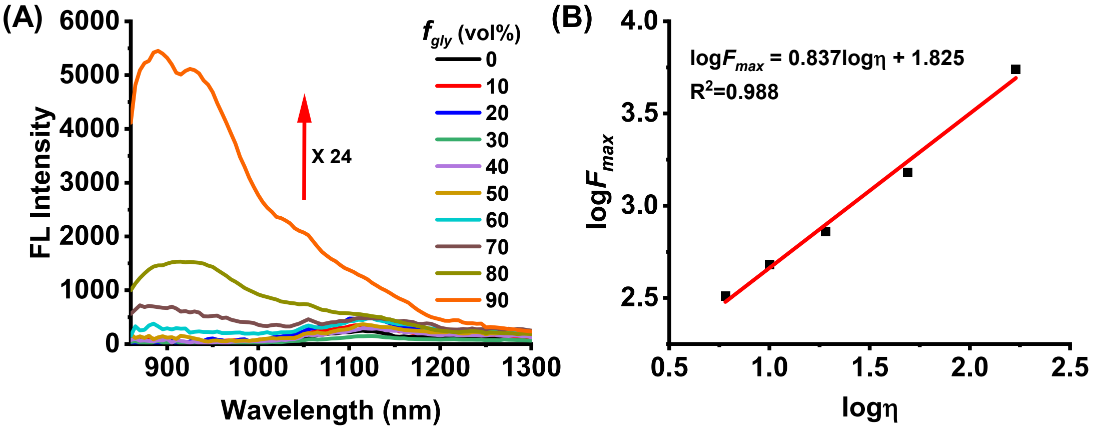

Figure S13. (A) NIR-II fluorescence emission spectra of DCy at different glycerol contents in Glycerpl-PBS systems. (B) Linear fitting curve of the maximum NIR-II fluorescence emission for

DCy plotted against glycerol-PBS concentration ratios (5.99 cP to 168.1 cP).

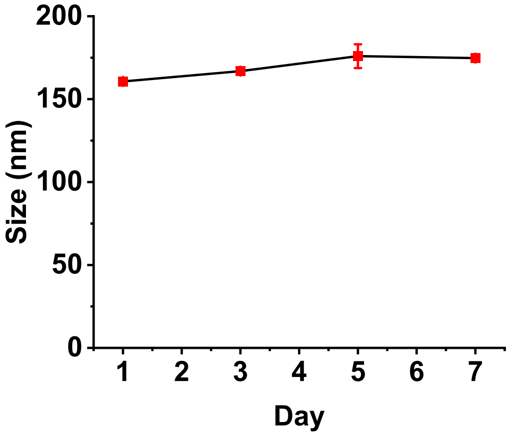

Figure S14. The stability of DCy-BOH@BSA-HA NPs in water.

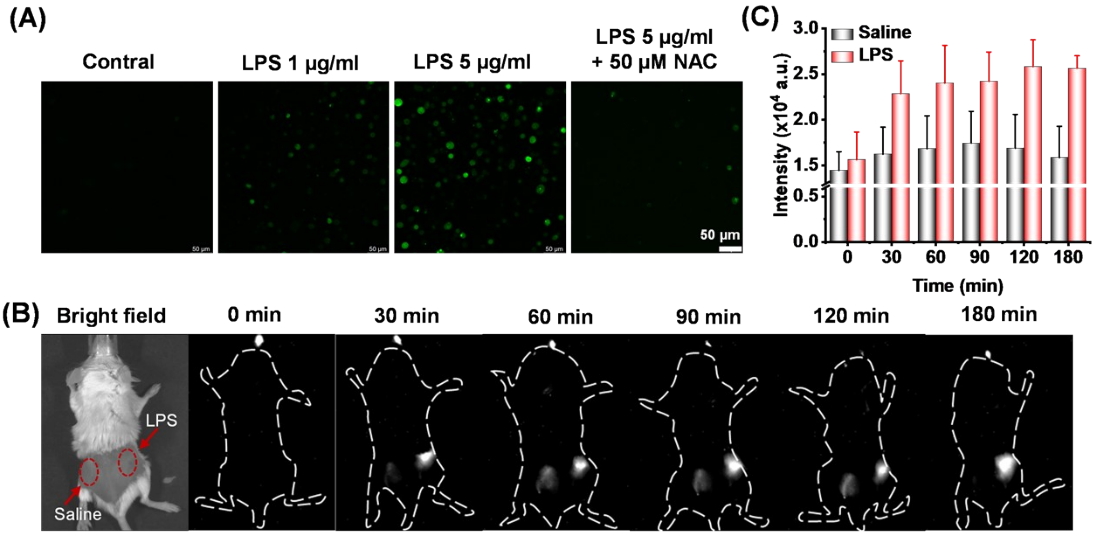

Figure S15. (A) Laser confocal images of reactive oxygen species (ROS) expression levels in LPS-induced RAW264.7 cells detected using the ROS indicator DCFH-DA. (B) Representative NIR-II FL images of the left thigh tissue (treated with normal saline) and right thigh tissue (treated with LPS) of mice at different time points (0, 30, 60, 90, 120, and 180 min) after subcutaneous injection of DCy-BOH@BSA (50 μL, 1 mg/mL). (Imaging parameters: 808 nm laser, 900 nm long-pass filter.) (C) Average fluorescence intensity of the corresponding left and right hindlimbs of mice at different time points in NIR-II FL imaging (n = 3).

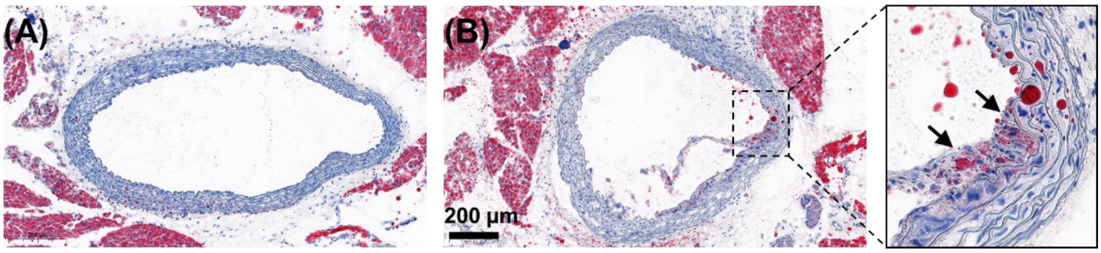

Figure S16. Oil red O staining images of aortic roots from (A) healthy control group and (B) AS group mice.

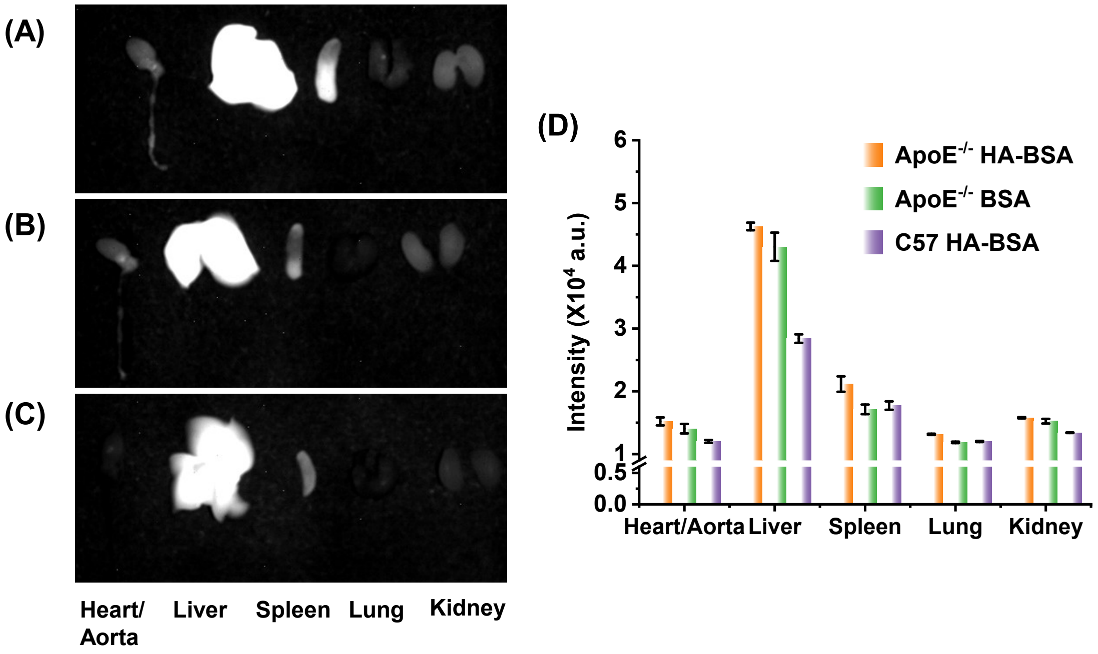

Figure S17. (A) NIR-II FL images of ex vivo organs and aortas from AS mice treated with DCy-BOH@BSA-HA, (B) AS mice treated with DCy-BOH@BSA, and (C) C57BL/6 mice treated with DCy-BOH@BSA-HA; (D) Relative fluorescence intensities.

1. K. W. Lee, Y. Gao, S. H. Chou, Y. Wan, A. C. H. Hsu, J. H. Tan, Y. Li, Z. Guan, H. Chen, S. Li, K. T. Wong and C. S. Lee, *Adv. Funct. Mater.*, 2024, **34**.