A novel water-soluble NIR-II cyanine dye for fast targeted through-skull fluorescence imaging of orthotopic glioma

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Table of contents

Experimental section

1. Materials and Reagents

N,*N*-Dimethylformamide (DMF), Dimethyl sulfoxide (DMSO), Anhydrous Tetrahydrofuran (THF), Methylmagnesium chloride (3M MeMgCl in THF), Potassium iodide (KI), Dithiothreitol (DTT), Benzo[c,d]indole-2(1H)-one and 2-chloro-3-hydroxymethylenecyclohexyl-1-ene-1-carbaldehyde were purchased from Shanghai Aladdin Biochemical Technology (China). 1-Iodopropane, Methanol (MeOH), Ethyl acetate (EA), Dichloromethane (DCM), Ethanol (EtOH), n-Hexane (Hex) and Ethyl ether were purchased from Shanghai Titan Scientific (China). Sodium hydride (NaH), Hydrochloric acid (HCl), Sulfuric acid (H_2SO_4), Potassium carbonate (K_2CO_3), Sodium hydroxide (NaOH), 18-Crown-6, Anhydrous sodium sulfate (Na_2SO_4), Sodium bicarbonate ($NaHCO_3$) and Sodium chloride (NaCl) were purchased from J&K Scientific (China). Human holo-Transferrin was obtained from Beyotime Biotechnology (China). Mouse glioma GL261 cells were obtained from Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences (China). Unless otherwise specified, all chemicals are purchased from Sinopharm and can be used directly without further purification.

2. Instrumentations

Nuclear magnetic resonance (NMR) spectra and electrospray ionization high-resolution mass spectrometry (ESI-HRMS) were obtained on Bruker Ascend 400 NMR spectrometer, Avance III 600 NMR spectrometer, and Bruker Maxis ESI-Q-TOF Mass spectrometer (Bruker Corporation, USA) respectively. Absorption spectra were measured using the Lambd-1050 UV-Vis spectrophotometer (Perkin-Elmer, USA). The

fluorescence emission spectra were acquired by an FLS-1000 fluorescence spectrometer with InGaAs detector for NIR-II spectra (Edinburgh, UK). The nano size was determined using a Tecnai G2 F20 transmission electron microscope (FEI, USA). The hydrodynamic size was determined using a Malvern Zetasizer Nano ZS particle size and zeta potential analyzer (UK). The circular dichroism spectrum was recorded on a Chirascan VX CD spectrometer (Applied Photophysics, UK). The bioluminescence imaging system was performed using Xenogen IVIS spectrum (Xenogen Corporation, USA). NIR-II images of cells were recorded by MicroVis-1000/I NIR-II microscope (Suzhou NIR-Optics Co., Ltd., China). In vivo NIR fluorescence images were recorded by a NIR-II small animal imaging system (Series III 900/1700 s) (Suzhou NIR-Optics Co., Ltd., China). Bioluminescence images were acquired using Xenogen Small Animal in Vivo Imaging System (Caliper Life Science, USA).

3. Synthesis of precursor compounds

3.1 1-propylbenzo[c,d]indol-2(1H)-one (compound 2): Under N_2 atmosphere, NaH (528 mg, 22 mmol) was added into a solution of benzo[c,d]indol-2(1H)-one (2.55 g, 15 mmol) in 30 mL of anhydrous DMF. Then the mixture solution was cooled to 0°C, and 1-iodopropane (1.5 g, 8.8 mmol) was added dropwise and further stirred at 0°C for 20 min. The mixture was continuous stirred at room temperature for 3 h. The reaction was terminated by slowly dropping ultrapure water until no bubble formed. Then reaction solution was extracted by ethyl acetate, washed with brine, and concentrated. The crude product was purified by column chromatography to give a yellow solid of compound 2 (2.78 g, 87.9 % yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.14 (d, J = 8.1 Hz, 1H), 8.03

(d, J = 6.9 Hz, 1H), 7.79 (dd, J = 8.1, 7.0 Hz, 1H), 7.60 (d, J = 8.4 Hz, 1H), 7.52 (dd, J = 8.4, 7.0 Hz, 1H), 7.17 (d, J = 7.0 Hz, 1H), 3.84 (t, J = 7.1 Hz, 2H), 1.70 (h, J = 7.3 Hz, 2H), 0.89 (t, J = 7.4 Hz, 3H).

3.2 2-oxo-1-propyl-1,2-dihydrobenzo[c,d]indole-6-sulfonic acid (compound 3): Compound 2 (844 mg, 4 mmol) was dissolved in acetic anhydride (2 mL) under N_2 atmosphere and then cooled to 0°C in ice bath. Then the solution was slowly added in concentrated sulfuric acid (0.4 g, 220 μL) dropwise and stir continuously for 10 min. The reaction mixture was heated to 55°C and stirred continuously for 3 hours. After, the reaction solution was cooled to room temperature, and K_2CO_3 (552 mg, 4 mmol) was added in with stirring for 10 minutes. The mixture solution was then poured directly into ethyl acetate, precipitated, filtered, and washed with ethyl acetate to obtain pure compound 3 (780 mg, yield 67%). ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.73 (d, J = 8.3 Hz, 1H), 8.00 (d, J = 7.0 Hz, 1H), 7.83 (d, J = 7.3 Hz, 1H), 7.79 (m, 1H), 7.10 (d, J = 7.3 Hz, 1H), 3.84 (t, J = 7.1 Hz, 2H), 1.71 (h, J = 7.3 Hz, 2H), 0.89 (t, J = 7.4 Hz, 3H). ^{13}C NMR (Methanol-d_4, 101 MHz) δ 170.06, 143.26, 138.29, 132.76, 131.03, 130.01, 128.11, 127.54, 127.20, 126.26, 106.88, 42.71, 23.63, 9.24. HR-MS (ESI, negative ion mode) calcd for $C_{14}H_{12}NO_4S^-$ 290.0493, found 290.0497.

3.3 2-methyl-1-propylbenzo[c,d]indol-1-ium-6-sulfonate (compound 5):

Compound 3 (425 mg, 1.42 mmol) was dissolved in 50 mL of ultrapure water under N_2 atmosphere. Then 18-Crown-6 (685 mg, 1.77 mmol) was added with stirring at room temperature for 12 h. Then, the solution was freeze-dried to solids. These solids were then dissolved in ether and centrifuged, and the supernatant was discarded. The precipitate was freeze-dried to yield yellow 2-oxo-1-propyl-1,2-

dihydrobenzo[c,d]indole-6-sulfonate) 18-crown-6 ether potassium (compound 4) (650 mg, 58% yield). Compound 4 was used directly in the next step synthesis. Compound 4 was dissolved in 30 mL dry THF under N_2 atmosphere. After cooling the mixture solution to 0℃ in an ice bath, MeMgCl (7.0 mL, 3 M in THF) was slowly added. The reaction solution was stirred in an ice bath for 10 min, then warmed to room temperature for 12 h. Hydrochloric acid (3 M, 10 mL) was added and stirred to stop the reaction. Then, after the THF was completely removed, the mixture solution was dried and washed with ethyl acetate to give compound 5 (200 mg, 70% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.27 (s, 1H), 8.48 (d, J = 10.5 Hz, 2H), 8.23 (s, 1H), 7.90 (s, 1H), 4.12 (s, 2H), 2.81 (d, J = 64.1 Hz, 1H), 1.73 (s, 2H), 1.02 (s, 3H).

4. Synthesis of Sulfo-1100

For the synthesis of Sulfo-1100, under N_2 atmosphere, compound 5 (116 mg, 0.4 mmol) was dissolved in 0.2 mL acetic acid and 0.1 mL triethylamine mixture solution with stirring. Then 2-chloro-3-hydroxymethylenecyclohexyl-1-ene-1-carbaldehyde (34.2 mg, 0.2 mmol) and 0.1 mL acetic anhydride was added in order. The reaction was carried out at 60 ℃ for 30 min with stirring. After cooling to room temperature, the reaction solution was added in 10 mL ethyl acetate and maintained for 10 min to produce a precipitate. Then the precipitate was washed 3 times with ethyl acetate to obtain pure black powder Sulfo-1100 (104 mg, 70% yield). ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.78 (d, J = 49.9 Hz, 3H), 8.44 (s, 2H), 7.96 (s, 3H), 7.52 (s, 1H), 7.29 (d, J = 15.1 Hz, 2H), 7.19 (s, 1H), 7.11 (s, 1H), 6.89 (s, 1H), 4.35 (d, J=8.1 Hz, 4H), 2.61 (s, 2H), 2.38 (s, 2H), 1.96 (s, 2H), 0.83 (s, 4H), 0.98 (t, J=7.1 Hz, 6H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 153.92, 149.05, 142.55, 140.94, 131.49, 131.38, 129.84, 129.70,

129.55, 129.23, 127.72, 124.89, 123.36, 110.16, 107.13, 77.11, 45.42, 26.48, 22.14, 18.73, 10.90.

5. Characterization of Sulfo-1100

5.1 Water-solubility detection. 1 mg of Sulfo-1100 was dissolved in 1 mL solvents with dielectric constant from small to large such as n-Hexane (Hex), dichloromethane (DCM), ethyl acetate (EA), ethanol (EtOH), methanol (MeOH), dimethyl sulfoxide (DMSO) and H_2O.

5.2 For determining the oil-water partition coefficient, Sulfo-1100 was dissolved in a mixture of water and 1-octanol (1:1 ratio V/V) at room temperature, followed by vortex for 10 min then achieved equilibrium between the two phases. The absorbance values of organic and aqueous phases were measured separately by an UV-vis spectrometer. The partition coefficient was expressed as Log$P$ = $\log_{10}$ [$C_{(\text{octanol})}$ / $C_{(\text{water})}$].

5.3 Photophysical property measurement. The spectral properties of Sulfo-1100 were determined in solvents including PBS, 10% FBS, DMSO, DMF, MeOH, and EtOH. 4.5 mg of Sulfo-1100 was dissolved in 2 mL of each solvent to obtain 3 mM stock solution. The UV absorption spectra, molar absorption coefficients, and fluorescence spectra of Sulfo-1100 (10 μM work solution) were determined on a Lambd-1050 UV-Vis Near-Infrared spectrophotometer and FLS1000 fluorescence spectrophotometer with an 808 nm exciter.

5.4 NIR-II fluorescence quantum yield calculation. The fluorescence quantum yield of Sulfo-1100 was measured according to the literature [S1] and IR-1061 was chosen as the reference (QY_{1061} = 1.7% in DCM). We measured the UV absorption and

fluorescence intensity of IR-1061 and Sulfo-1100 at absorbance values of 0.02, 0.04, 0.06, 0.08, and 0.1. The transient fluorescence spectrometer was used with a 980 nm exciter, a power of 1 W and a slit width of 14 nm. The quantum yield was calculated as follows:

$$QY = QY_{ref} \times \frac{n^2}{n_{ref}^2} \left(\frac{A_{ref}}{Iref}\right) \frac{I_{sample}}{A_{sample}}$$

5.5 Photostability assay. Sulfo-1100 solution (50 μM, 10% FBS) was incubated at 37°C for 2 h followed by continuous irradiation under 808 nm laser light for 1 h. Images were acquired at 5 min intervals by a NIR-II small animal imaging system. The fluorescence intensity at t = 0 was normalized to obtain the intensity. The excitation light wavelength was 808 nm with the filter LP1000 nm, exposure time 100 ms, and the power at 10 W.

6. Preparation of hT-Sulfo-1100 NPs

The hT-Sulfo-1100 NPs were prepared by self-assembly. Briefly, holo-Tf was dissolved in 1×PBS (1 mg/mL) and mixed with DTT. Then Sulfo-1100 solution (2 mg/mL, in H_2O) was added while stirring vigorously for 12 h at room temperature. After that, the mixed solution was dialyzed in water for 36 h to remove the redundant DTT and free Sulfo-1100. Finally, the hT-Sulfo-1100 NPs are stored at 4°C. All experiments were conducted in the dark. The characterization of hT-Sulfo-1100 NPs was showed in supporting information.

7. Characterization of hT-Sulfo-1100 NPs

7.1 Particle size measurement. The hT-Sulfo-1100 NPs solution was diluted and 10 μL of the diluted solution was dropped on a copper grid. The particle size was

determined using a transmission electron microscope. The hydrodynamic size in water was determined by a particle size and zeta potential analyzer.

7.2 Optical spectra detection. CD spectra were recorded on a CD spectrometer at ambient temperature. The holo-Tf and hT-Sulfo-1100 NPs were dissolved in deionized water (10 μM), and the UV absorption was recorded from 260 nm to 190 nm, as well as the CD spectra were determined. Absorption spectra were measured with a Lambd-1050 UV-Vis Near-Infrared spectrometer, and fluorescence spectra were measured with a FLS1000 fluorescence spectrophotometer with an 808 nm exciter.

7.3 Molecular docking. The crystal structure of holo-Tf was obtained from the PDB protein crystal database encoded 3V83. KingDraw tool was used to construct the molecular structure of Sulfo-1100. The ground state geometry was optimized at PBE0/def2-TZVP level with tight convergence criteria by using the ORCA 5.0.2 program package [S2, S3]. Molecules were visualized with the Avogadro 1.2.0 program package. PDBQT files for receptors and ligands were prepared using Autodock Tools 1.5.7 [S4]. The molecular docking was performed by using Autodock Vina. Molecules were visualized with PyMOL 2.6 (Open-Source Build) [S5].

7.4 Loading efficacy calculation. We measured UV-vis-NIR absorbance values of Sulfo-1100 (10 μM) and hT-Sulfo-1100 NPs (1.2 μM) solutions (in PBS with 10% FBS). In this assay, the concentration of hT-Sulfo-1100 NPs was estimated according to h the concentration of holo-Tf, whose molecular weight was regarded as 80 kD. Based on the ratio of absorbance values between Sulfo-1100 and hT-Sulfo-1100 NPs, the concentration of Sulfo-1100, which was binded with holo-Tf could be calculated by the formula: $c = \frac{10 \times A_{NPs}}{A_{Sulfo-1100}}$. To investigate the effect of holo-Tf on the fluorescence

emission spectra of hT-Sulfo-1100 NPs, the fluorescence spectra were measured by a fluorescence spectrophotometer, and the transient fluorescence spectrometer was operated with an 808 nm exciter with a power of 0.90 W and a slit width of 10 nm.

7.5 Photostability detection. hT-Sulfo-1100 NPs were dissolved in 10% FBS (50 μM) followed by continuous irradiation under 808 nm laser light with various time. The excitation wavelength was 808 nm with the filter LP 1000 nm. The exposure time was 500 ms, and the power was 10 W.

7.6 Penetration depth test. 200 μL of 100 μM hT-Sulfo-1100 NPs solution (10% FBS) was taken to a three-well plate and bacon was used to mimic the animal tissues. An infrared in vivo imaging system was applied for continuous irradiation with an excitation wavelength of 808 nm, a filter of LP 1000 nm, an exposure time of 500 ms, and a power selection of 10 W. The thickness of the overlapping bacon was determined by a graduated straightedge.

8. In vitro cytotoxicity and anti-interference properties detection

8.1 Cytotoxicity of Sulfo-1100 and hT-Sulfo-1100 NPs to cells. The cytotoxicity was evaluated using the MTT assay. GL261 cells were seeded in a 96-well plate with 1×10^4 cells/well and incubated for 18 h. Then, the cells were incubated in 200 μL DMEM with different concentrations of Sulfo-1100 or hT-Sulfo-1100 NPs for 24 h (0, 10, 20, 30, 40, and 50 μg/mL). After that, MTT solution (1 mg/mL) was added to each well, and the plates were incubated at 37°C for another 4 h. The purple formazan crystal in each well was dissolved in 150 μL DMSO, and the absorbance was determined at 450 nm using a multifunctional microplate reader.

8.2 Anti-interference properties detection. Normal interfering substances were selected for the anti-interference study including $CaCl_2$ (2 mM), $MgCl_2$ (2 mM), $CuSO_4$ (50 μM), $ZnCl_2$ (100 μM), glucose (10 mM), cysteine (1 mM), serine (1 mM), and hydrogen peroxide (100 μM). The interfering substances were added to hT-Sulfo-1100 NPs solution (20 ug/mL, in PBS) respectively, and detected under 808 nm excitation by infrared in vivo imaging system with the excitation wavelength of 808 nm, the filter of LP 1000 nm, the exposure time of 1000 ms, and the power of 10 W.

9. In vitro active targeting ability detection

GL261 cells were incubated in DMEM medium supplemented with 1% penicillin-streptomycin (v/v) and 10% (v/v) FBS at 37°C under a humidified atmosphere with 5% CO_2 in a carbon dioxide incubator. GL261 cells were seeded on a round slide (10^5 cells/slide) and incubated in a glass-bottom dish for 12 h, then 20 μg/mL of Sulfo-1100 and hT-Sulfo-1100 NPs were added for the next 4 h. After discarding the medium and washing glass-bottom dish three times with 1×PBS, cells were fixed in 4% paraformaldehyde at room temperature for 10 min, followed by PBS washing. The cells were placed under a NIR II microscope for imaging. The excitation wavelength was 808 nm, the filter was LP 900 nm, the exposure time was 200 ms, and the power density was about 15 mW/cm^2.

10. In vivo glioma model construction

All animal experiments were approved by the Animal Care and Use Committee of Shaanxi Normal University and were conducted in accordance with the Ethical Principles of Animal Use and Care in China (GB/T 35892-2018). 5-6 weeks female BALB/c-nu mice and 6-8 weeks female C57BL/6 mice were obtained from the

Laboratory Animal Center of Shaanxi Normal University (Xi'an, China). The mice were housed under climate-controlled (24°C, 50-60% humidity) conditions. The mice were fed with an ordinary solid diet and distilled water and were subjected to 12 h light-12 h dark cycles. The health status of the mice was monitored daily.

GL261 cells which carried luciferase-encoding gene (Luc) plasmids were used to construct the glioma model. GL261 cells (2×10^5 cells per mouse) were slowly injected into the right striatum of C57BL/6 mice to establish gliomas. The position is bregma +0.6 mm, -1.8 mm, depth 4.0 mm, return to 3.5 mm. After 7-8 days of modeling, the luciferin was intraperitoneally injected into mouse at 150 mg/kg, and in vivo bioluminescence imaging was performed by Xenogen IVIS spectrum after 10 minutes post injection.

11. In vivo glioma NIR-II imaging and metabolism observation

11.1 In vivo NIR-II imaging. After 14 days post the orthotopic glioma established, the hT-Sulfo-1100 NPs or Sulfo-1100 (1 mg/kg of Sulfo-1100) were intravenously injected into mice and NIR-II images at different time points were acquired on the NIR-II in vivo imaging system. The excitation wavelength was 808 nm, the filter was LP 1000 nm, the exposure time was 500 ms, and the power was 5 W.

11.2 Drug metabolism assay. Sulfo-1100 and hT-Sulfo-1100 NPs (1 mg/kg of Sulfo-1100) were injected intravenously into normal BALB/c mice with abdominal hair removal for whole-body imaging. The excitation wavelength was 808 nm, the filter was LP 1000 nm, the exposure time was 500 ms, and the power was 5 W.

12. Hematoxylin and eosin tissue staining

For hematoxylin and eosin (H&E) staining, briefly, fresh organs were embedded with optimal cutting temperature compound (O.C.T.) and sliced by cryostat microtome, dehydrated, and performed H&E staining. Tissues were obtained at 24 h after injection.

13. Statistical analysis

Unless stated otherwise, all results are shown as mean ± SD. Statistical analyses of data were done using Student's t test. Here, three asterisks (***) indicated $P < 0.001$.

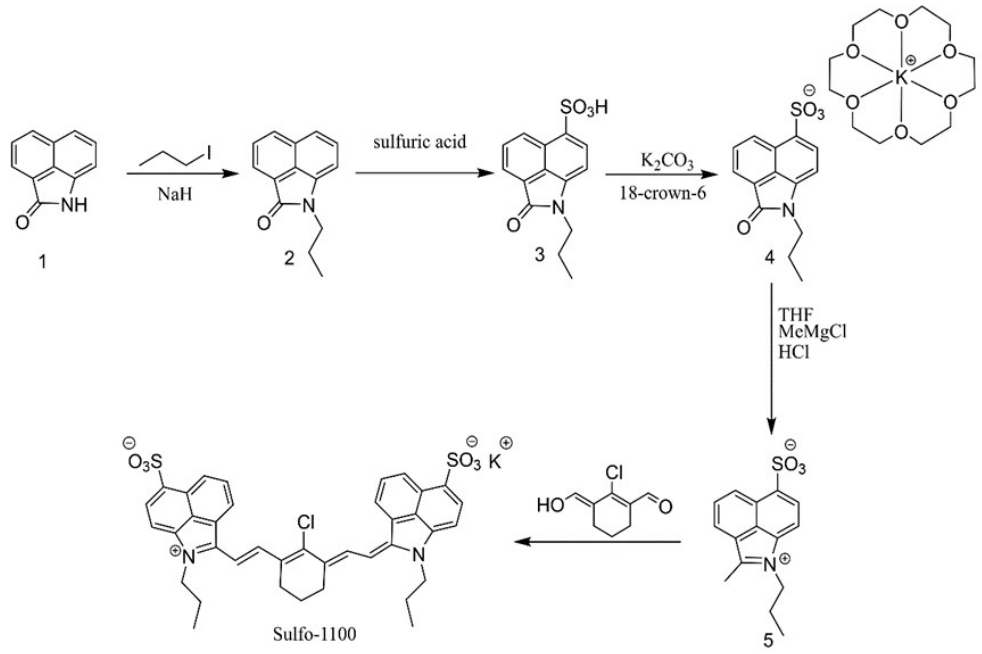

Figure S1. Synthetic routes of Sulfo-1100.

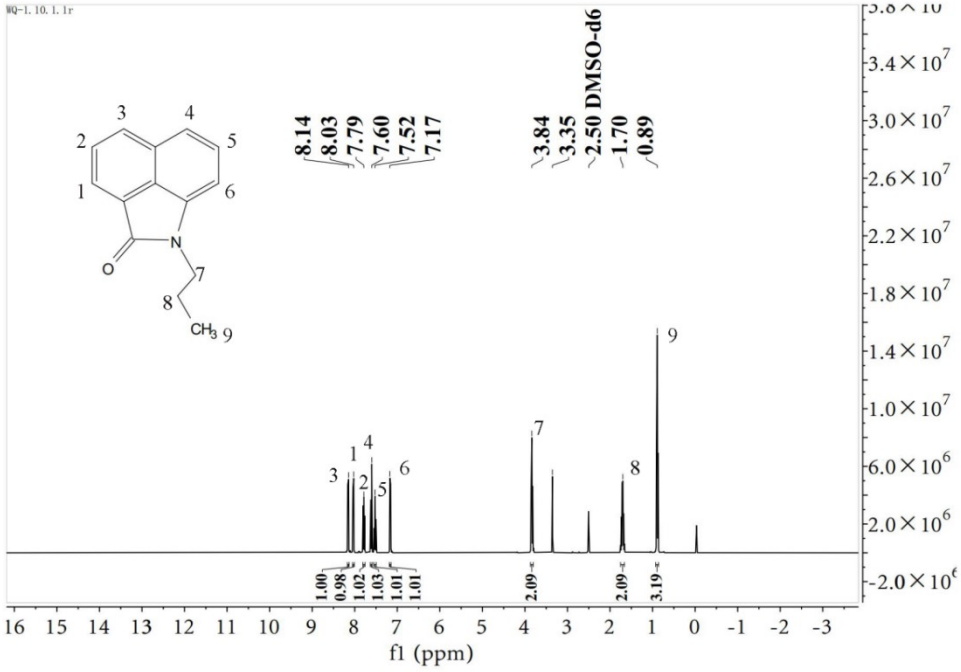

Figure S2. ^{1}H NMR spectrum of compound 2 (DMSO-d_6, 298K, 400 MHz)

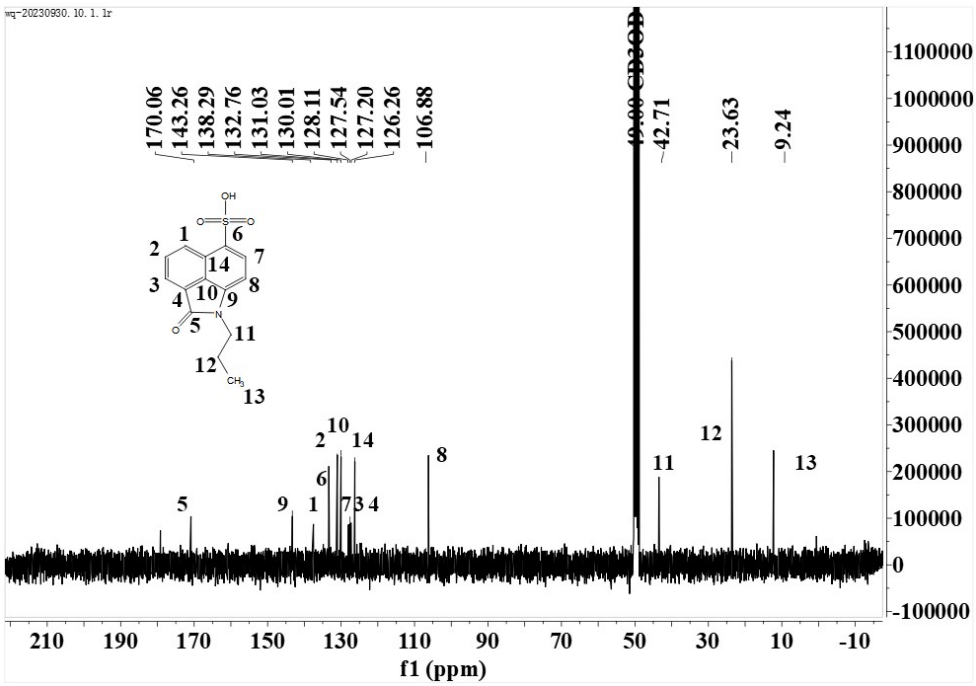

Figure S3. ^{1}H NMR spectrum of compound 3 (DMSO-d_6, 298K, 600 MHz).

Figure S4. ^{13}C NMR spectrum of compound 3 (Methanol-d_4, 298K, 101 MHz).

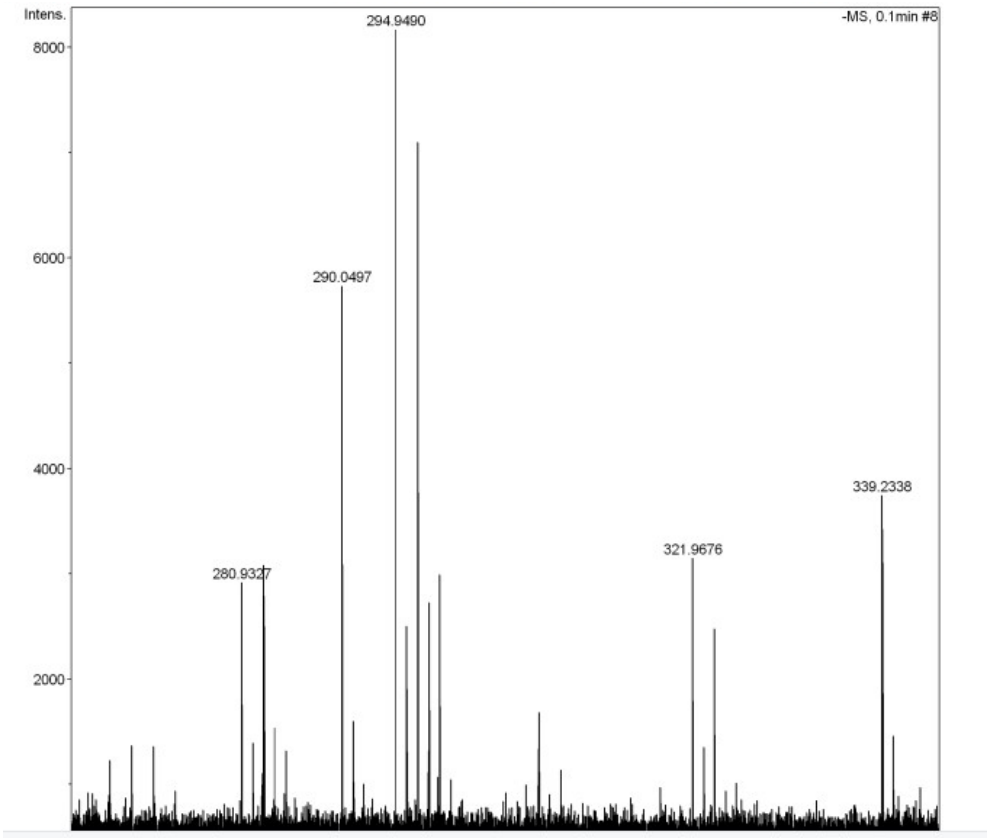

Figure S5. HR MS of compound 3.

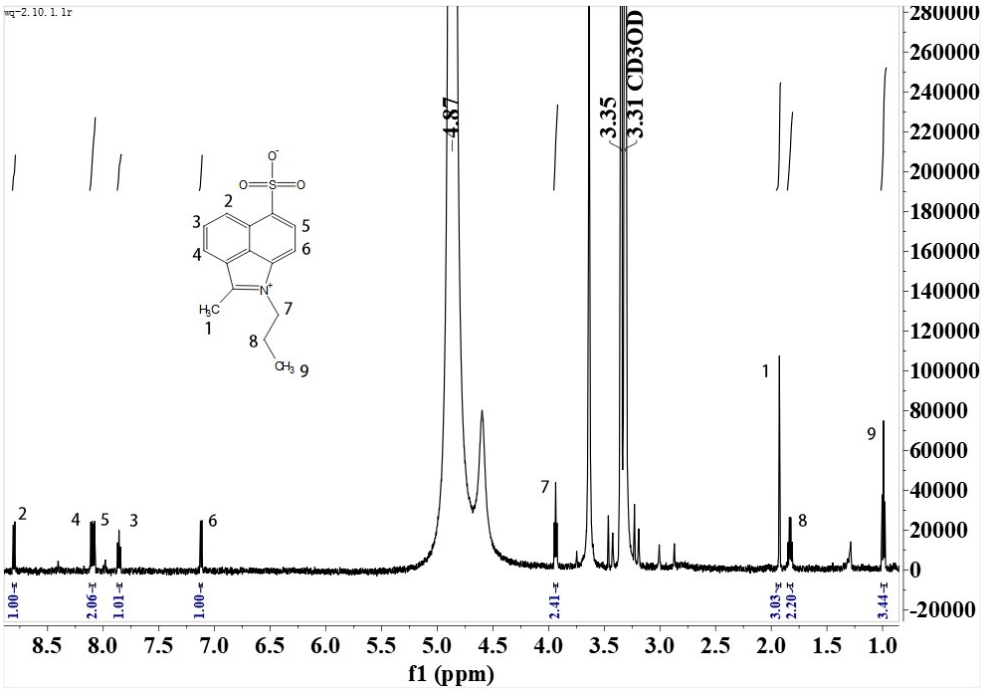

Figure S6. ^{1}H NMR spectrum of compound 5 (DMSO-d_6, 298K, 400 MHz).

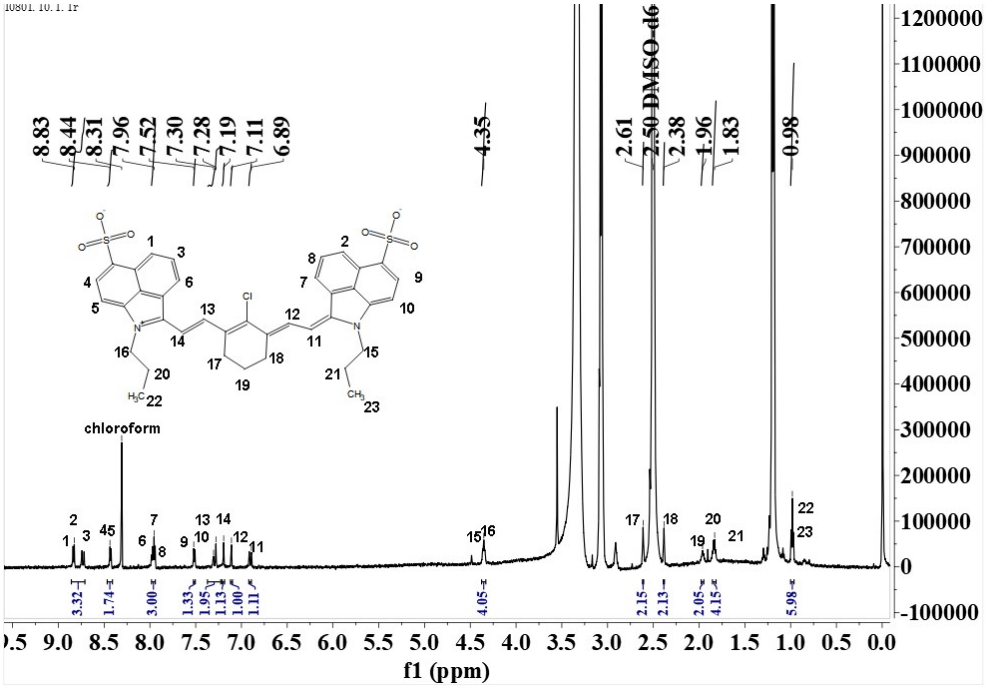

Figure S7. ^{1}H NMR spectrum of Sulfo-1100 (DMSO-d_6, 298K, 600 MHz).

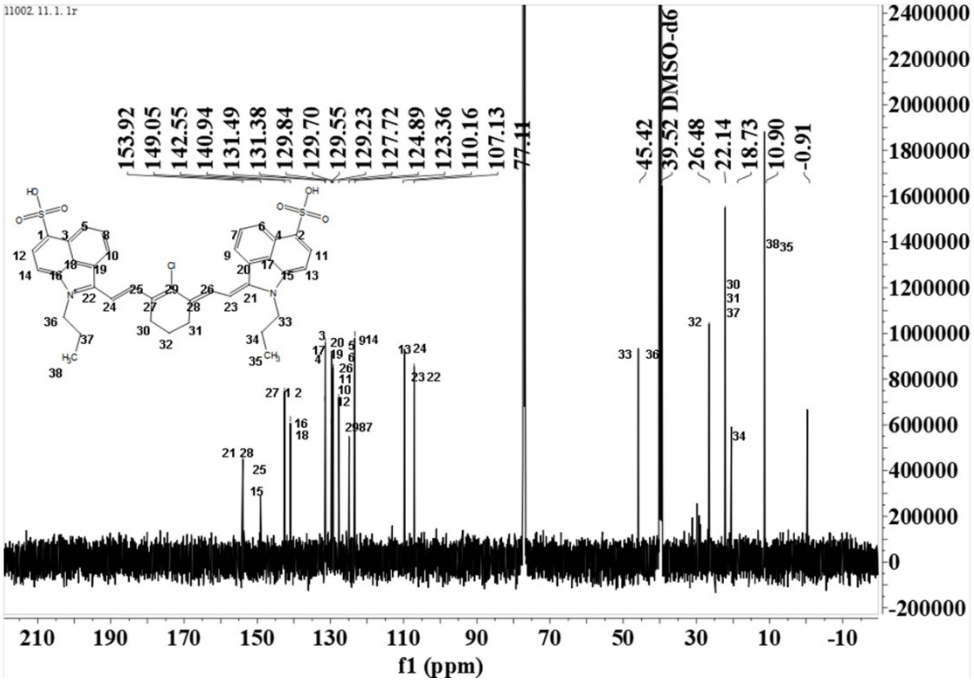

Figure S8. ^{13}C NMR spectrum of Sulfo-1100 (DMSO-d_6, 298K, 151 MHz)

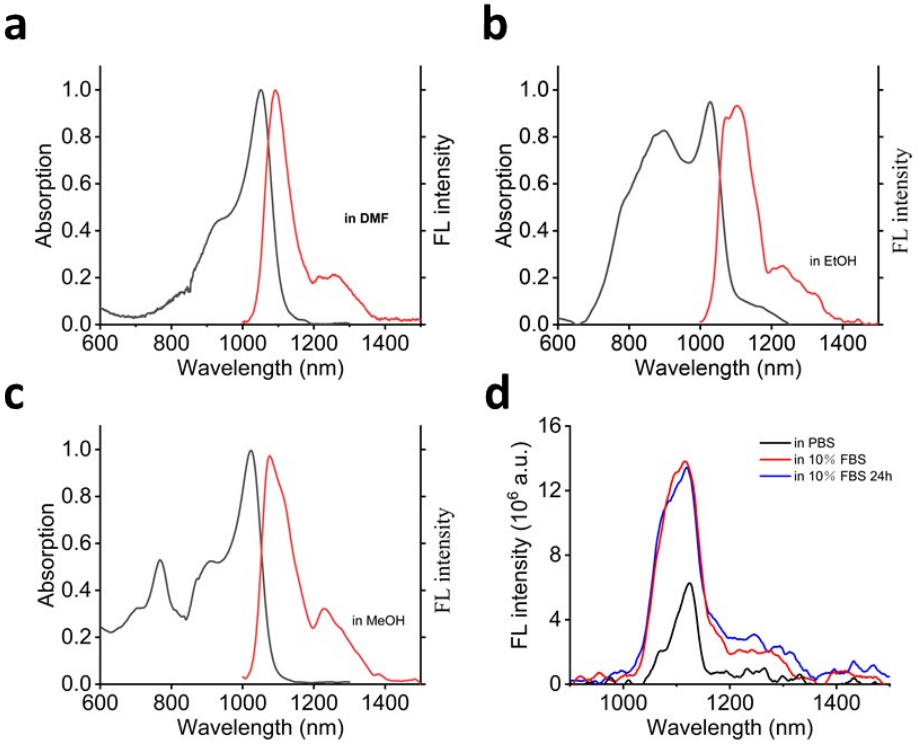
a
b
c
d
Absorption
FL intensity
Wavelength (nm)
in DMF
in EtOH
in MeOH
FL intensity (10^6 a.u.)
in PBS
in 10% FBS
in 10% FBS 24h

Figure S9. UV-Vis-NIR absorption spectra (black lines) and NIR fluorescence emission spectra (red lines) of Sulfo-1100 in different solvents: (a) in DMF, (b) in EtOH and (c) in MeOH. (d) NIR fluorescence emission spectra of Sulfo-1100 in PBS, 10% FBS, and 10% FBS for 24h.

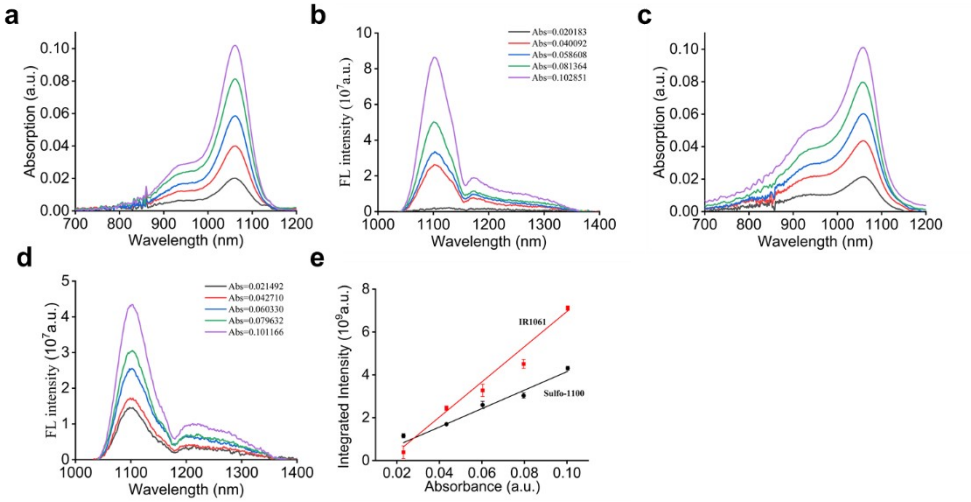

Figure S10. Fluorescence quantum yield of Sulfo-1100. (a) Absorption spectra of IR1061. (b) Fluorescence intensity of IR1061 at absorbance values of 0.02, 0.04, 0.06, 0.08 and 0.1. (c) Absorption spectra of Sulfo-1100. (d) Fluorescence intensity of Sulfo-1100 at absorbance values of 0.02, 0.04, 0.06, 0.08 and 0.1. (e) Integrated emission area of IR-1061 (red line) and Sulfo-1100 (black line) as a function of absorbance.

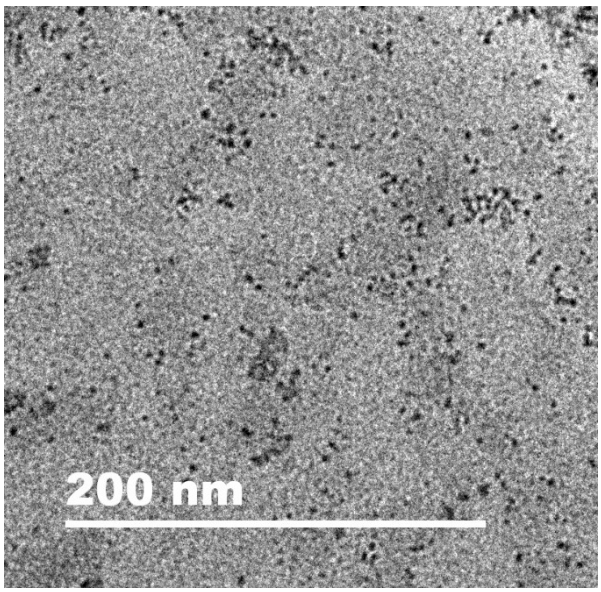

Figure S11. TEM image of hT-Sulfo-1100 NPs.

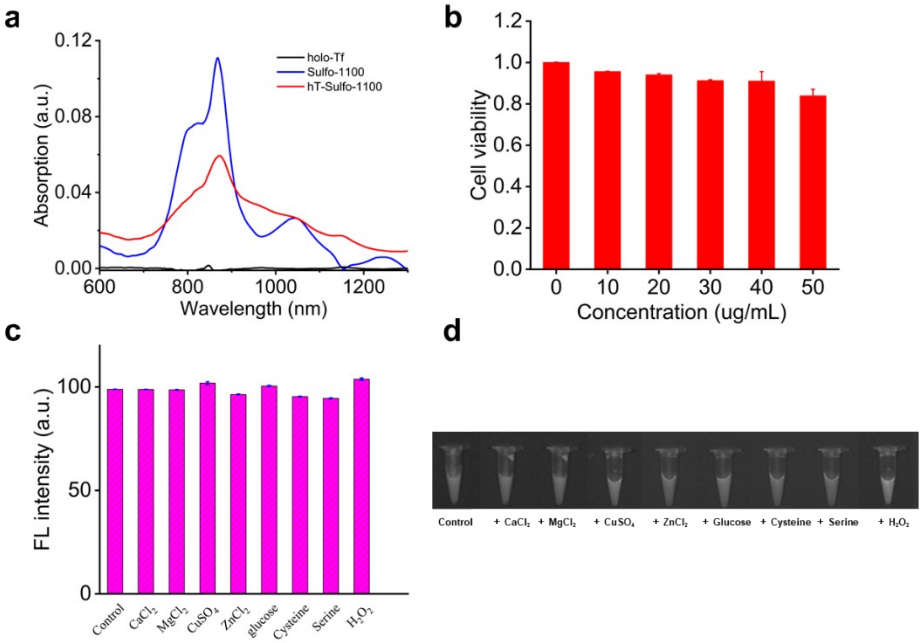

Figure S12. (a) Absorption spectra of holo-Tf, Sulfo-1100, and hT-Sulfo-1100 NPs. Both concentrations were 10 μM. (b) Cytotoxicity of hT-Sulfo-1100 NPs. (c) Detection of the anti-interference ability of hT-Sulfo-1100 NPs. (d) NIR-II imaging of interference experiment. Normal interfering substances were selected for the anti-interference study including $CaCl_2$ (2 mM), $MgCl_2$ (2 mM), $CuSO_4$ (50 μM), $ZnCl_2$ (100 μM), glucose (10 mM), cysteine (1 mM), serine (1 mM), and hydrogen peroxide (100 μM).

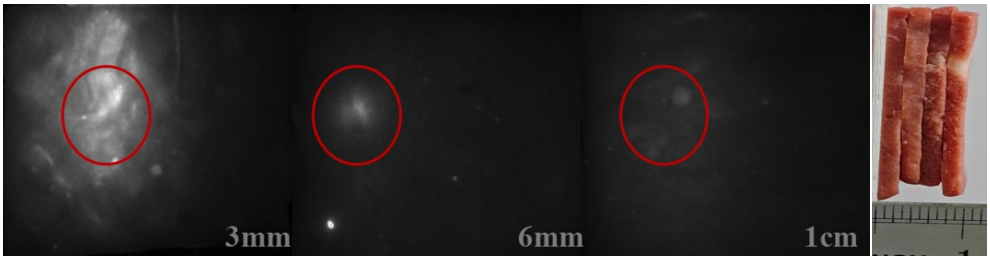

Figure S13. Depth of penetration determination of hT-Sulfo-1100 NPs. The raw bacon was used to mimic the animal tissues by irradiation with an excitation wavelength of 808 nm, a filter of LP 1000 nm, an exposure time of 500 ms, and a power selection of 10 W.

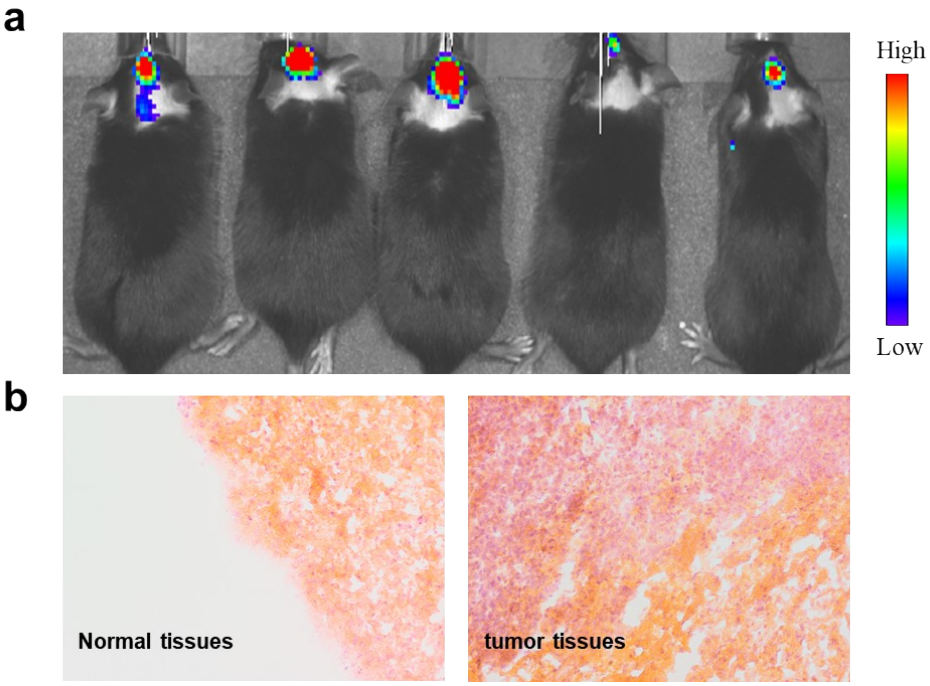

Figure S14. Verification of orthotopic glioma model. (a) Bioluminescence imaging of glioma models. (b) Histological images of mouse brains.

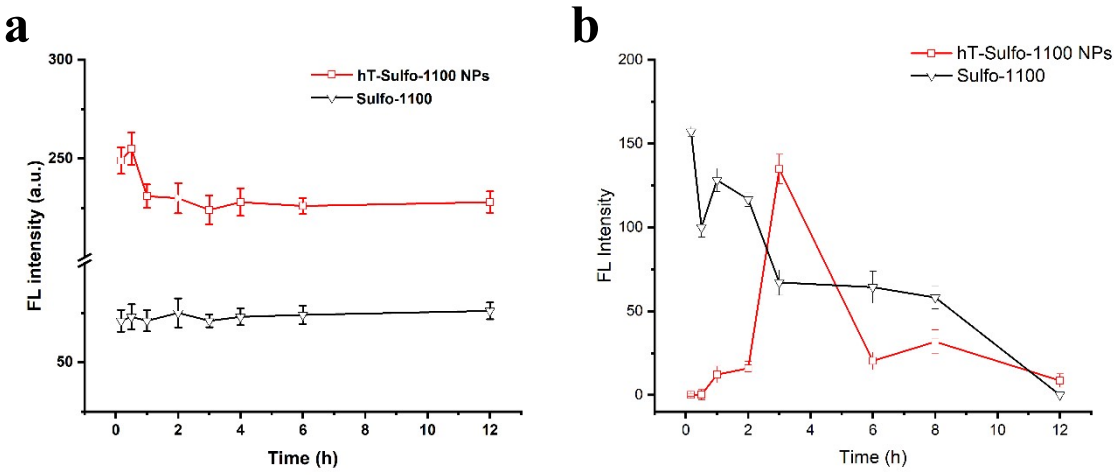

Figure S15. Fluorescence intensity of tumor and bladder in figure 4a (a) and 4d (b). Error bars represent the standard deviation (n = 3).

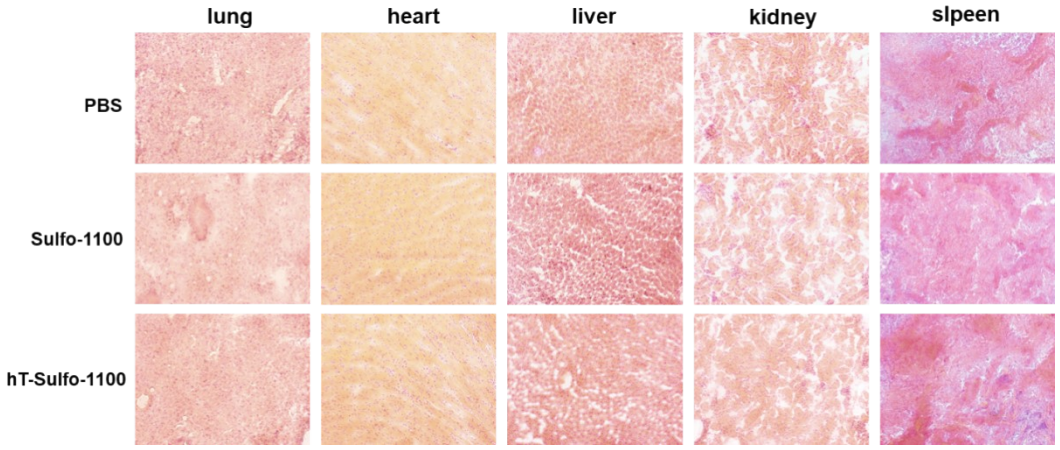

Figure S16. Histological images of major organs after 24 h post-injection of PBS, Sulfo-1100 and hT-Sulfo-1100 NPs by H&E staining.

Table S1. Optical properties of Sulfo-1100 in different solvents

Solvent	λ_{abs} (nm)	λ_{em} (nm)	ε_{max} ($cm^{-1}M^{-1}\cdot10^4$)
DMSO	1059	1100	1.0960
DMF	1049	1091	1.1619
EtOH	1030	1099	1.3351
MeOH	1021	1075	1.1015
10% FBS	866	1114	1.1149
PBS	776	1120	0.6737

CRediT authorship contribution statement

Q. Wei: investigation, visualization and resources; H. Jia: methodology, resources and validation; H. Zhao: investigation and resources; W. Tang: methodology, investigation, and writing-original draft; X. Duan: conceptualization, supervision, writing- review & editing.

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