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Electronic Supplementary Information

Biomarker-responsive Nanoprobe with Aggregation-induced Emission for Locating and Guiding Resection of Deep-Seated Tumors via Optoacoustic and NIR Fluorescence Imaging

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Experimental section

Reagents

The solvents including dimethyl formamide (DMF), cyclohexanone, dioxane, ethanol, methanol, dichloromethane (DCM), ethyl acetate and toluene were purchased from Aladdin Regents and dried with molecular sieve before use. Other solvents used in this study were analytical grade reagents and used without further purification. The water used in the experiments was the triple-distilled water.

1,2,3,3-Tetramethyl-3H-indolium iodide, 4-bromo-2-hydroxybenzaldehyde, 4-methoxydiphenylamine, DavePhos, $Pd_2(dba)_3$, boron tribromide, trimethylhydroquinone, methyl 3,3-dimethylacrylate, sulfamic acid, N-bromosuccinimide (NBS), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride, 4-dimethylaminopyridine (DMAP) were purchased from Aladdin Regents.

DMEM, RPMI-1640 penicillin, Penicillin-Streptomycin Solution, Luciferase-labeled human hepatocellular carcinoma cells (HCC-LM3-fLuc), luciferase expressing breast cancer cells (4T1-Luc) and 10% phosphate buffer saline (PBS) were purchased from KeyGen Bio-Tech. Fetal Bovine Serum (FBS) was purchased from Life Technologies.

Apparatus

Nuclear magnetic resonance (NMR) spectra were measured on AVANCE III HD 600 NMR spectrometer. ^{1}H NMR and ^{13}C NMR spectra were conducted at 600 MHz and 151 MHz, respectively. High resolution mass spectra were recorded on Bruker MAXIS IMPACT mass spectrometer. Non-high resolution mass spectra were measured with Bruker Esquire HCT Plus mass spectrometer. HPLC was carried out on Agilent 1260 Infinity liquid chromatograph (with DAD). Absorption spectra and fluorescence spectra were obtained on Hitachi UH-5300 UV-Vis spectrophotometer and Hitachi F-4700 fluorescence spectro-photometer. Particle size/distribution was determined with Malvern Nano-ZS90 particle size analyzer via dynamic light scattering (DLS) mechanism. Transmission electronic microscopy experiment (TEM) was conducted on a JEM-2010HR transmission electron microscopy. Animal bioluminescence, and the near-infrared fluorescence (in vivo and ex vivo) imaging were performed by using Ami small animal imaging system (Spectral Instruments Imaging Co.). Optoacoustic imaging was conducted with inVision128 multispectral optoacoustic tomographic (MSOT) imaging system (iThera Medical

GmbH).

Synthesis

Synthesis of Compound 1

PBr_3 was slowly added to a solution of $CHCl_3$ (50 mL) and DMF (12 mL, 155 mmol) at 0 °C, after 1 h, cyclohexanone (4 mL, 39 mmol) was injected and the mixture was stirred for 18 h at room temperature. The resulting mixture was poured into ice water and then solid $NaHCO_3$ was added slowly until pH = 7. The aqueous phase was extracted with CH_2Cl_2 and the combined organic layers dried with anhydrous Na_2SO_4 and concentrated under reduced pressure to afford an intermediate as a yellow oil, the product was used for next step without further purification.

To a solution of the above intermediate compound (0.39 g, 1.9 mmol) and Cs_2CO_3 (1.8 g, 4 mmol) in dry DMF (15 mL) was added a solution of 4-bromo-2-hydroxybenzaldehyde (0.73 g, 3.84 mmol) in DMF (1 mL) at room temperature. The reaction mixture is stirred at this temperature for 48 h until an intense yellow spot appeared on the reaction TLC (TLC monitoring: hexane / ethyl acetate (4:1, v/v). At the end of the reaction this precipitate is filtered, washed twice with water and DCM. The combined organic layers were dried with anhydrous Na_2SO_4 and evaporated under reduced pressure. The residue was purified using silica gel chromatography CH_2Cl_2 / AcOEt (10:1, v/v) as eluent to obtain compound 1 as a yellow powder. Yield: 220 mg (60 %). ^{1}H NMR (600 MHz, $CDCl_3$) δ 10.32 (s, 1H), 7.28 (d, J = 1.3 Hz, 1H), 7.20 (dd, J = 8.1, 1.8 Hz, 1H), 7.02 (d, J = 8.1 Hz, 1H), 6.62 (s, 1H), 2.60 – 2.55 (m, 2H), 2.44 (t, J = 6.1 Hz, 2H), 1.75 – 1.69 (m, 2H). MS (ESI): $C_{14}H_{11}BrO_2^+$ [M^+], *m/z* 291.11.

Synthesis of Compound 2

Compound 1 (200 mg, 0.68 mmol), 4-methoxydiphenylamine (200 mg, 0.68 mmol), $Pd_2(dba)_3$ (4 mg, 0.1 mmol), DavePhos (4 mg, 0.1 mmol) and Cs_2CO_3 (554 mg, 1.7 mmol) were mixed in dioxane (15 mL) under argon. The reaction mixture is stirred at 95 °C for 18 h until an intense orange spot appeared on the reaction TLC (TLC monitoring: hexane / ethyl acetate (1:1, v/v). The mixture was evaporated under reduced pressure and was purified by silica gel chromatography (hexane / ethyl acetate (1:1, v/v)) to obtain compound 2 as an orange powder. Yield 70 % 217 mg. ^{1}H NMR (600 MHz, $CDCl_3$) δ 10.18 (s, 1H), 7.29 (t, J = 7.5 Hz, 2H), 7.14 – 7.05 (m, 6H), 6.95 (d,

J = 8.0 Hz, 1H), 6.91 – 6.86 (m, 2H), 6.66 (s, 1H), 6.62 (s, 1H), 3.82 (s, 3H), 2.58 – 2.52 (m, 2H), 2.43 (t, *J* = 6.0 Hz, 2H), 1.70 (m, 2H). MS (ESI): $C_{27}H_{23}NO_3^+$ [M^+], *m/z* 408.2.

Synthesis of Compound 3

To a solution of compound 2 (328 mg, 0.8 mmol) in anhydrous Ac_2O (10 mL) were added K_2CO_3 (220 mg, 1.6 mmol) and indolium (427 mg, 1 mmol). After 16 h at 80 °C, the deep-blue solution was obtained and concentrated, and then the resulting residue was dissolved in CH_2Cl_2 and washed with H_2O. The organic layer was dried over Na_2SO_4, filtered and concentrated *in vacuo*. The residue was purified using silica gel chromatography CH_2Cl_2 / MeOH (90:1, v/v) as eluent to obtain compound 3 as a dark blue powder. Yield 58 %, 427 mg. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.44 (d, *J* = 14.3 Hz, 1H), 7.47 – 7.42 (m, 1H), 7.41 – 7.36 (m, 3H), 7.34 (d, J = 1.7 Hz, 1H), 7.35 – 7.31 (m, 2H), 7.28 (t, J = 4.4 Hz, 2H), 7.22 (d, J = 1.3 Hz, 1H), 7.21 (s, 1H), 7.18 – 7.15 (m, 2H), 6.98 – 6.92 (m, 2H), 6.84 (dd, *J* = 8.7, 2.2 Hz, 1H), 6.72 (d, *J* = 1.9 Hz, 1H), 6.39 (d, *J* = 14.5 Hz, 1H), 3.92 (s, 3H), 3.86 (s, 3H), 2.82 – 2.71 (m, 4H), 1.93 (m, 2H), 1.64 (s, 6H). MS (ESI): $C_{39}H_{37}N_2O_2^+$ [M^+], *m/z* 565.3.

Synthesis of Compound 4

Trimethylhydroquinone (3.0 g, 20 mmol) and methyl 3,3-dimethylacrylate (2.74 g, 24 mmol) were added to methanesulfonic acid (20 mL) and heated to 70 °C. The resulting solution was reacted with vigorous stirring for 2 hours. The reaction solution was then poured into water (100 mL) and then extracted with DCM, and the organic phase was washed with water and brine, dried over anhydrous Na_2SO_4, and the solvent was concentrated to give a mediate as a brown powder for the next step.

The above intermediate compound (2.34 g, 10 mmol) and N-bromosuccinimide (1.78 g, 10 mmol) were dissolved in 10% aqueous acetonitrile. The resulting solution was stirred at room temperature for 4 h. The solvent was removed and diluted with DCM and the organic phase was washed with water and brine, dried over anhydrous Na_2SO_4, and concentrated in vacuo. The crude product was purified by column chromatography on silica gel (DCM) to give compound 4 (1.93 g, 77%) as a yellow powder. ^{1}H NMR (600 MHz, CDCl3) δ 3.02 (s, 2H), 2.14 (s, 3H), 1.94 (dd, *J* = 18.2, 1.0 Hz, 6H), 1.43 (s, 6H). MS (ESI): $C_{14}H_{18}O_4^+$ [M^+], *m/z* 250.1

Synthesis of DHXI-FL

In a flask, compound 3 (565 mg, 1.0 mmol) was dissolved in dry CH_2Cl_2 (10 mL) and then BBr_3 (20 eq.) was added dropwise at -40 °C. The mixture was stirred at room temperature for 16 h. Then, the reaction was quenched with a saturated solution of $NaHCO_3$ at 0 °C and extracted with CH_2Cl_2. The organic layer was then washed with H_2O, dried with anhydrous Na_2SO_4 and evaporated under reduced pressure. The residue was purified using silica gel chromatography CH_2Cl_2 / MeOH (10:1, v/v) as eluent to obtain DHXI-FL as a dark green powder. Yield 90 % 495 mg. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.44 (d, *J* = 12.4 Hz, 1H), 7.63 (s, 1H), 7.63 (s, 1H), 7.38 (s, 1H), 7.33 (m, 3H), 7.29 – 7.29 (m, 2H), 7.24 – 7.15 (m, 6H), 7.00 (d, *J* = 8.5 Hz, 2H), 6.87 (d, *J* = 6.9 Hz, 1H), 6.59 (s, 1H), 6.18 (d, *J* = 12.8 Hz, 1H), 3.79 (s, 3H), 2.74 – 2.70 (m, 2H), 2.68 (t, *J* = 5.9 Hz, 2H), 1.92 – 1.86 (m, 2H), 1.64 (s, 6H). HR-MS (ESI) $C_{38}H_{35}N_2O_2^+$ [M^+], *m/z* 551.2690.

Synthesis of DHXI

DHXI-FL (200 mg, 0.36 mmol) and compound 4 (110 mg, 0.45 mmol) were dissolved in 10 mL anhydrous DCM, and then EDC (86 mg, 0.45 mmol) and a catalytic amount of DMAP were added to the solution. After stirring for 24 h at room temperature under nitrogen, the solvent was removed and diluted with DCM. The organic phase was washed with water and brine, dried over anhydrous Na_2SO_4, and concentrated in vacuo. The crude product was purified by column chromatography on silica gel (DCM: methanol = 20: 1) to give DHXI as a dark green powder. Yield 78 %, 227 mg. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.45 (d, *J* = 14.8 Hz, 1H), 7.48 – 7.44 (m, 1H), 7.40 – 7.33 (m, 4H), 7.25 – 7.20 (m, 2H), 7.19 – 7.15 (m, 6H), 7.02 – 6.97 (m, 2H), 6.85 (dd, *J* = 8.6, 2.2 Hz, 1H), 6.75 (d, *J* = 1.9 Hz, 1H), 6.58 (d, *J* = 14.8 Hz, 1H), 3.28 (s, 2H), 2.81 (t, *J* = 6.2 Hz, 2H), 2.75 – 2.70 (m, 2H), 2.19 (s, 3H), 1.93 (dd, *J* = 14.3, 1.0 Hz, 9H), 1.63 (s, 6H), 1.54 (s, 6H), 1.26 (t, *J* = 7.8 Hz, 2H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 190.70, 187.39, 176.80, 171.33, 161.85, 157.23, 154.42, 151.51, 147.55, 145.59, 144.97, 143.35, 142.69, 142.28, 141.36, 139.56, 138.81, 134.17, 129.90, 129.14 128.54, 127.18, 126.84, 126.00, 125.57, 122.86, 122.10, 119.16, 118.62, 116.32, 115.80, 115.38, 112.45, 105.64, 103.87, 50.13, 47.70, 38.49, 33.57, 29.69, 29.37, 28.97, 28.00, 27.21, 25.60, 24.54, 22.68, 20.42, 14.44, 14.12, 12.70, 12.19 ppm. HR-MS (ESI) $C_{52}H_{51}N_2O_5^+$ [M^+], *m/z* 783.3817.

Optical response of DHXI@HSA toward NQO1

The optical response of the nanoprobe DHXI@HSA toward the biomarker NQO1 activity was carried out according to the following procedures. NQO1 with different activities from 0.1 to 20 $\mu g \cdot mL^{-1}$ was added into the prepared nanoprobe DHXI@HSA solution in 10 mM PBS, pH 7.4 containing 100 μM NADH (the nanoprobe's final concentration: 0.6 $mg \cdot mL^{-1}$). After the addition of NQO1, the solutions were incubated at 37 °C for a certain amount of time and then the absorption and fluorescence spectra of each solutions was measured. For time-dependent experiments, the solutions were kept at 37 °C for different time period before spectral measurements.

For measurement of fluorescence spectra, the excitation wavelength is 680 nm.

Cell experiment

Cell culture

The luciferase-expressing HCC cells (HCC-LM3-fLuc cells) were cultured in 90% RPMI-1640 supplemented with 10% fetal bovine serum (FBS) at 37 ºC in a 5% CO_2 atmosphere. The luciferase-expressing 4T1 cells (4T1-Luc cells) were cultured in 90% DMEM supplemented with 10% fetal bovine serum (FBS) at 37 ºC in a 5% CO_2 atmosphere.

Cytotoxicity evaluation

Cells were seeded in 96-well plate (5000 cells/well), the cells were incubated with varied concentration of different formulations. After incubation, the plates were washed with PBS three times then treated with 0.5 $mg \cdot mL^{-1}$ MTT (in DMEM) for 4 h, the resulting formazan crystals were dissolved in DMSO and then measured the absorption at 570 nm.

Ex vivo fluorescence imaging

Under anaesthesia the skin and peritoneum of mice with liver tumor was cut open to reveal the liver, the nanoprobe DHXI@HSA solution (0.06 $g \cdot kg^{-1}$) was sprayed onto the liver, 30 min later, the mice were sacrificed by CO_2 exposure, the liver and other organs were dissected and underwent imaging. For fluorescence imaging, the experiments were conducted with the excitation filter of 710 nm and the emission filter of 750 nm.

Phantom optoacoustic imaging

Phantom optoacoustic imaging was performed on MSOT system (inVision 128 from iThera Medical GmbH). Briefly, the test solutions containing DHXI@HSA (0.6 mg·mL^{-1}), NQO1 (0 - 20 μg·mL^{-1}) and NADH (100 μM) were stirred for different time at 37 °C, and then added into NMR sample tubes for phantom optoacoustic imaging. Relative OA intensity was used to reveal the nanoprobe's response towards varied concentrations of NQO1. It was calculated with the equation: Relative OA intensity = $[(OA_{710})_{NQO1} - (OA_{710})_{control}] / (OA_{710})_{control}$.

In vivo imaging

Bioluminescence imaging

For bioluminescence imaging, the tumor bearing mice were given an intraperitoneal injection of D-luciferin (150 mg·kg^{-1}). After 10 min, the mice were anesthetized with continuous isoflurane and imaged.

Fluorescence imaging

For fluorescence imaging, the liver tumor bearing mice were injected through tail vein with the dosage of DHXI@HSA (0.3 g·kg^{-1} for i.v. injection). The experiments were conducted with the excitation filter of 710 nm and the emission filter of 750 nm.

Optoacoustic imaging

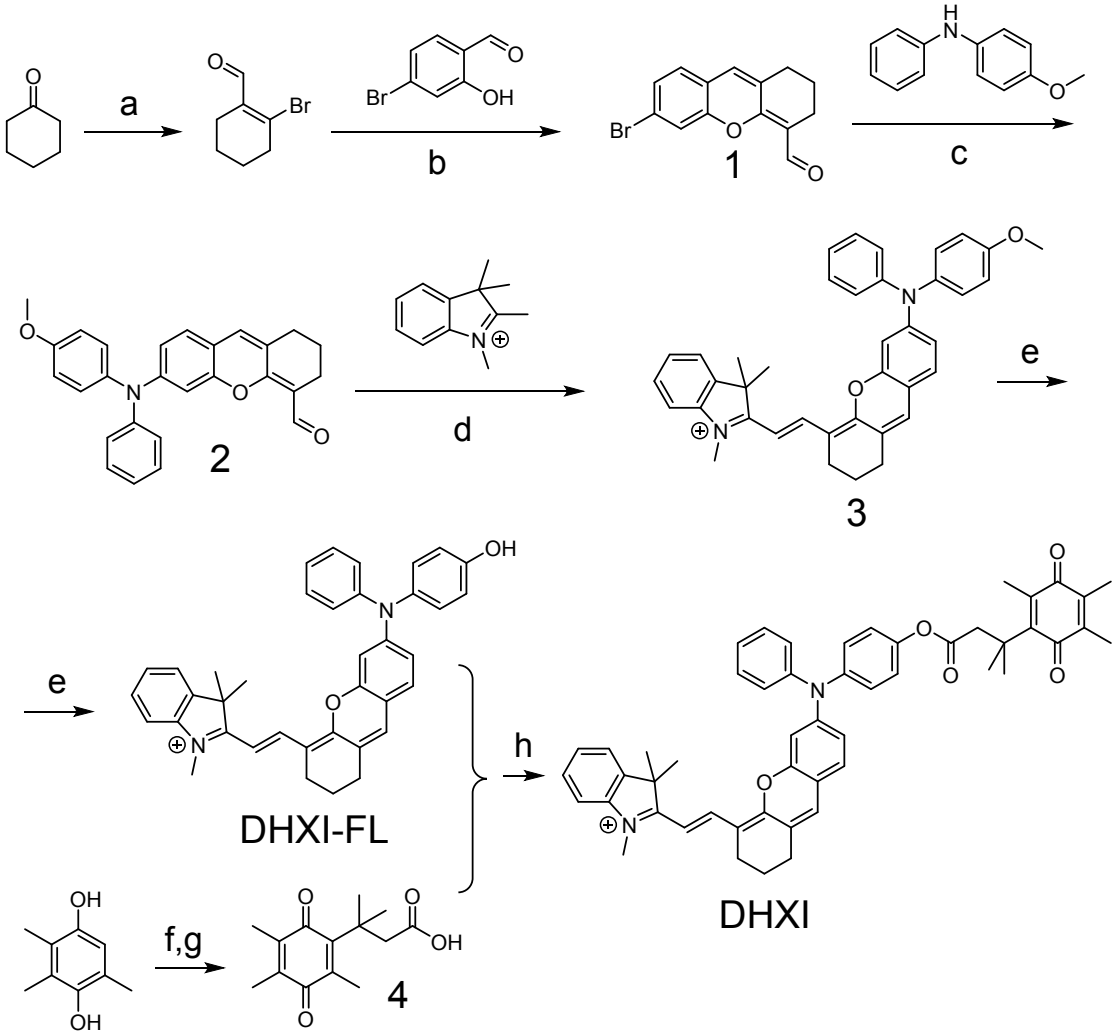
Animal optoacoustic imaging was carried out by using MSOT system (inVision 128 from iThera Medical GmbH). Mice were anesthetized with continuous oxygen and 1% isoflurane via mouse breathing mask and put in the prone position in a 34 °C water bath. For the liver-tumor-bearing mice, the dosage of DHXI@HSA (0.3 g·kg^{-1}) was administered through veil tail. The mice were anesthetized and placed in the prone position in animal holder for imaging. Cross-sectional images were acquired with a step size of 0.3 mm. Imaging wavelengths selected conforming to the major turning points in DHXI-FL's and hemoglobin's absorption spectra included 680 nm, 690 nm, 700 nm, 710 nm, 730 nm, 760 nm, 800 nm and 850 nm (background). We recorded 5 frames at every imaging wavelength. After the MSOT images were generated, the z-stack was rendered as orthogonal-view three-dimensional images (guided ICA spectral unmixing was utilized to separate

signals coming from the generated fluorophore and those from the photo-absorbing tissue elements in the body (e.g. hemoglobin)).

Histological evaluation

For biosafety study, healthy mice were intravenously injected with DHXI@HSA (0.3 $g \cdot kg^{-1}$) or pH 7.4 PBS, respectively. After 24 h, these mice were euthanized and the corresponding organs (heart, liver, spleen, lung and kidney) were excised and embedded in paraffin and then sectioned to 4 μm for H&E staining.

Synthetic procedures



Scheme S1. Synthetic routes of DHXI. Reagents and conditions: a) $CHCl_3$, PBr_3, DMF, r.t., 18 h; b) Cs_2CO_3, DMF, r.t., 48 h; c) $Pd_2(dba)_3$, DavePhos, Cs_2CO_3, dioxane, 95 °C, 18 h; d) Ac_2O, K_2CO_3 80 °C, 16 h; e) BBr_3, CH_2Cl_2, -40 °C, 16 h; f,g) methyl 3,3-dimethylacrylate, methanesulfonic acid, 70 °C, 1.5 h; 10% aqueous acetonitrile solution, NBS, r.t., 1 h; h) DCM, EDC, DMAP, N_2, r.t., 24 h.

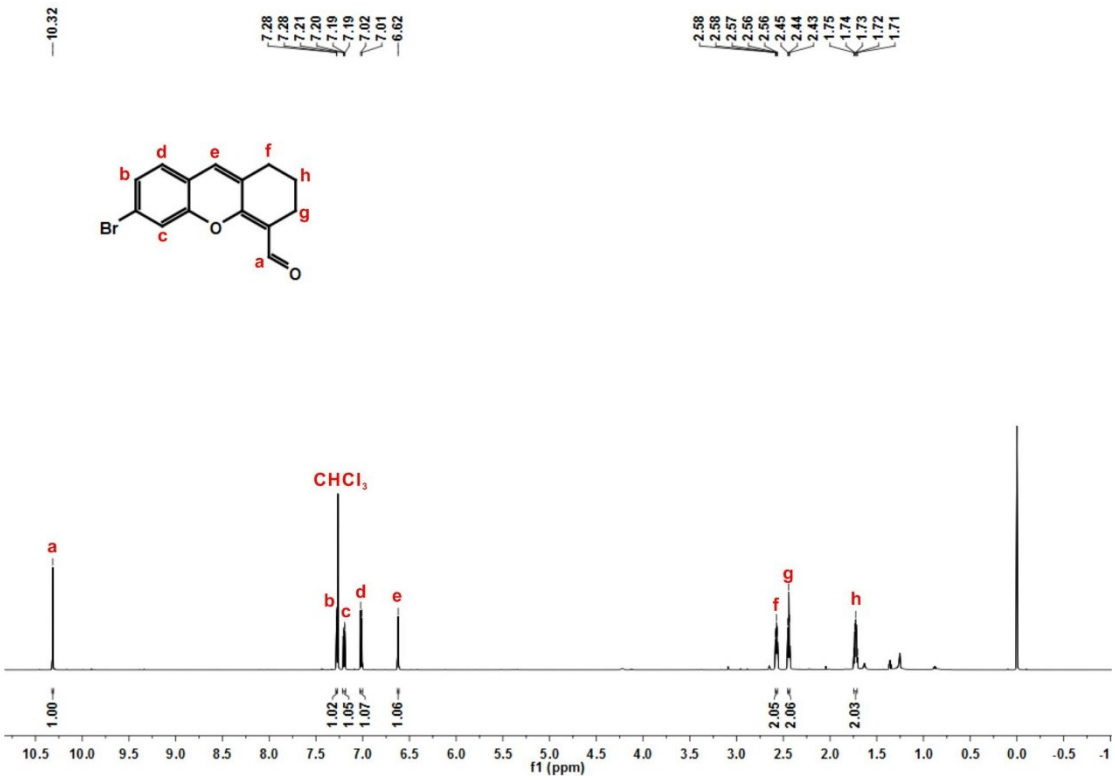


Figure S1. ^{1}H NMR spectrum of compound 1 (in $CDCl_3$).

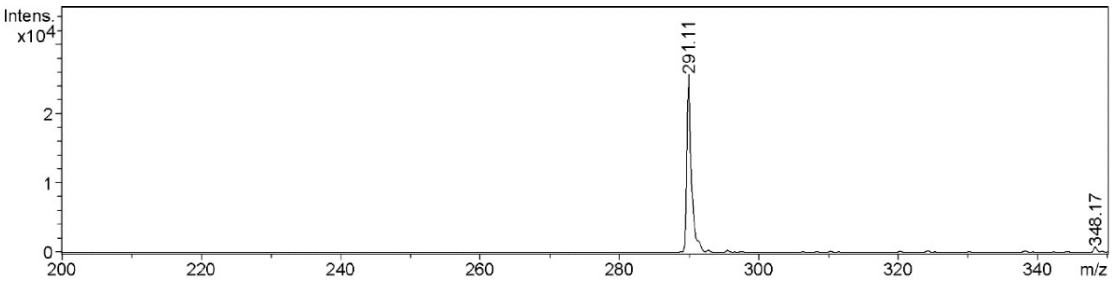


Figure S2. MS spectrum of compound 1.

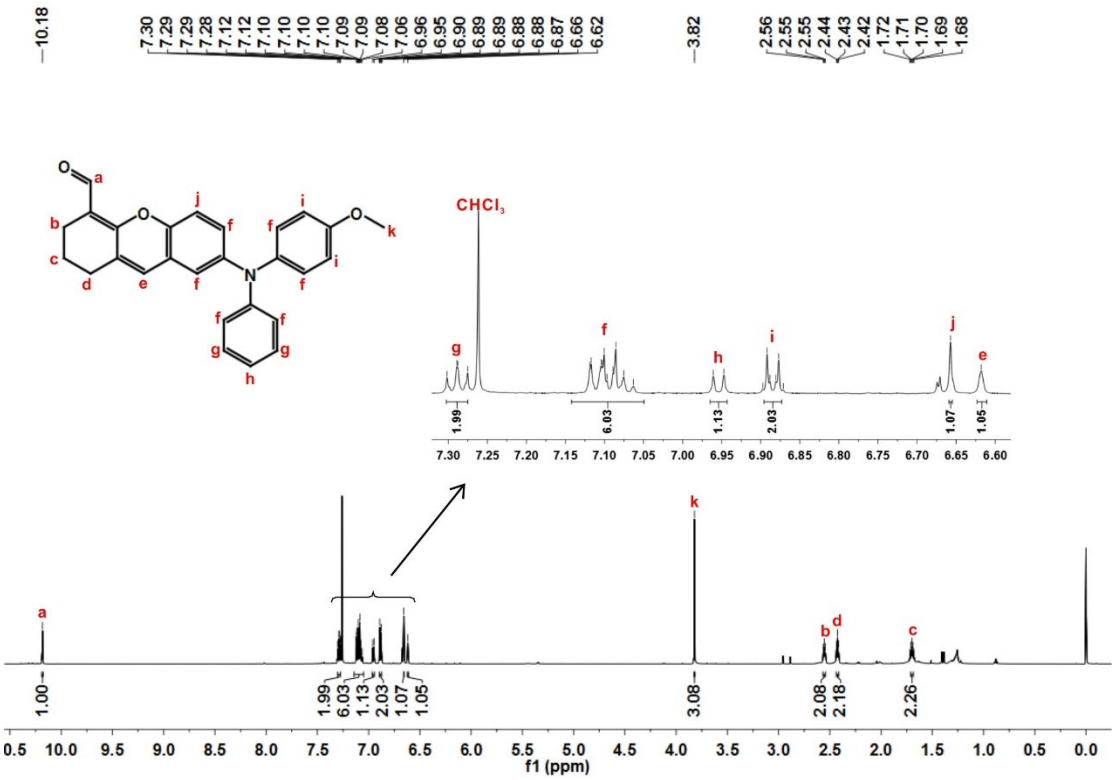


Figure S3. ^{1}H NMR spectrum of compound 2 (in $CDCl_3$).

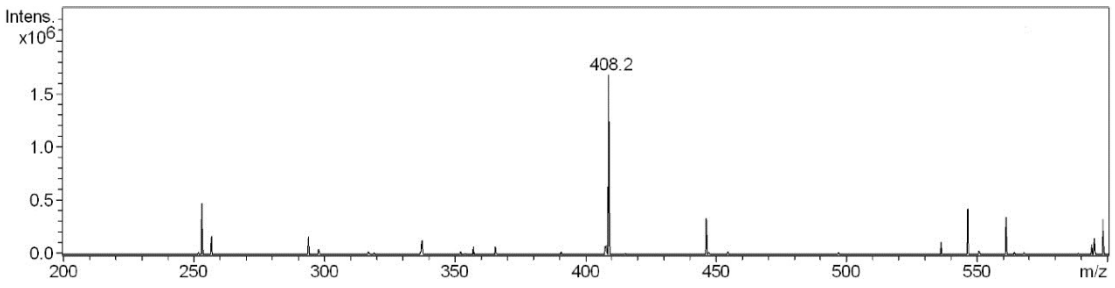


Figure S4. MS spectrum of compound 2.

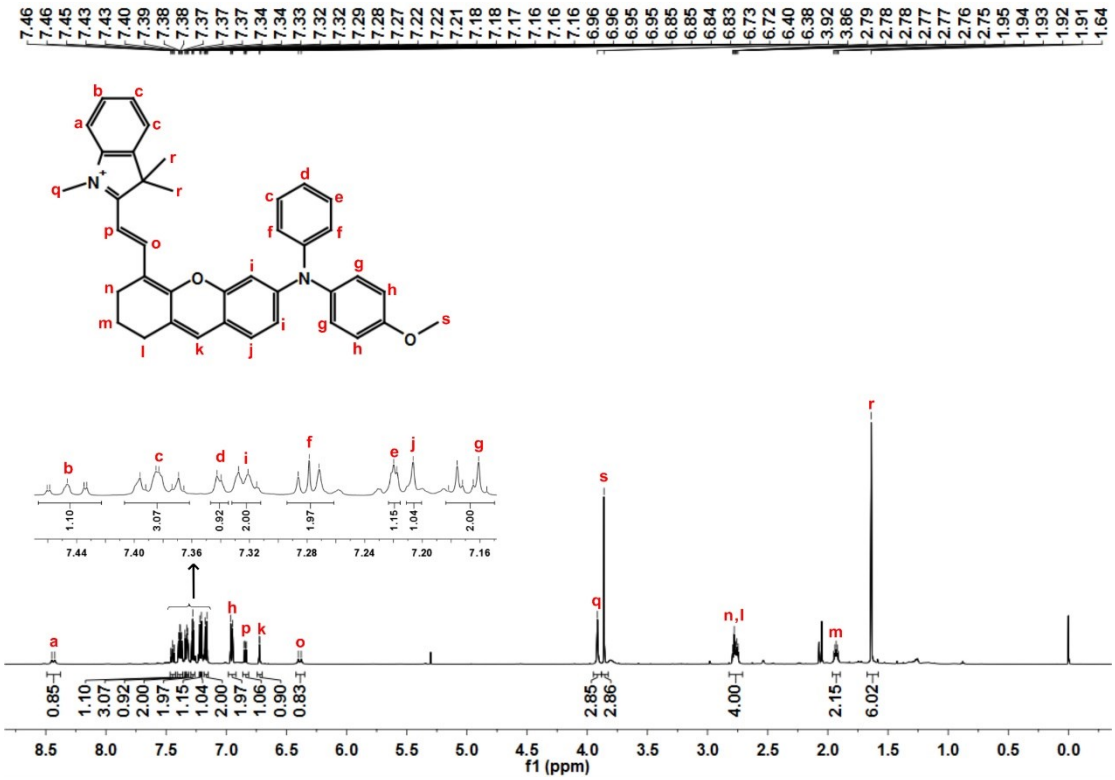


Figure S5. ^{1}H NMR spectra of compound 3 (in $CDCl_3$).

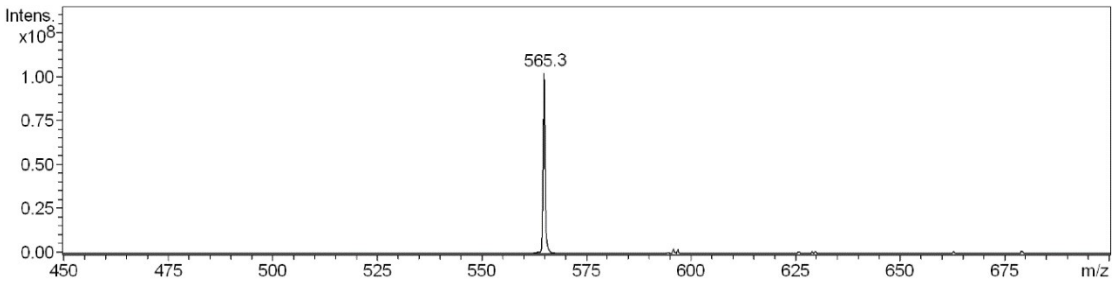


Figure S6. MS spectrum of compound 3.

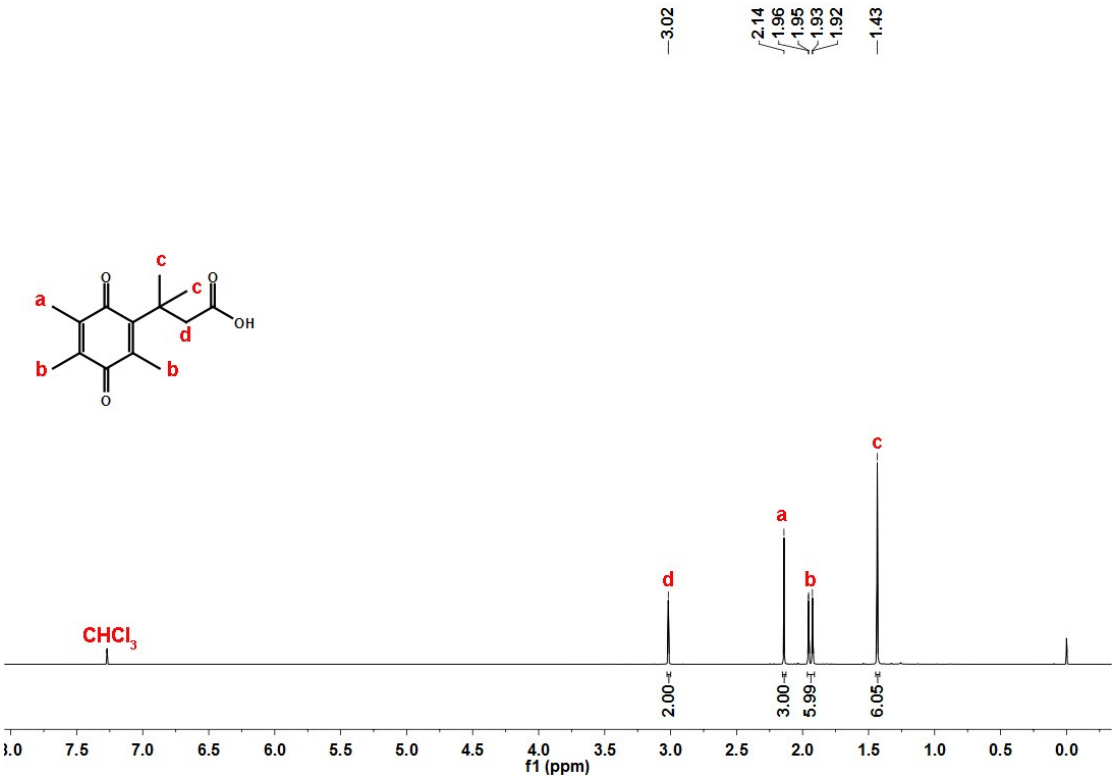


Figure S7. ^{1}H NMR spectra of compound 5 (in $CDCl_3$).

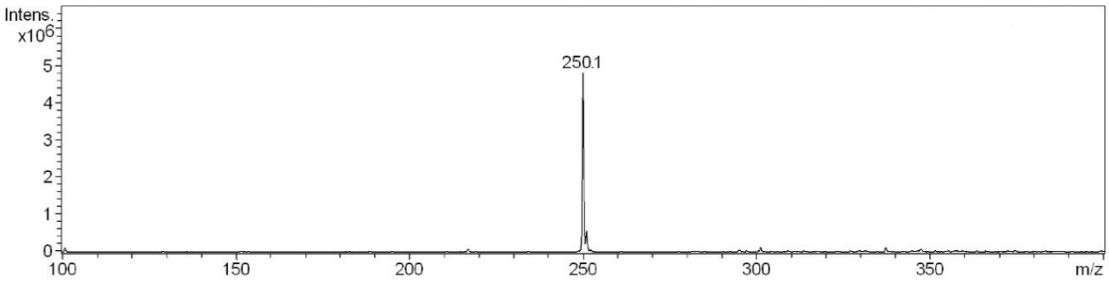


Figure S8. MS spectrum of compound 5.

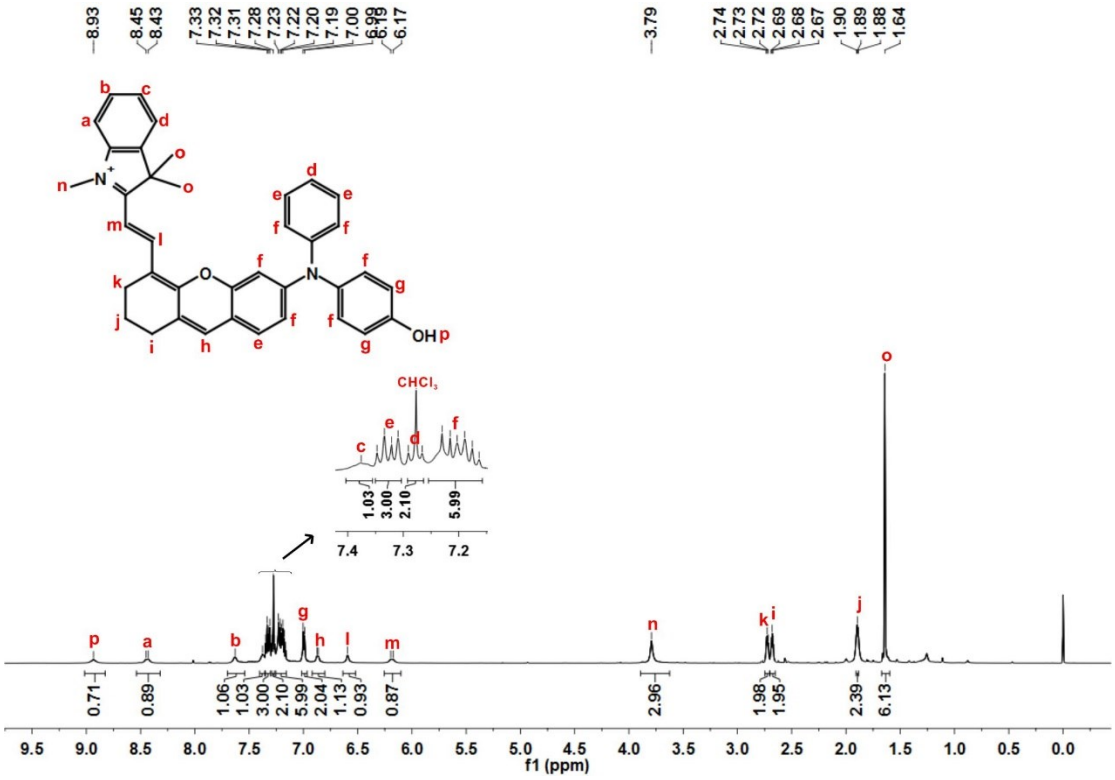


Figure S9. ^{1}H NMR spectra of DHXI-FL (in $CDCl_3$).

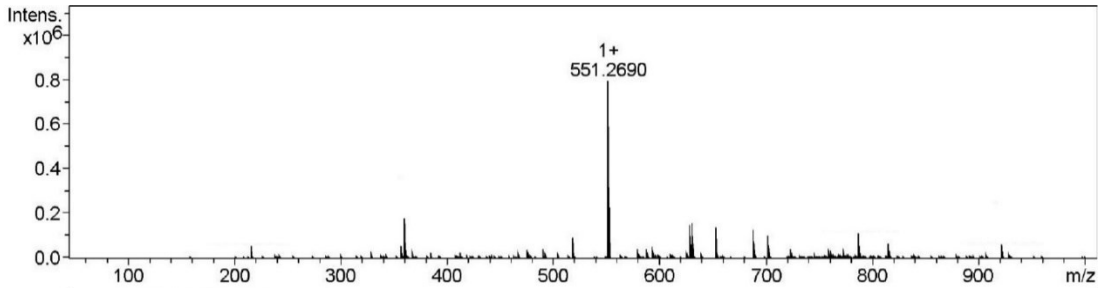


Figure S10. HR-MS spectrum of DHXI-FL.

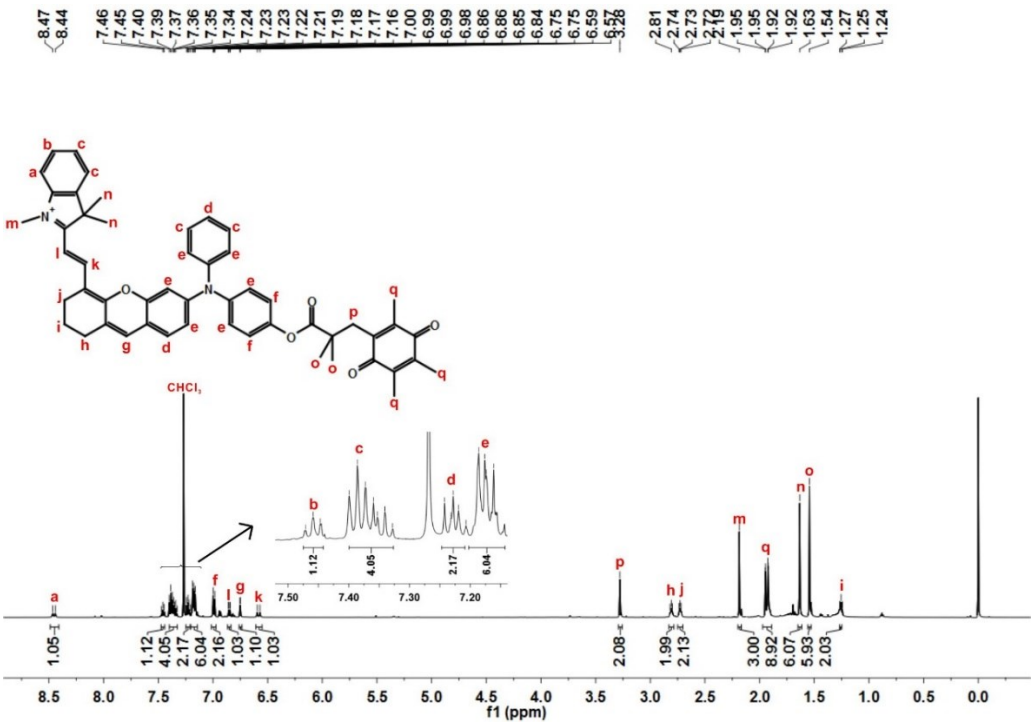


Figure S11 A. ^{1}H NMR spectra of DHXI (in $CDCl_3$).

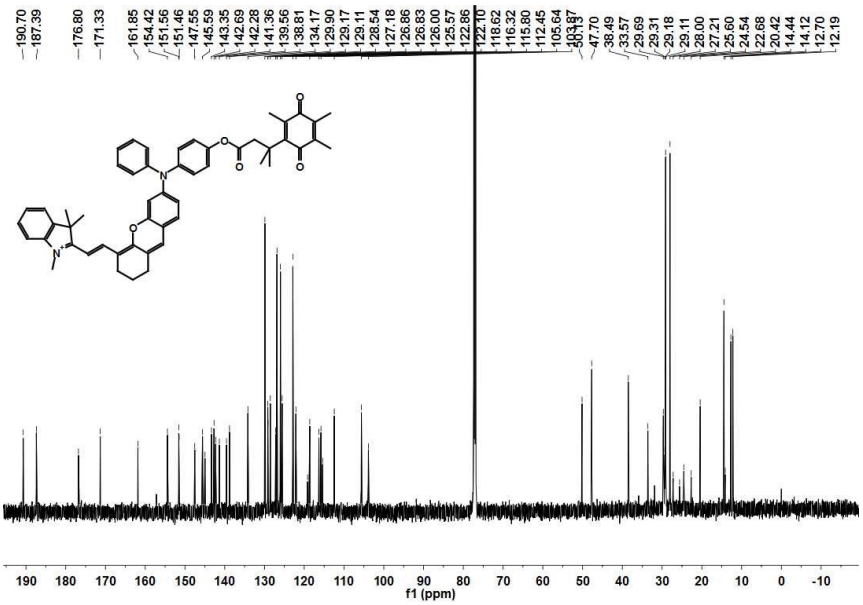


Figure S11 B. ^{13}C NMR spectra of DHXI (in $CDCl_3$).

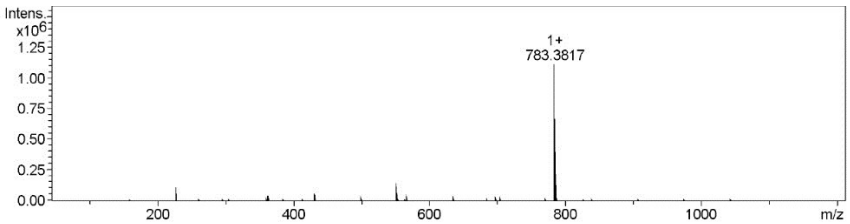


Figure S12. HR-MS spectrum of DHXI.

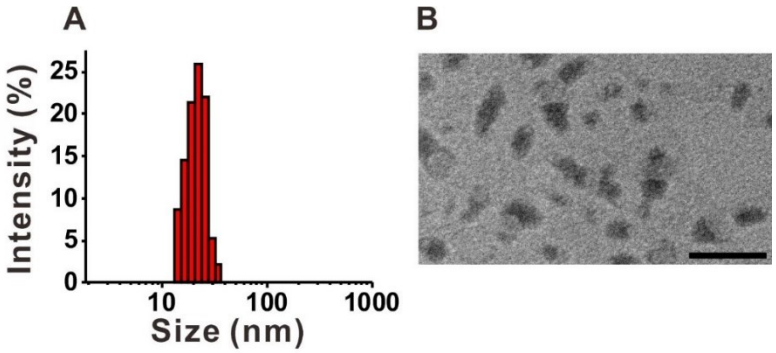


Figure S13 (A) Particle size distribution of DHXI@HSA measured by DLS. (B) TEM image of DHXI@HSA particles. Scale bar: 50 nm.

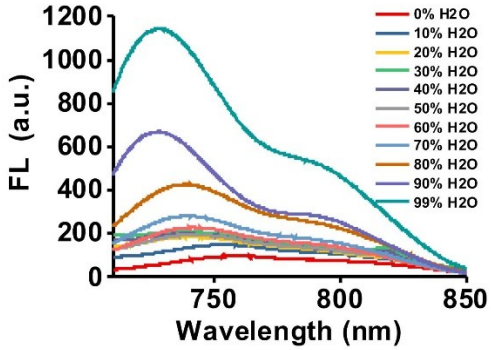


Figure S14 Fluorescence spectra of DHXI-FL in poor solvent/good solvent mixtures (good solvent: THF, poor solvent: water, DHXI-FL concentration: 10 μM, HSA: 5 mg·mL^{-1}). Excitation wavelength: 680 nm.

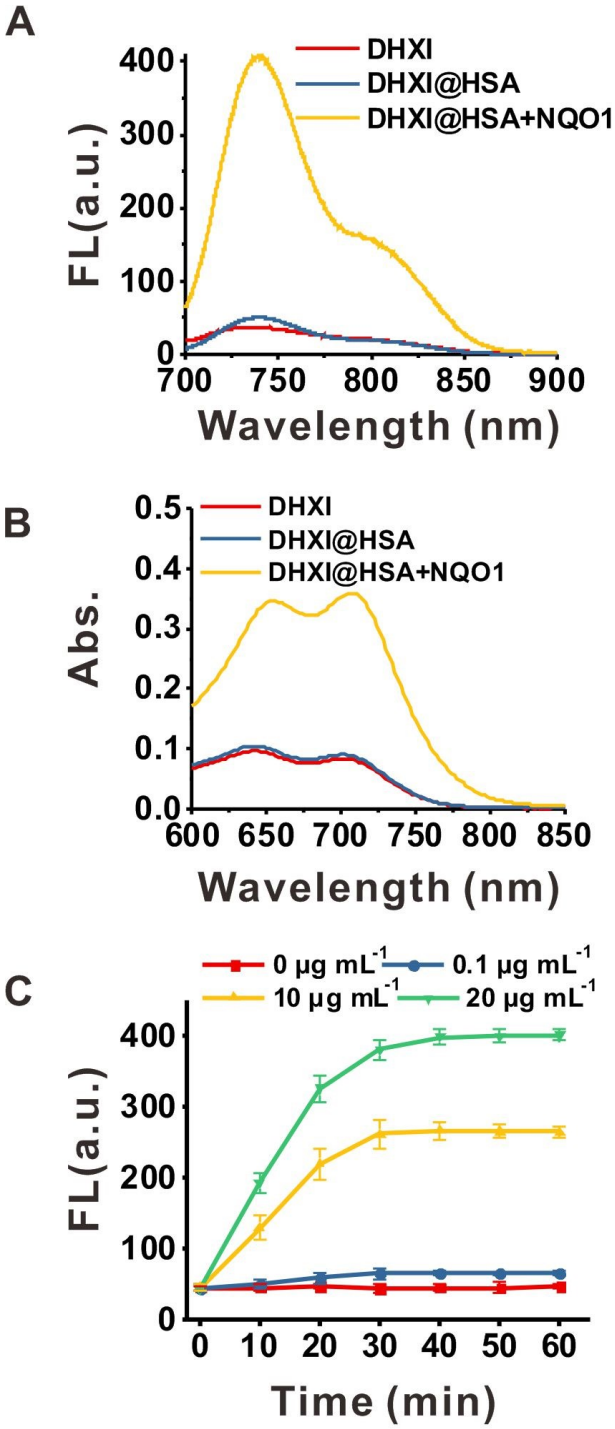


Figure S15 (A) Fluorescence spectra for DHXI, DHXI@HSA solution, and DHXI@HSA solution after incubation with NQO1 (20 μg·mL^{-1}) respectively. (B) Absorption spectra for DHXI, DHXI@HSA solution, and DHXI@HSA solution after incubation with NQO1 (20 μg·mL^{-1}) respectively. (C) Fluorescence intensity at 730 nm of the nanoprobe DHXI@HSA (0.6 mg·mL^{-1})

in pH 7.4 PBS upon addition of NQO1 (0, 0.1, 10, 20, μg·mL^{-1}) for different time. Excitation: 680 nm.

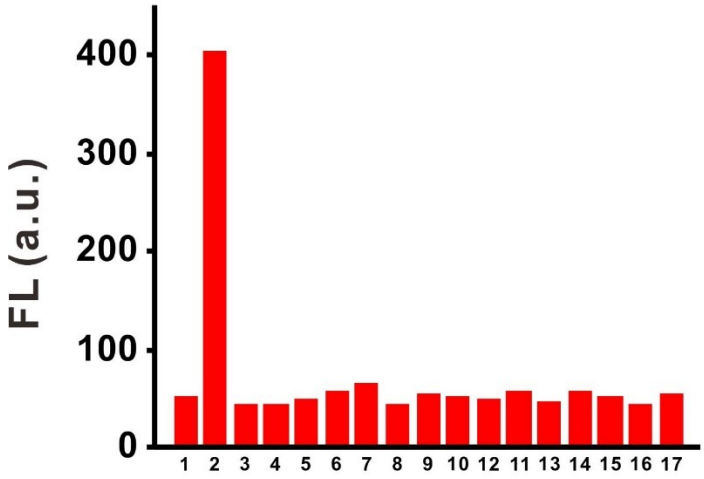


Figure S16. Selectivity experiment for DXHI@HSA (0.6 mg·mL^{-1}) in the presence of various species. 1 blank, 2 NQO1 (20 μg·mL^{-1}), 3 Fe^{3+} (1 mM), 4, GSH (1 mM), 5 KO_2 (1 mM), 6 HOCl (1 mM), 7 NO^{2-} (1 mM), 8 Cys (1 mM), 9 Hcy (1 mM), 10 vitamin C, 11. Na^+ (1 mM), 12. K^+ (1 mM), 13. Ca^{2+} (1 mM), 14 ALP (100 U·L^{-1}), 15 Nitroreductase (20 μg·mL^{-1}), 16 β-galactosidase (20 μg·mL^{-1}), 17 Acetylcholinesterase (20 μg·mL^{-1}).

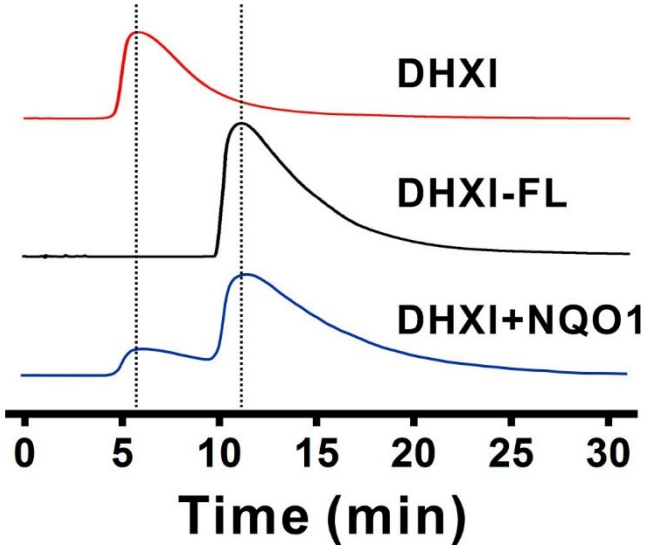


Figure S17 HPLC profile for pure DHXI (red line), pure DHXI-FL (black line) and DHXI upon treated with NQO1 (Blue line). The mobile phase was 80/20 methanol / H_2O and the flow rate was 1.0 mL / min.

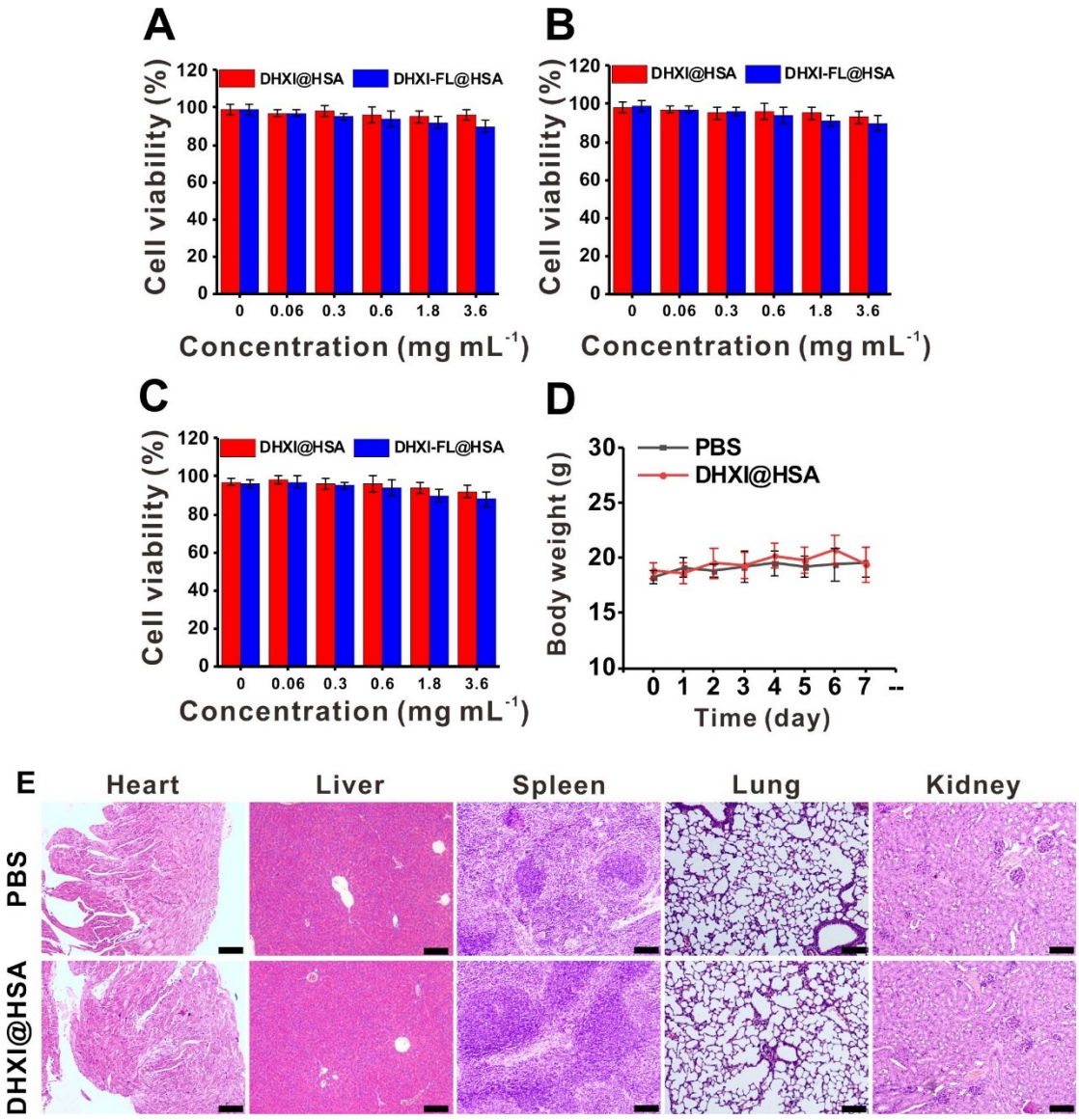


Figure S18. Viabilities of L-O2 (A) and HCC-LM3-fLuc (B) and 4T1 (C) cells upon 24 hours of incubation with DHXI@HSA, DHXI-FL@HSA of different concentrations (0, 0.06, 0.3, 0.6, 1.8, 3.6 mg·mL^{-1} DHXI@HSA or DHXI-FL@HSA). (D) Time-dependent changes in mice' body weight (n = 6 per group). (E) Representative histological sections (H&E staining) for main organs of the mice one day after intravenous injection of PBS or DHXI@HSA. Scale bar = 100 μm.

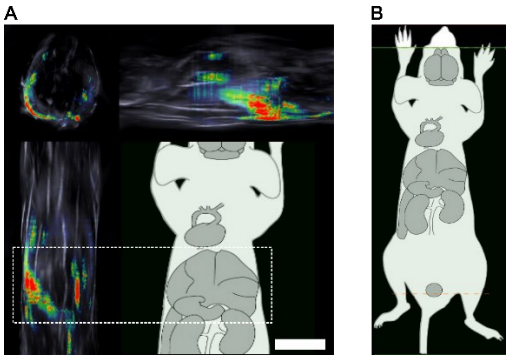


Figure S19. Illustration of the lying posture (the mouse is lying on its stomach) and the part of the trunk of the mouse for MSOT imaging.

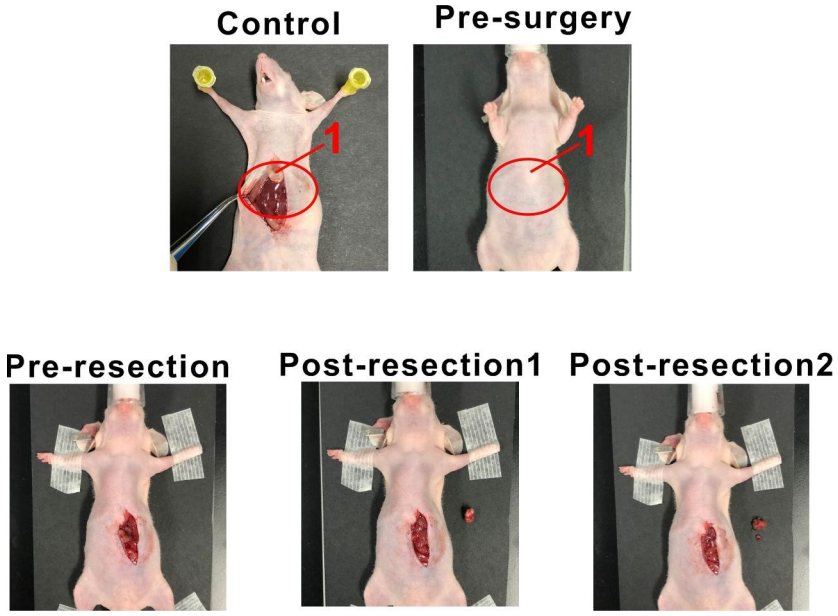


Figure S20 White-light photographs of the surgery process showing the lying posture of the mouse during surgery (the mouse lay on its back).

Control: healthy mouse underwent a sham-surgery; the label 1 points at the xiphoid process which is usually used as positioning landmark.

Surgical resection of orthotopic liver tumor. Pre-resection: the skin and peritoneum were removed and the liver region was exposed. Post-resection 1: the tumor was excised and placed beside the mouse body, Post-resection 2: the residual tissue was excised and placed beside the mouse body along with the tumor.

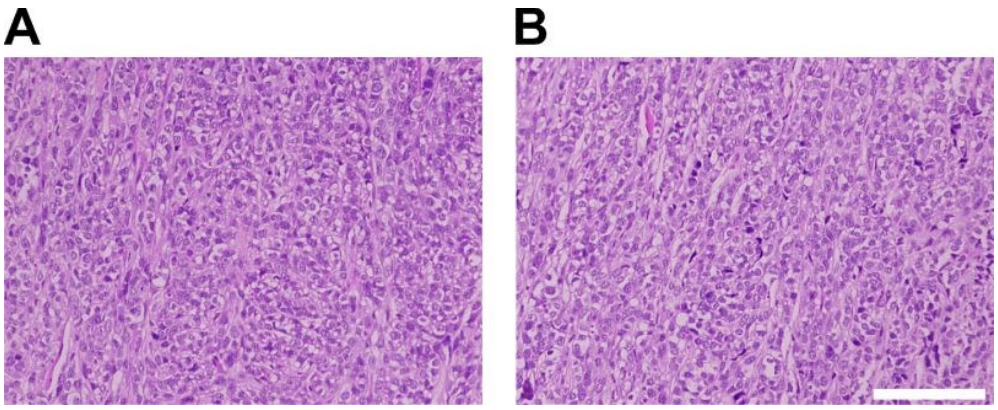
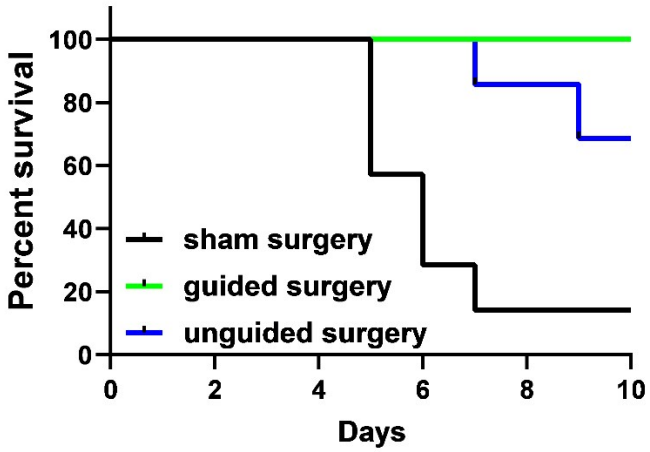


Figure S21 H&E staining of the tissue sections of the two tumors resected from abdominal cavity. Scale bar: 100 μm.

(A)



(B)

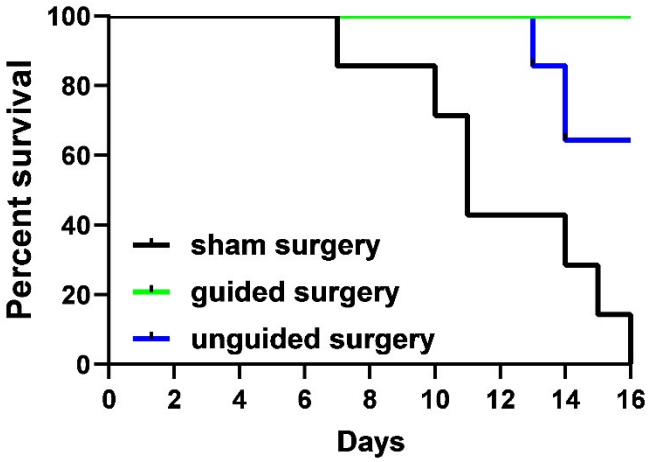


Figure S22 (A) Survival curve of mice in each group during surgical resection of liver tumors for 10 days. (B) Survival curve of mice in each group during surgical resection of abdominal tumors for 16 days.