Supporting Information

A bright NIR-II fluorescent probe for breast carcinoma imaging and image-guided surgery

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Author contributions

X. C. Hong, M. F. Wu, and Y. L. Xiao conceived and designed the experiments. X. D. Zeng, L. R. Xie, D. L. Chen, S. S. Li, J. X. Nong, B. Wang performed the experiments. X. D. Zeng, L. Tang, Q. Q. Li, Y. Li, Z. X. Deng, X. C. Hong, M. F. Wu, Y. L. Xiao analyzed the data and wrote the manuscript. All authors discussed the results and commented on the manuscript.

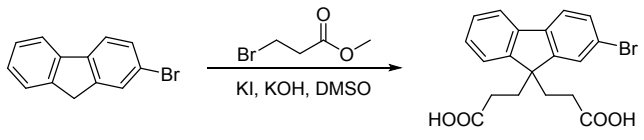
Materials and General Procedure

mPEG-NH_2 (2 kDa) was purchased from Ponsure Biotechnology CO., Ltd. The synthesis reagents were purchased from commercial suppliers (such as Aldrich, Energy Chemical, Sinopharm Group Co., Ltd.) and used without further purification unless otherwise noted. 4,7-bis(7-bromo-2,3-dihydrothieno[3,4-*b*][1,4]dioxin-5-yl)-5,6-dinitrobenzo[*c*][1,2,5]thiadiazole (compound **4**) was synthesized according to our previous report.[1] Tetrahydrofuran (THF) was freshly distilled from sodium/benzophenone. Anhydrous pyridine was freshly distilled using calcium hydride. ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ and DMSO-d_6 at room temperature using a Bruker AV400 magnetic resonance spectrometer. MALDI-TOF-MS characteristics were performed on an AB SCIEX 5800 MALDI TOF mass spectrometer. UV-vis-NIR spectra were tested with a SHIMADZU UV-2600 or PerkinElmer Lambda 25 spectrophotometer. NIR fluorescence spectrum was performed on an Applied NanoFluorescence spectrometer at room temperature with an excitation laser source of 785 nm or 808 nm. The NIR-II in vivo imaging system was purchased from Suzhou NIR-Optics Technologies CO., Ltd. Analytical and preparative TLC were performed on silica gel plates, and column chromatography was conducted over silica gel (mesh 200-300), both of which were obtained from the Qingdao Ocean Chemicals.

Synthesis and characterization

The preparation procedure of **H3** was performed according the previous work. [2, 3]

Synthesis of 3,3'-(2-bromo-9*H*-fluorene-9,9-diyl)dipropionic acid (1)



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2-bromofluorene (4.4 g, 18 mmol) and potassium iodide (299 mg, 1.8 mmol) were dissolved in dimethyl sulfoxide (41 mL) under an argon atmosphere. Methyl 3-bromopropionate (4.32 mL, 39.6 mmol, 6.613 g) was added to the reaction mixture. Potassium hydroxide (5.05 g, 90 mmol) was added in 10 portions to the solution. The green reaction mixture was stirred at room temperature for 24 h and quenched with water. The mixture was acidified to pH 5 with 2 M aq. HCl solution, extracted with EtOAc (3×150 mL). The combined organic layers were dried with anhydrous

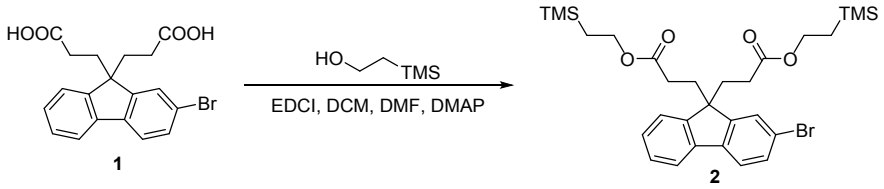
magnesium sulfate, filtered and concentrated by rotary evaporator. Compound **1** (5.94 g, 85%) was obtained as a yellow solid which can be used in the next step without further purification.

^{1}H NMR (400 MHz, DMSO-d_6) δ 11.95 (s, 2H), 7.84 (dt, J = 15.7, 5.3 Hz, 3H), 7.61 – 7.48 (m, 2H), 7.44 – 7.34 (m, 2H), 2.35 (dd, J = 17.1, 9.1 Hz, 4H), 1.45 – 1.25 (m, 4H).

^{13}C NMR (101 MHz, DMSO-d_6) δ 174.3, 151.0, 148.2, 140.3, 140.0, 131.0, 128.6, 128.2, 126.9, 123.7, 122.4, 121.3, 120.8, 54.2, 34.1, 29.2.

ESI-MS m/z: $[M+H]^+$ calcd for $C_{19}H_{18}BrO_4^+$, 389.0; found, 389.4.

Synthesis of bis(2-(trimethylsilyl)ethyl) 3,3'-(2-bromo-9*H*-fluorene-9,9-diyl)dipropionate (2)



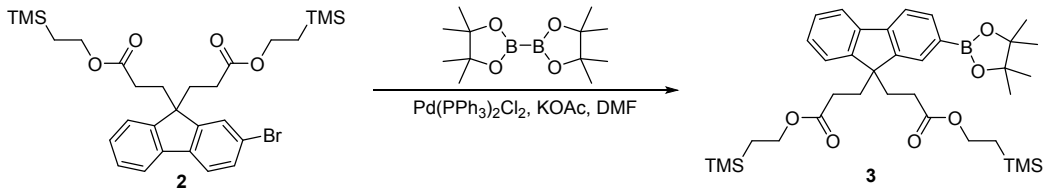
To a solution of compound **1** (3.88 g, 10 mmol) and 2-trimethylsilylethanol (7.75 mL, 54 mmol, 6.39 g) in DCM (24 mL) and DMF (36 mL) was cooled at -15 °C under an argon atmosphere. EDCI (9.59 g, 50 mmol) and DMAP (1.96 g, 16 mmol) were added in a single portion and the reaction mixture was stirred at -15 °C for 5 h. The reaction mixture was then allowed to warm at ambient temperature. After dilution with dichloromethane (300 mL), the organic layer was washed with saturated NH_4Cl solution (200 mL), water (4×200 mL), saturated aqueous brine (100 mL), dried over anhydrous magnesium sulfate, filtered and evaporated. The residue was crystalized by MeOH to yield compound **2** (3.53 g, 60%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.70 – 7.66 (m, 1H), 7.58 (d, J = 7.9 Hz, 1H), 7.49 (d, J = 7.8 Hz, 2H), 7.35 (s, 3H), 4.01 – 3.92 (m, 4H), 2.45 – 2.33 (m, 4H), 1.61 – 1.50 (m, 4H), 0.88 – 0.76 (m, 4H), -0.01 (s, 18H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 173.3, 149.9, 147.3, 140.3, 140.2, 130.9, 128.1, 127.9, 126.4, 123.1, 121.5, 121.4, 120.2, 62.5, 54.0, 34.6, 29.2, 17.2, -1.5.

ESI-MS m/z: $[M+Na]^+$ calcd for $C_{29}H_{41}BrNaOSi_2^+$, 611.2; found, 611.2.

Synthesis of bis(2-(trimethylsilyl)ethyl) 3,3'-(2-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-9*H*-fluorene-9,9-diyl)dipropionate (3)



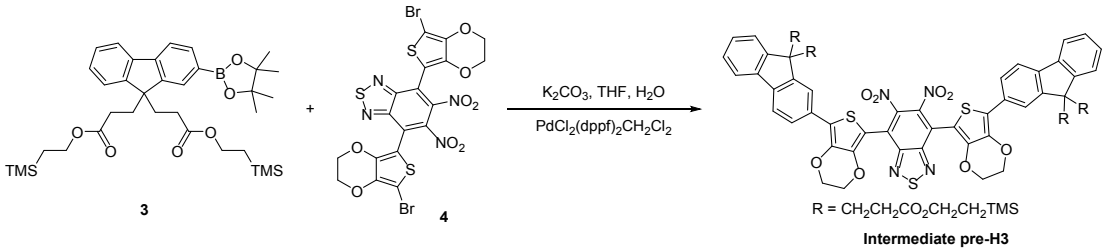
To a solution of compound **2** (1.76 g, 3 mmol), bis(pinacolate)diboron (0.914 g, 3.6 mmol) and KOAc (0.706 g, 7.2 mmol) in DMF (60 mL) was added bis(triphenylphosphine)palladium(II) dichloride (210 mg, 0.3 mmol) under an argon atmosphere. Then the reaction mixture was heated in an oil bath at 80 °C for 2 h. After the reaction mixture was cooled down to room temperature, 60 mL ethyl acetate was added and solid was removed by filtration. The solution was dilute with water (120 mL) and extracted with *n*-hexane (3×60 mL). The combined organic layers were washed with water (4×60 mL), saturated aqueous brine (150 mL) and dried with anhydrous $MgSO_4$. The solvents were concentrated using rotary evaporator and pumped vacuum to obtain colourless and transparent liquid **3** (0.86 g, 45%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.84 – 7.73 (m, 2H), 7.72 – 7.62 (m, 2H), 7.39 – 7.23 (m, 3H), 3.99 – 3.87 (m, 4H), 2.42 (d, *J* = 4.5 Hz, 4H), 1.60 – 1.43 (m, 4H), 1.34 (s, 12H), 0.84 – 0.73 (m, 4H), -0.07 (s, 18H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 173.4, 148.2, 146.9, 144.1, 141.0, 134.5, 128.9, 128.1, 127.6, 123.1, 120.4, 119.3, 83.7, 62.2, 53.5, 34.6, 29.2, 24.9, 17.1, -1.5.

ESI-MS m/z: $[M+Na]^+$ calcd for $C_{35}H_{53}BNaO_6Si_2$+, 659.3; found, 659.4.

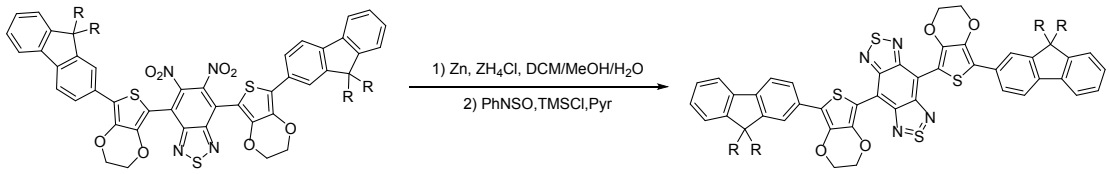
Synthesis of intermediate pre-H3



To a solution of compound **3** (64 mg, 0.1 mmol) and 4,7-bis(7-bromo-2,3-dihydrothieno[3,4-b][1,4]dioxin-5-yl)-5,6-dinitrobenzo[c][1,2,5]thiadiazole compound **4** (33 mg, 0.05 mmol) in THF (2.5 mL) was bubbled with argon for 5 min. Potassium carbonate (18 mg, 0.125 mmol) in 0.5 mL distilled water and 1,1'-Bis(diphenylphosphino)ferrocenepalladium(II)dichloride dichloromethane complex (9 mg, 0.01 mmol) were added to the above reaction mixture under an argon atmosphere. The mixture was heated in an oil bath at 75 °C for 14 h. After cooling to room temperature, the

solvent was removed in vacuo. The residue was dissolved in dichloromethane, and the resulting solution was washed with water, saturated aqueous brine. After drying over anhydrous magnesium sulfate and removal of the solvents under reduced pressure, product intermediate **pre-H3** (65 mg, 85% yield) was obtained as a purple solid which can be used in the next step without further purification.

Synthesis of compound H3



Zinc dust (392 mg, 6 mmol) and NH_4Cl (96.3 mg, 1.8 mmol) were added to a stirred solution of intermediate **pre-H3** (76 mg, 0.05 mmol) in dichloromethane (7.2 mL) and 90% methanol (11.4 mL) under an argon atmosphere. After being stirred at room temperature for 4 h, the solution was filtered through Celite pad, diluted with dichloromethane, and washed with water, saturated aqueous $NaHCO_3$, and saturated aqueous brine. The organic phase was dried over anhydrous $MgSO_4$, filtered and concentrated under vacuum to afford a yellow solid which was utilized for the next step without further purification.

To a dark yellow solution in anhydrous pyridine (1 mL) was added N-thionylaniline (0.2 mL, 1.8 mmol, 247 mg) and chlorotrimethylsilane (0.3 mL, 3.5 mmol, 377 mg). The mixture was heated in an oil bath at 80°C for 20 h. The reaction mixture was allowed to cool down to room temperature, poured into iced water, extracted with dichloromethane. The combined organic layer was washed with water, saturated aqueous brine, dried over anhydrous $MgSO_4$, and concentrated in vacuo. The residue was purified by flash column chromatography on silica gel (15:1 petroleum ether: ethyl acetate) to yield the product **H3** as dark green solid (25 mg, two step 34% yield).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.94 (dd, J = 8.0, 1.4 Hz, 2H), 7.83 (s, 2H), 7.75 (t, J = 8.0 Hz, 4H), 7.36 (dq, J = 8.7, 6.3 Hz, 6H), 4.58 – 4.52 (m, 4H), 4.44 – 4.37 (m, 4H), 3.99 (ddd, J = 9.0, 7.4, 3.6 Hz, 8H), 2.48 (t, J = 8.3 Hz, 8H), 1.63 (ddd, J = 21.5, 9.8, 6.6 Hz, 8H), 0.88 – 0.81 (m, 8H), -0.01 (s, 36H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 173.7, 152.6, 148.3, 147.9, 141.9, 141.0, 140.3, 138.6, 132.4, 127.7, 126.1, 123.1, 122.1, 120.6, 120.3, 120.1, 113.1, 110.0, 109.1, 64.8, 64.6, 62.5, 53.7, 34.7, 29.2, 17.2, -1.5.

MALDI-TOF-MS Calcd for: $C_{76}H_{90}N_4O_{12}S_4Si_4^+$ ($[M]^+$): 1490.4515, found: 1490.7948.

Synthesis of NIR-II probe H3-PEG2K

The preparation procedure of **NIR-II probe H3-PEG2K** was shown in Fig. 1A. To a solution of **H3** (2 mg, 1.34 μmol) in DCM (1 mL) was added trifluoroacetic acid (1 mL) and the reaction mixture was stirred at room temperature for 7.5 hours. The solvent was removed in vacuo and the crude product was washed by dichloromethane to yield the desired **H3** derivative with four carboxylic acid groups as a dark green solid which was characterized by MALDI-TOF-MS and used for the next step without further purification. MALDI-TOF-MS Calcd for: $C_{56}H_{42}N_4O_{12}S_4^+$ ($[M]^+$): 1090.1682, found: 1090.4248.

To a solution of **H3** derivative with four carboxylic acid groups (1.46 mg, 1.34 μmol) in anhydrous DMF (0.5 mL) was added DIPEA (6 μL) and HATU (4.08 mg, 10.72 μmol), and then stirred for 20 min at room temperature under an argon atmosphere. The reaction mixture was added mPEG2k-NH_2 (21.44 mg, 10.72 μmol) and further stirred for 24 h at room temperature. The crude product was precipitated in cold diethyl ether. The crude product was the dissolved in DCM and purified by preparative thin-layer chromatography. The final product **H3-PEG2K** (3.8 mg, 40%) as a dark green solid was confirmed using ^1H NMR and MALDI-TOF-MS shown in Figure S13 and S14.

Cell Culture and Animal Model

Mouse mammary tumor cell line 4T1 and mouse fibroblastic cell line L929 were purchased from the China Center for Type Culture Collection (CCTCC). All cells were grown in a humidified atmosphere at 37 °C with 5% CO_2 atmosphere. 4T1 Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco), and L929 cells were maintained in Mimumum Essentiul Medium (MEM, Gibco), supplemented with 10% fetal bovine serum, 100 IU mL^{-1} penicillin, 100 μg mL^{-1} streptomycin. For 4T1 breast tumor model establishment, 4T1 cells (roughly 2×10^6 in 75 μL of FBS-free DMEM medium) were subcutaneously injected into the right back leg of the 6-week-old female Balb/c nude mice which are purchased from Charles River Laboratories (Beijing, China).

The tumors were allowed to reach ~500 mm^3 for small animal fluorescent imaging studies (tumor volume = Length*Width*Width/2). For spontaneous DMBA-induced mammary carcinoma rat model establishment, female rats with approximately 150 g body weight were obtained from the Center for Disease Prevention and Control in Hubei Province, China and given DMBA suspended in soya bean oil (20 mg/mL) at a dosage of 200 mg/kg by oral administration, keep feeding for 10-15 weeks. At 10 weeks after oral administration of DMBA soya bean oil suspension, palpable mammary carcinoma was observed in the breast position of several rats. Multiple mammary tumors were monitored and some primary tumors reached approximately 10 mm at 13 weeks post administration of DMBA. These rats with mammary carcinomas were used for NIR-II fluorescence imaging and image-guided surgery. All animal experiments were performed according to the Chinese Regulations for the Administration of Affairs Concerning Experimental Animals and approved by the Institutional Animal Care and Use Committee (IACUC) of Wuhan University.

The cell viability assessment of H3-PEG2K.

The cytotoxicity of **H3-PEG2K** NIR-II probe was investigated by a standard MTT assay. The cell viability was measured using normal cells L929 and tumor cells 4T1 for 24 h incubation. The L929, and 4T1 cells were seeded in a 96-well plate (around 2000 cells per well). After 12 h, the medium was substituted with the fresh medium contained **H3-PEG2K** with different concentrations. Followed by incubation for 24 h, then a standard MTT method was performed for measuring the cell viability.

***In Vivo* Toxicity Studies.**

For the evaluation of the *in vivo* toxicity of **H3-PEG2K**, the healthy KM mice purchased from the Center for Disease Prevention and Control in Hubei Province were intravenously injected via the tail vein with PBS (0.2 mL), **H3-PEG2K** (0.2 mL, 10 mg kg^{-1}) PBS solution, n = 5 per group. At 24 hours post-injection, the serum was obtained from mice fundus artery and then for biochemistry test including CREA, BUN, TBIL, ALT, AST, and ALP.

***In Vivo* NIR-II Fluorescence Imaging.**

All NIR-II fluorescent images were collected using a NIR-II imaging system with the indium-

gallium-arsenide (InGaAs) camera (Princeton Instruments). The excitation light source was an 808 nm diode laser. The laser power density was 90 mW cm^{-2} with a 1,000 nm long-pass filter during *in vivo* imaging. The mice were anesthesized by intraperitoneal injection of pentobarbital sodium solution (50 mg kg^{-1}) during the NIR-II imaging. For tumor imaging, the Balb/c nude mice bearing subcutaneous 4T1 tumors were given **H3-PEG2K** (0.2 mL, 0.2 mg) via tail vein injection. After injection, the mice were mounted in the prone position beneath the laser for imaging at various time points. For rat mammary carcinoma imaging, **H3-PEG2K** (1 mL, 2 mg) were injected into rats via the tail vein. And animals were mounted in the NIR-II imaging system at different time points. After 8.5 hours intravenous injection, the image-guided tumor resection surgery was operated in the same NIR-II imaging system.

***Ex Vivo* Biodistribution Analysis.**

For 4T1 breast tumor imaging, at 24 h after injection of **H3-PEG2K** into tumor-bearing Balb/c nude mice, mice were sacrificed, then the major organs and tumor tissues were collected for imaging study. For DMBA-induced mammary rat carcinoma imaging, the rats were sacrificed after completion of image-guided tumor resection surgery post tail vein injection of **H3-PEG2K**. *Ex vivo* organs and tumor tissues were imaged in the NIR-II imaging system with the laser power density of 90 mW cm^{-2}, which was used for *in vivo* fluorescent imaging.

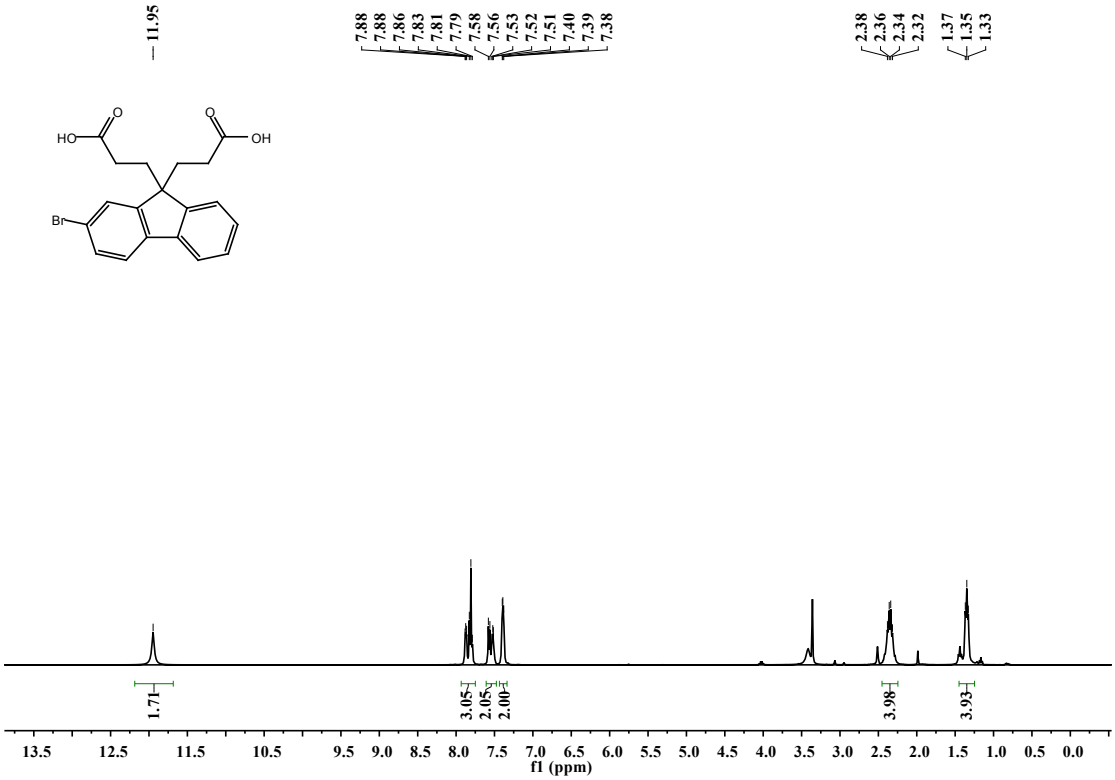


Figure S1. ^{1}H NMR of compound **1**

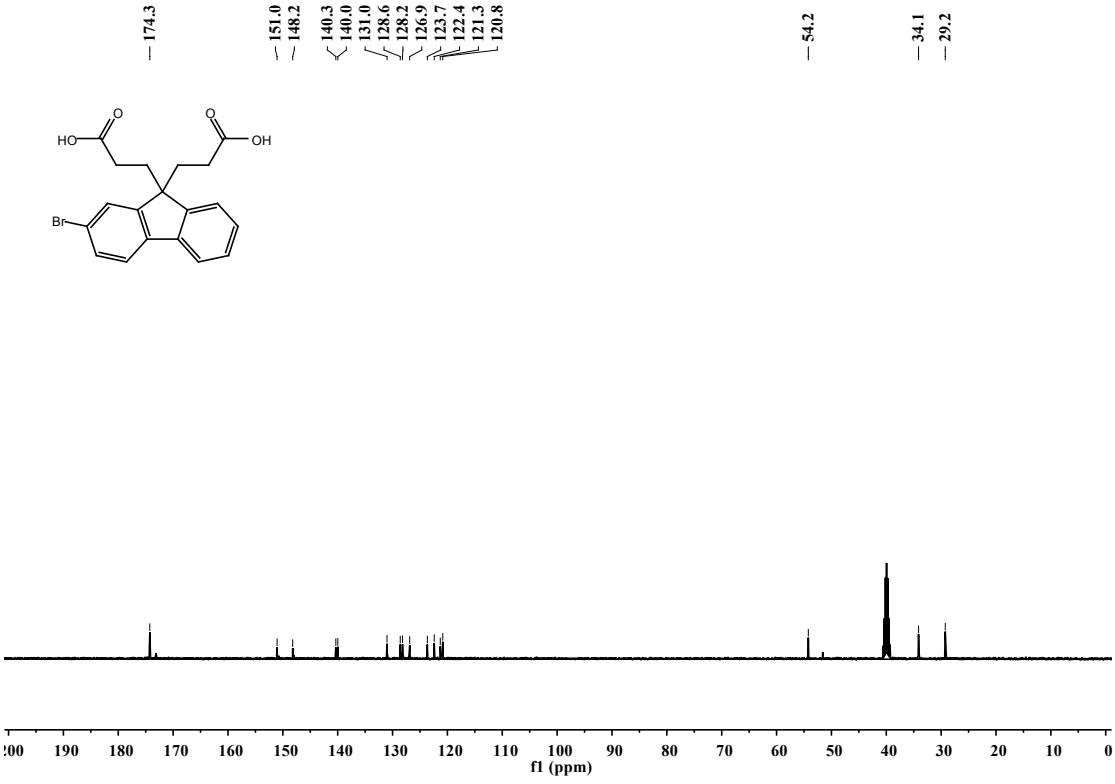


Figure S2. ^{13}C NMR of compound **1**

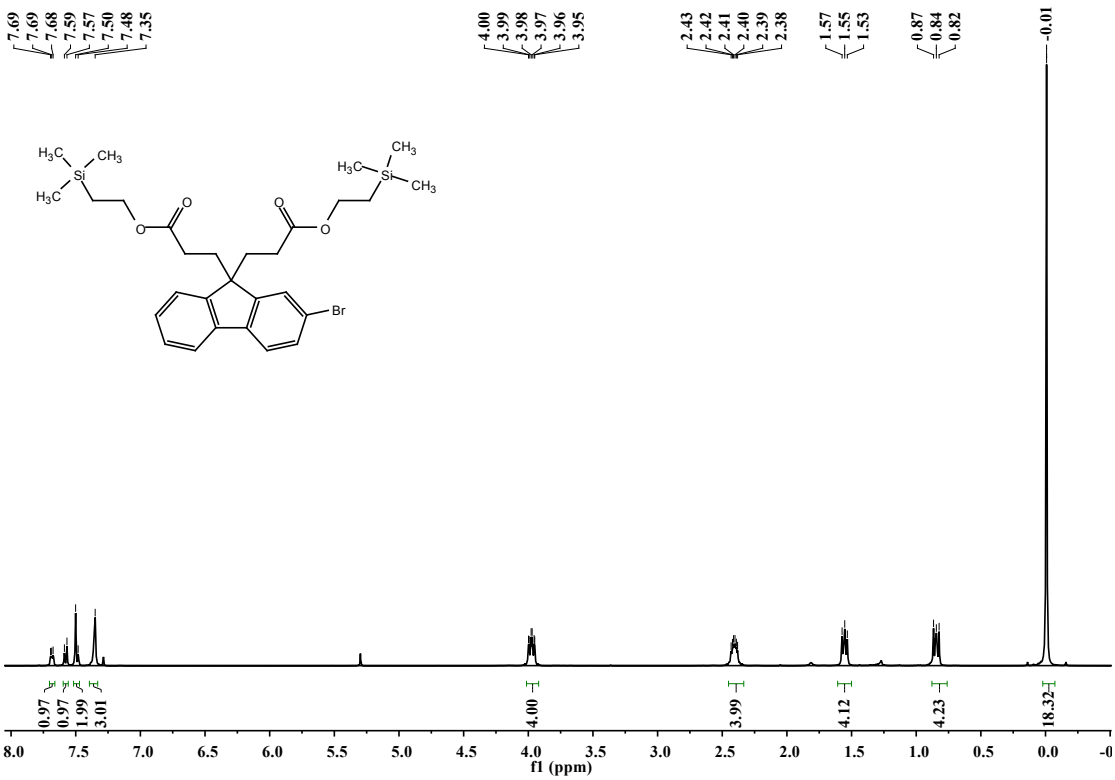


Figure S3. ^{1}H NMR of compound **2**

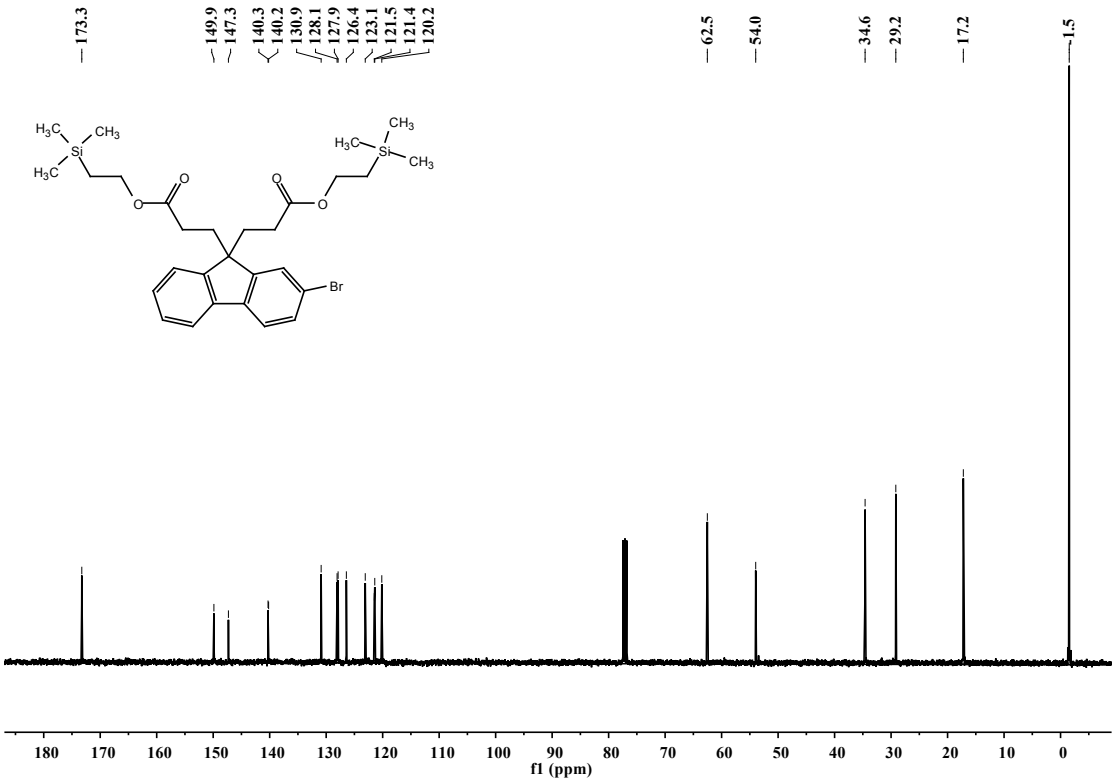


Figure S4. ^{13}C NMR of compound **2**

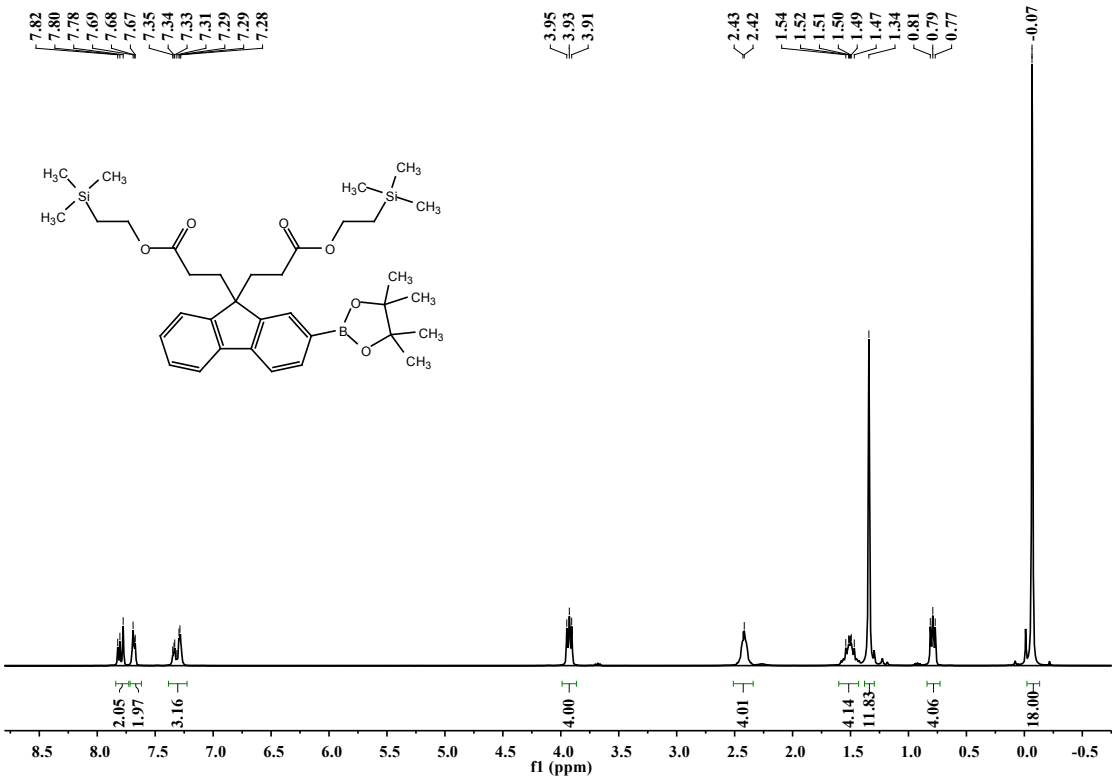


Figure S5. ^{1}H NMR of compound **3**

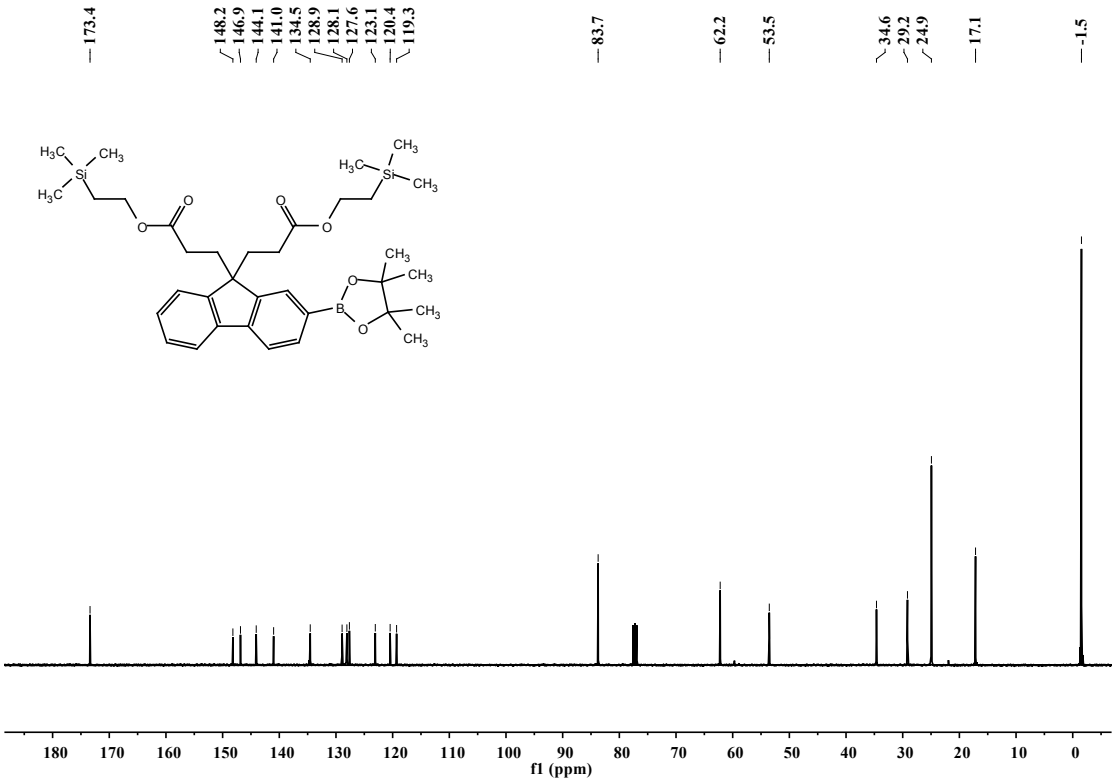


Figure S6. ^{13}C NMR of compound **3**

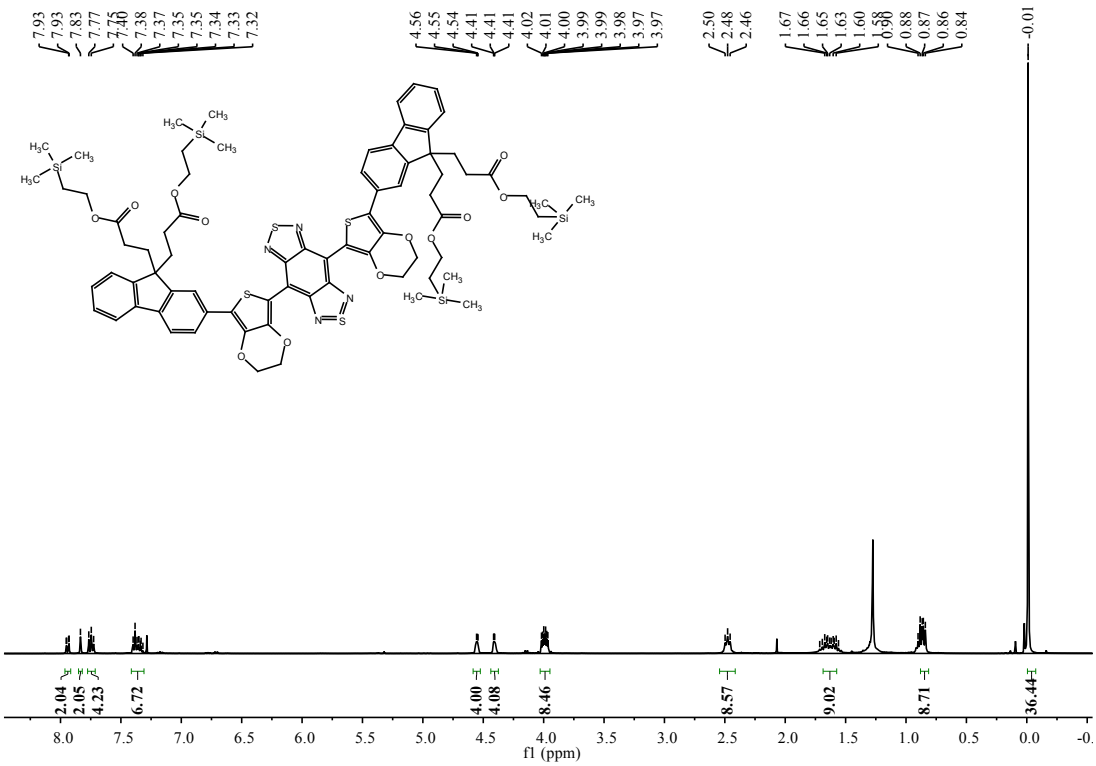


Figure S7. ^{1}H NMR of **H3**

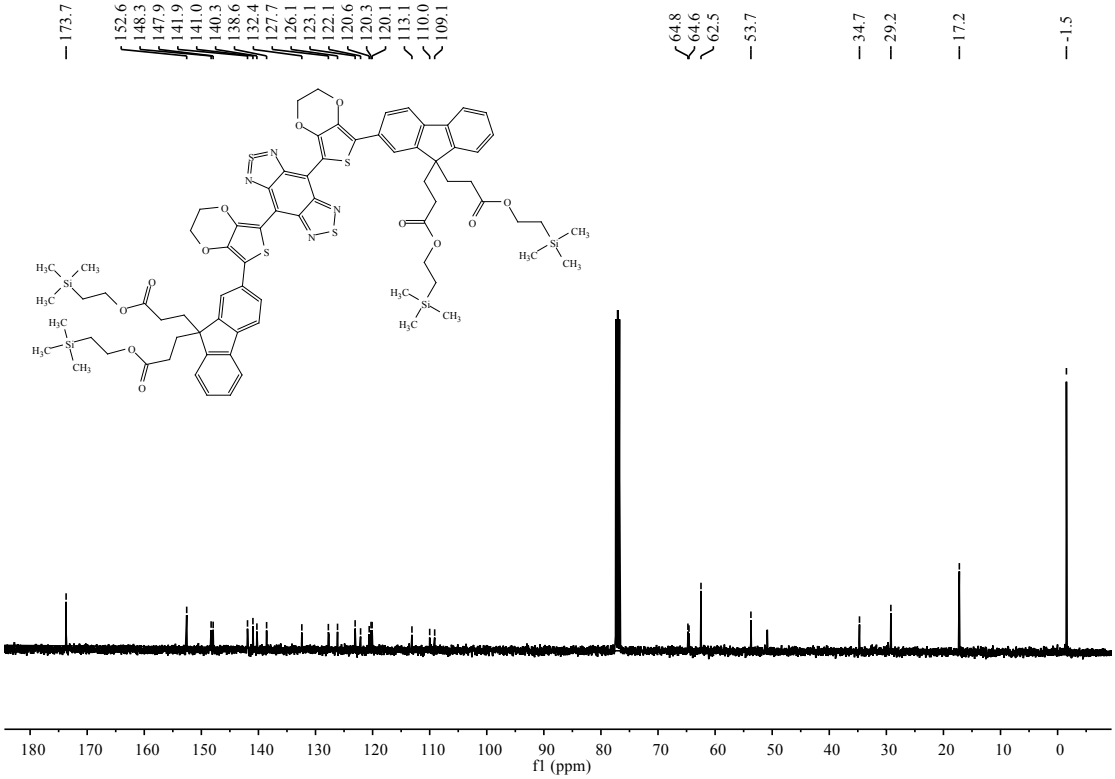


Figure S8. ^{13}C NMR of **H3**

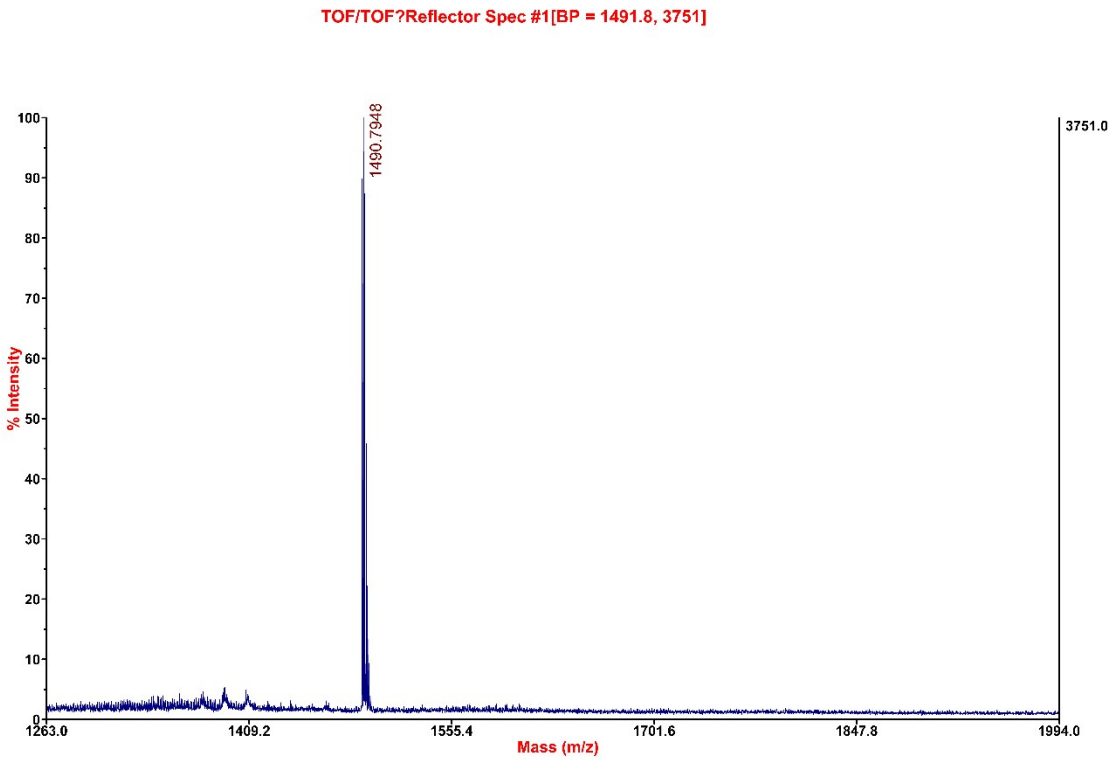


Figure S9. The MALDI-TOF-MS spectrum of **H3**.

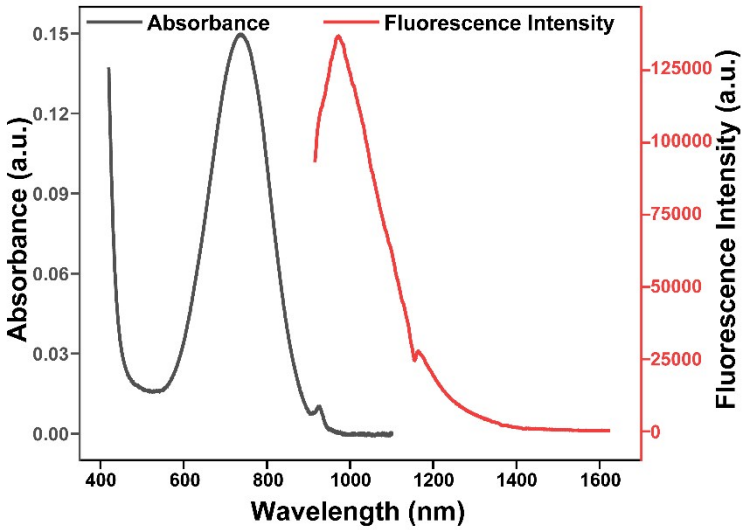


Figure S10. The UV-vis-NIR absorbance spectrum and NIR-II fluorescence spectrum of **H3** in DCM.

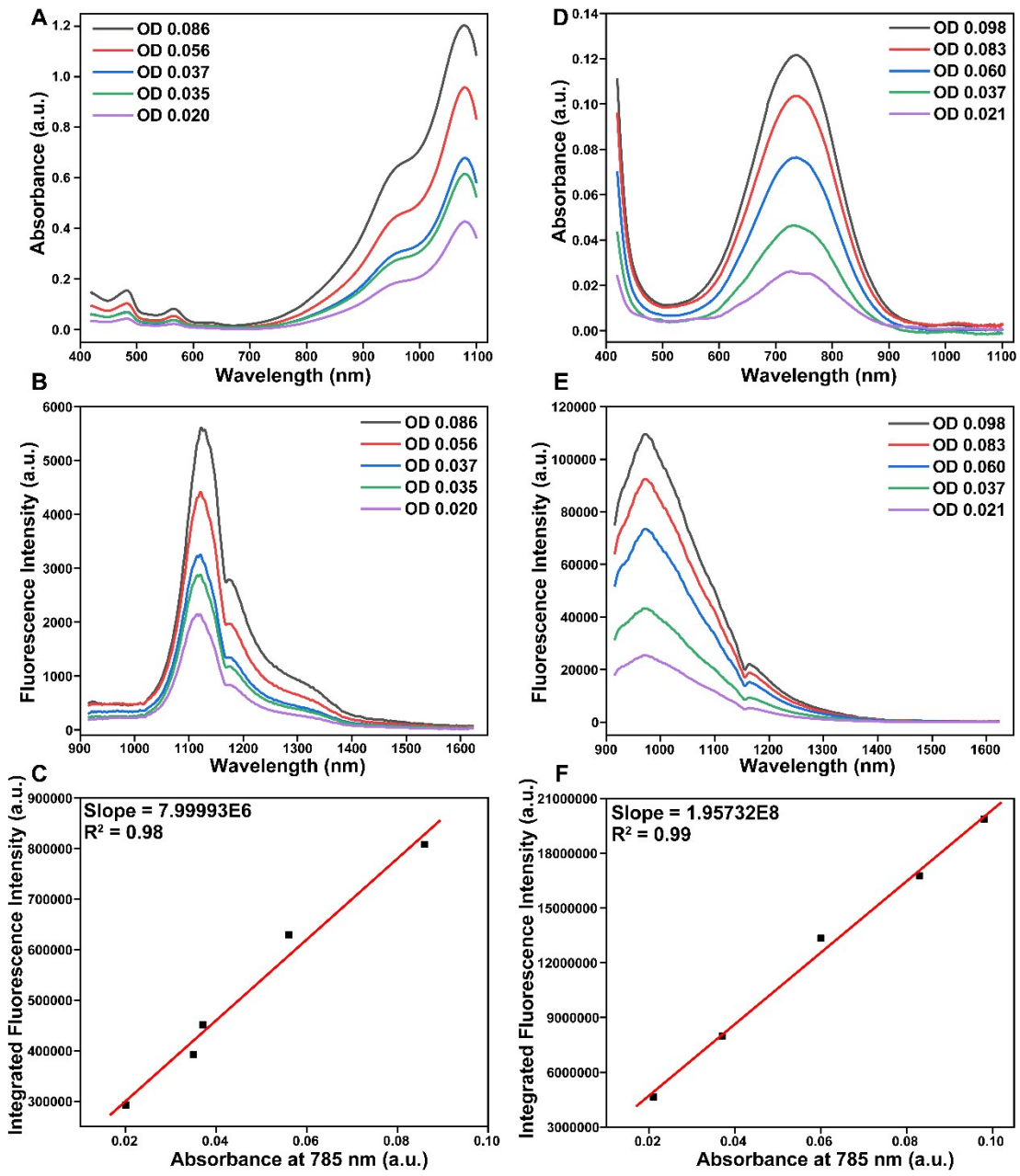


Figure S11. Fluorescence quantum yield measurements of **H3** in DCM. The method was reported in our previous work [1] and IR-26 is the reference with 0.5% quantum yield in DCE. (A-C) The absorbance spectra, fluorescence spectra and slope of IR-26 reference in DCE. (D-F) The absorbance spectra, fluorescence spectra and slope of **H3** in DCM.

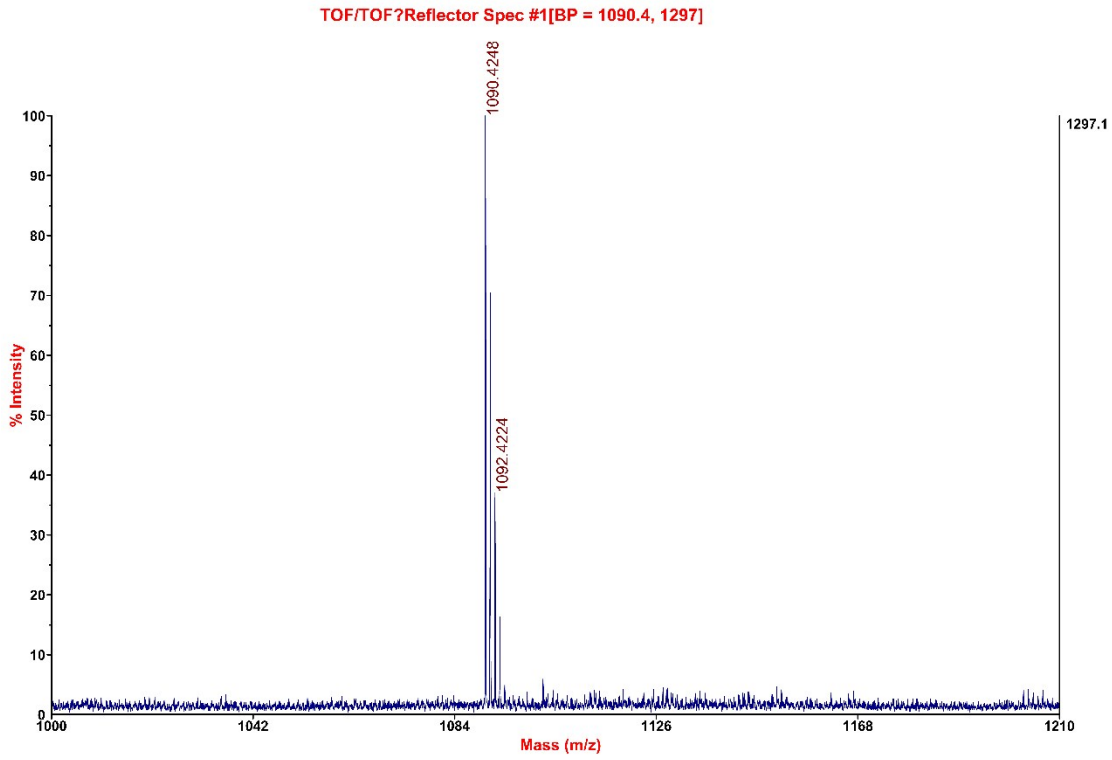


Figure S12. The MALDI-TOF-MS spectrum of **H3** derivative with four carboxylic acid groups from TFA de-protection of **H3**.

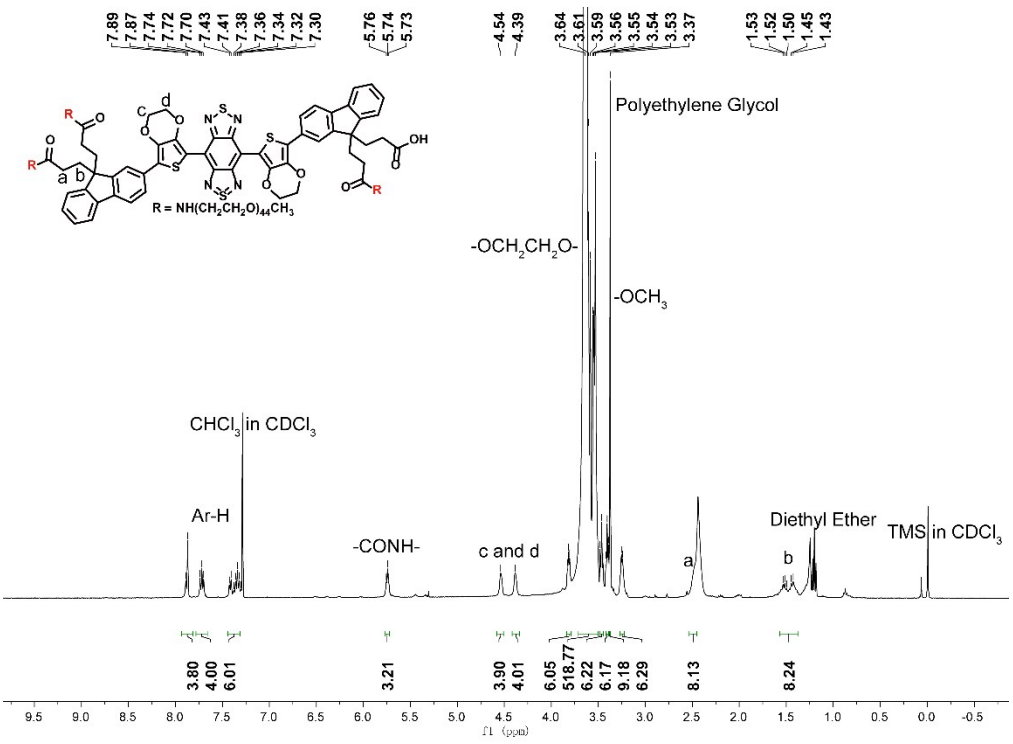


Figure S13. The ^{1}H NMR characterization of **H3-PEG2K**.

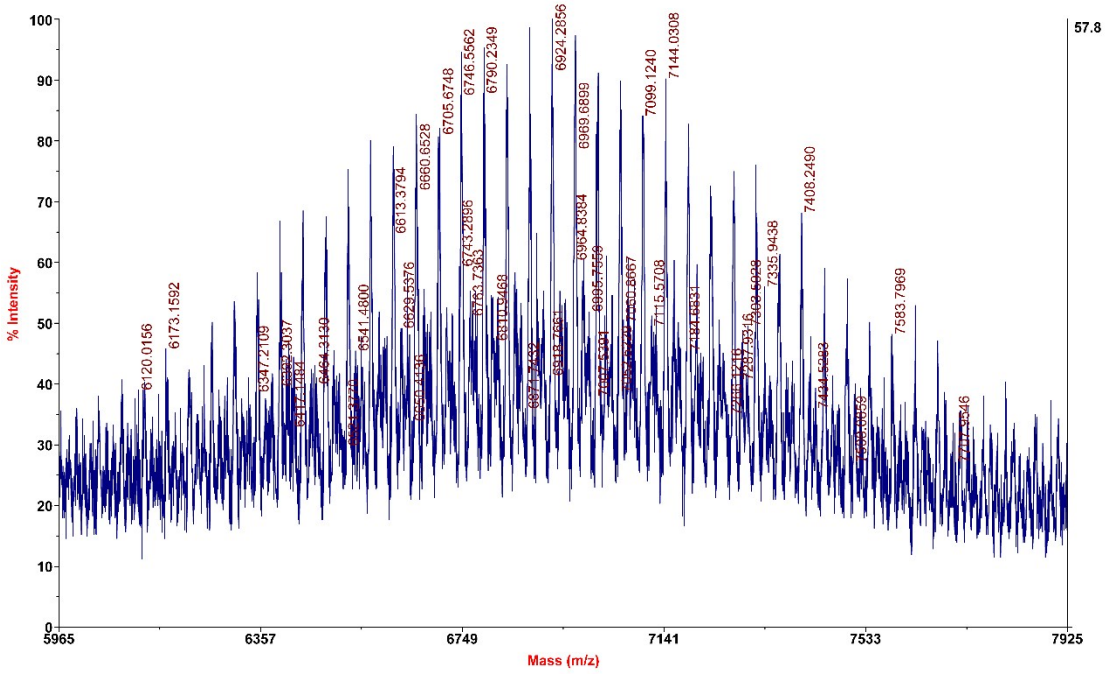


Figure S14. The MALDI-TOF-MS spectrum of **H3-PEG2K.**

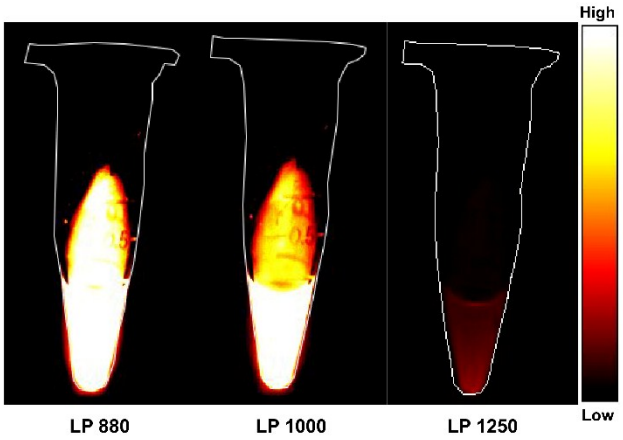


Figure S15. Comparison of NIR-II signals of **H3-PEG2K** PBS solution (95 μM) under various long-pass (LP) filters (880, 1000 and 1250 nm) using 25 ms exposure time and an 808 nm laser excitation.

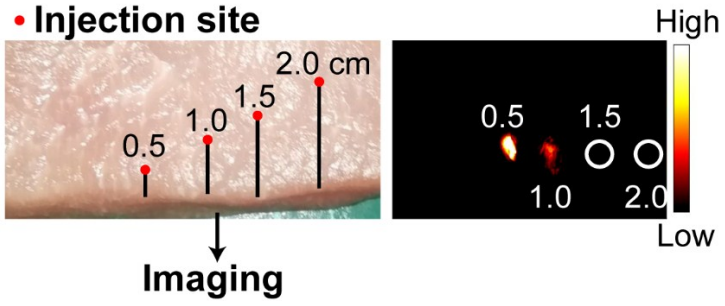


Figure S16. Penetration depth of **H3-PEG2K** in pork muscle. The optical photograph (left) of pork

muscle and NIR-II image (right, 80 ms, 90 mW cm^{-2}) after injection of **H3-PEG2K** (20 μL, 20 μg mL^{-1}) at different depths (0.5, 1.0, 1.5, and 2.0 cm).

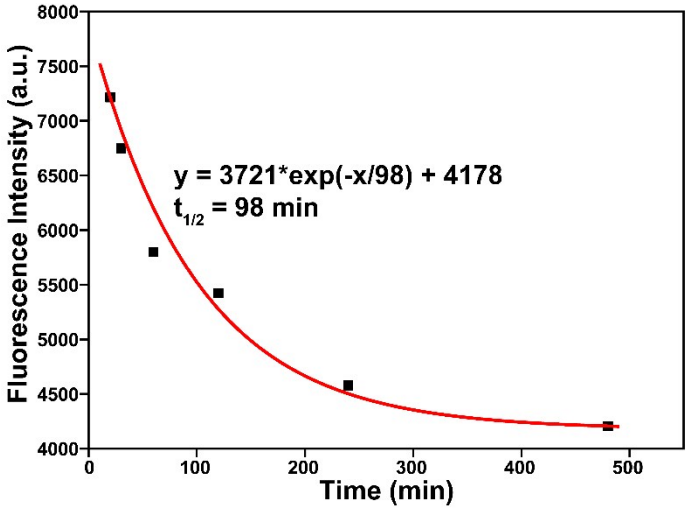


Figure S17. The blood half-life circulation curve of **H3-PEG2K** in female KM mice was determined to be 98 minutes by fitting the data from the mean fluorescence intensity of blood sample at particular time points after tail vein injection of probe using a first-order exponential decay.

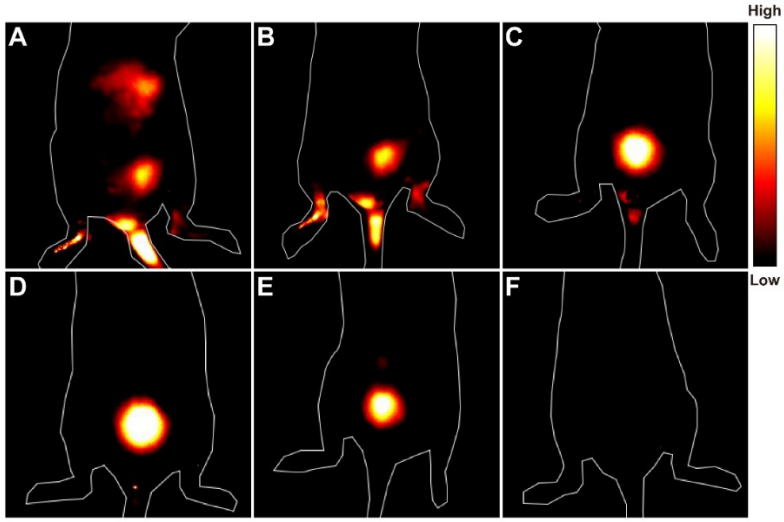


Figure S18. The NIR-II fluorescence images (20 ms exposure time and 1000 nm long-pass filter under an 808 nm laser excitation) of a mouse in the supine position at various time points of 0.5 h (A), 1 h (B), 2 h (C), 4 h (D), 6 h (E), and 24 h (F) after an intravenous injection of **H3-PEG2K** (0.2 mg in 0.2 mL PBS) showed different bladder fluorescent signals at different time points from weak to strong and disappearance in the end.

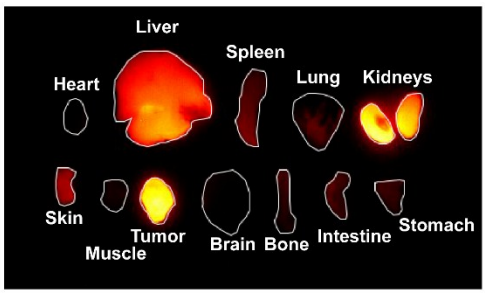


Figure S19. The *ex vivo* bio-distribution by NIR-II fluorescence imaging (1000 nm long-pass filter, 50 ms exposure time) of **H3-PEG2K** (0.2 mg in 0.2 mL PBS) within intravenous injection into 4T1 tumor-bearing nude mice at 24 h under an 808 nm laser excitation (90 mW cm^{-2}).

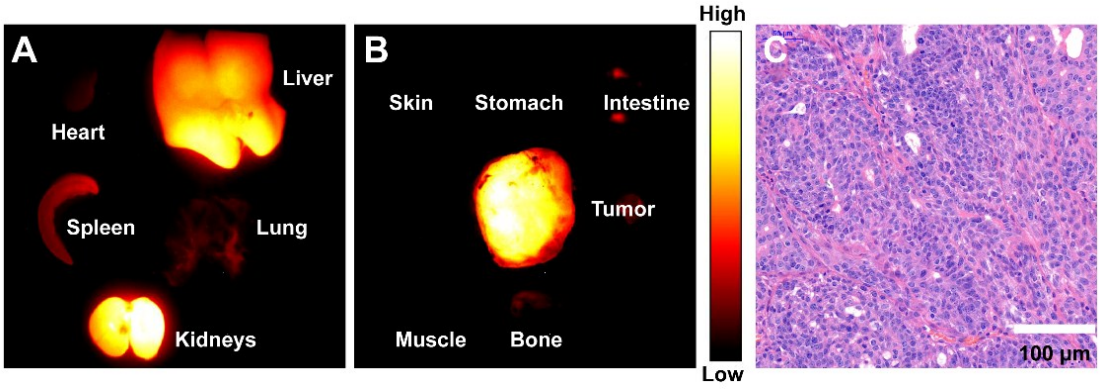


Figure S20. (A-B) The *ex vivo* bio-distribution including rat heart, liver, spleen, lung, kidneys, skin, stomach, intestine, tumor, muscle and bone by NIR-II fluorescence imaging within 100 ms exposure time and 1000 nm long-pass filter under an 808 nm laser irradiation at 8.5 h post intravenous injection of **H3-PEG2K** (2 mg in 1 mL PBS) into DMBA-induced mammary carcinoma rat. (C) Hematoxylin-eosin (H&E) staining analysis of rat mammary carcinoma. The scale bar in Fig. S18 C is 100 μm.

Reference

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