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Electronic Supplementary Information

Rational design of super-contrast NIR-II fluorophore affords high-performance NIR-II molecular imaging guided microsurgery

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Author contributions. S.Z., R.T., Y.L. and X.C. conceived and designed the study. S.Z. and R.T. performed all the in vivo and cellular experiments. H.S. performed the theoretical calculations. H.M., Q.Y. and H.W. contributed to the synthesis of the compounds. R.T., S.Z., S.C., D.O.K., G.N. Y.L. and X.C. wrote the manuscript.

Materials. Unless otherwise noted, all reagents were obtained commercially and used without further purification. Tetrahydrofuran (THF), toluene, and dimethylformamide (DMF) used for reactions were purified by a solvent purification system (Innovative Technology, Inc.) before use. All air and moisture sensitive reactions were carried out in flame-dried glassware under a nitrogen atmosphere. PbS quantum dots with >1500 nm emission were purchased from Nirmidas Biotech. TATE peptide containing Dde protecting group, TATE(Dde), was purchased from CSBio Company, Inc. (Menlo Park, CA). Follicle Stimulating Hormone (FSH) from human pituitary ~7,000 IU/mg (vs. W.H.O. reference preparation 83/575) was purchased from Sigma-Aldrich (F4021-1MG). DBCO-PEG_4-NHS was purchased from Click chemistry tools Bioconjugate technology company.

NIR-II emission spectra and Quantum yield test. The fluorescence quantum yields of the fluorophores were measured in a way similar to that which was reported previously.[1] Generally, a home built NIR-II set-up was used to measure the fluorescence spectra in the region of 900-2200 nm using an InGaAs array detector (Princeton OMA-V, 1 dimension) and a spectrometer (Acton SP2300i) under an 808 nm diode laser excitation (RMPC lasers, 160 mW). The filter sets included one 850 nm short-pass (SP) filter (Thorlabs), one 1000 nm SP filter (Thorlabs), one 1100 nm SP filter (Omega), and one 1300 nm SP filter (Omega) were used as the excitation filters, and one 900 nm long-pass filter (Thorlabs) was used as an emission filter. The obtained emission spectra were further corrected by the detector sensitivity profile and the absorbance features of the filter. The fluorescence quantum yields were determined against the reference fluorophore HIPCO SWCNT with a known quantum yield of 0.40% in aqueous solutions. All samples were measured at 25°C, and the reference fluorophore HIPCO SWCNT was used with an optical density (*OD*) of 0.08 at 808 nm. The NIR-II fluorescence emission intensities were measured under the same 808 nm excitation. The intensity read out from the InGaAs camera was a spectrally integrated total emission intensity in the 900-1500 nm region.

Animals.

All animal studies were conducted in accordance with the principles and procedures outlined in the National Institutes of Health (NIH) Guide for the Care and Use of Animals, and under protocols approved by the NIH Clinical Center Animal Care and Use Committee (CC-ACUC, protocol number: NIBIB 16-03). Some of animal experiments are performed under the Stanford University's Administrative Panel on Laboratory Animal Care (APLAC). All animal experiments Nude mice were purchased from Jackson's Laboratory (Bar Harbor, ME). Bedding, nesting material, food, and water were provided ad libitum. Ambient temperature was controlled at 20 to 22°C with 12-hour light/12-hour dark cycles.

U87 and AR42J tumor imaging.

Tumors were inoculated with $3*10^6$ U87 or AR42J cells subcutaneously, and a NIR-II image was taken when the tumor size reached ~1*1 cm^2. The deep brain tumor was inoculated by craniotomy operation with a small volume of U87 cells.

NIR-II imaging.

Mice were shaved using Nair hair removal cream (Nair™ Lotion with Aloe & Lanolin) and

anesthetized using isoflurane before placing them on a stage with a venous catheter for injection of imaging agents. For each imaging experiment, at least 3 mice were used per group. All NIR-II images were collected on a two-dimensional InGaAs array (Princeton Instruments). The excitation laser was an 808 nm laser diode at a power density of ~0.15 W/cm^2. Emission was typically collected with different long pass filters. A lens set was used for obtaining tunable magnifications, ranging from 1x (whole body) to 2.5x (high magnification) by changing the relative position of two NIR achromats (200 mm and 75 mm, Thorlabs). A variable exposure time was used for the InGaAs camera to capture images in the NIR-II window.

PET imaging.

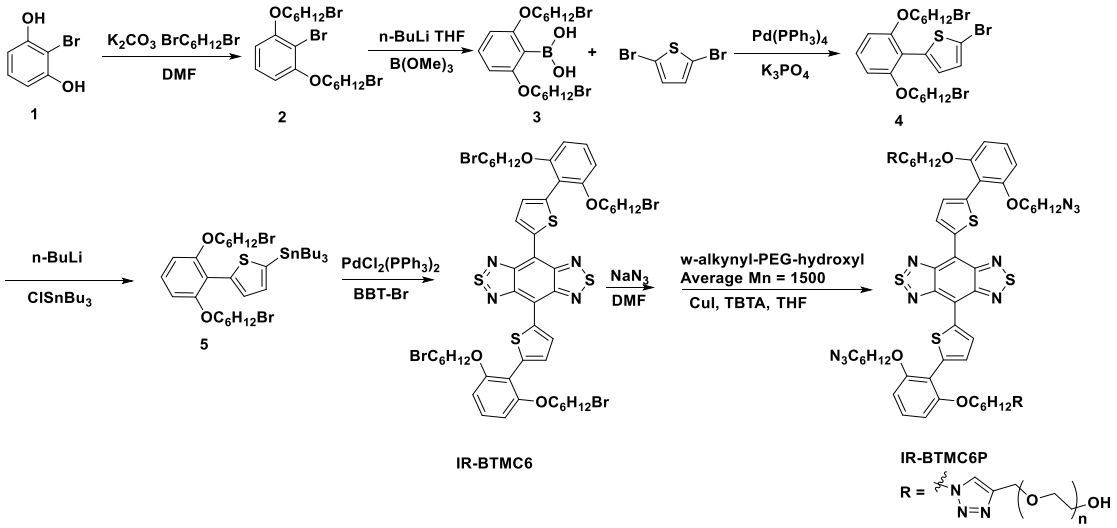
The PET imaging for AR42J tumor was followed by our previous report.[2] PET imaging was performed at ~3 weeks post tumor cells inoculation when the tumor volume reached about 200-350 mm^3. PET studies were acquired on Inveon (Siemens) scanners. Images were reconstructed using a 3D ordered subset expectation maximum algorithm, and ROI were drawn using IRW (Siemens) and VivoQuant (INVICRO).

Density functional theory calculations.

All the calculations were performed using the Gaussian 09 software.[3] The ground-state (S0) geometries of the simplified structures IR-BTM, IR-BEM, and IR-BGM were first optimized using the B3LYP/6-31G(d) method and re-optimized at the tuned-ωB97XD*/6-31G(d) level. The corresponding range-separation parameter (ω, in Bohr^{-1}) for each molecule was optimally tuned according to the GAP-tuning method and listed in Table S2. The excited-state (S_1) geometries of these molecules were optimized using a time dependent (TD)-ωB97XD*/6-31G(d) method. The HOMOs, LUMOs, and absorption excitation energies of these molecules were obtained at the ωB97XD*/6-31G(d) level based on their optimized S_0 geometries. The emission energies of these molecules were calculated at the TD-ωB97XD*/6-31G(d) level based on their optimized S_1 geometries.

Measurements. ^{1}H and ^{13}C NMR spectra were performed on 500 MHz and 400 MHz NMR spectrometers (Bruker AVANCE) using $CDCl_3$. Mass spectra were in general recorded on QSTAR Elite (ABI). Ultraviolet-visible-near infrared (UV-VIS-NIR) absorption spectra were recorded on Shimadzu UV-3600Plus. Size exclusion chromatography (SEC) was performed on Malvern Viscotek 270 max with 10 μm PLgel 600 × 7.5 mm column. THF was used as the mobile phase at a flow rate of 1.0 mL/min at 40 ˚C. A UV-Vis-NIR spectrophotometer (Cary 6000i) with background correction was employed to measure the optical absorption spectrum. Fluorescence spectrophotometry was carried out on a Hitachi F-7000 fluorescence spectrophotometer.

Synthesis of S-D-A-D-S dyes with donor engineering



Synthesis of compound 2. A mixture of 2-bromobenzene-1,3-diol (10 g, 52.9 mmol), 1,6-dibromohexane (21.5 mL, 132.3 mmol), and K_2CO_3 (21.9 g, 158.7 mmol) in dry DMF (80 mL) under N_2 was stirred at room temperature (RT). After 20 h, the mixture was diluted with H_2O, and the aqueous solution was extracted with Et_2O. The organic layer dried over $MgSO_4$, and concentrated. The residue was purified by silica gel column chromatography (hexane/DCM=5:1) to yield compound **2** as a white solid (20.3 g, 74%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.19 (t, J = 8.3 Hz, 1H), 6.55 (d, J = 8.3 Hz, 2H), 4.04 (t, J = 6.3 Hz, 4H), 3.45 (t, J = 6.4 Hz, 4H), 1.89 (m, 8H), 1.56 (m, 8H); ^{13}C NMR (100 MHz, CDCl3): δ 156.7, 128.1, 105.7, 102.1, 68.9, 33.9, 32.7, 28.9, 27.9, 25.3; HRMS (ESI) calcd for $C_{18}H_{28}Br_3O_2^+$ ([M+H$^+$]), 512.9639 Found 512.9637.

Synthesis of compound 3. At -78 ℃, n-BuLi (7 mL, 2.4 M) was added to the solution of compound **1** in dry THF (80 mL) under N_2. After stirring for 2 h at -78 ℃, $B(OMe)_3$ was added and the mixture was stirred overnight at RT. To the solution, water (100 mL) was added and was extracted by ethyl acetate. The organic phase was dried by $NaSO_4$, filtered, and removed by vacuum to yield compound **3** as a white solid. Compound **3** can be used without further purification.

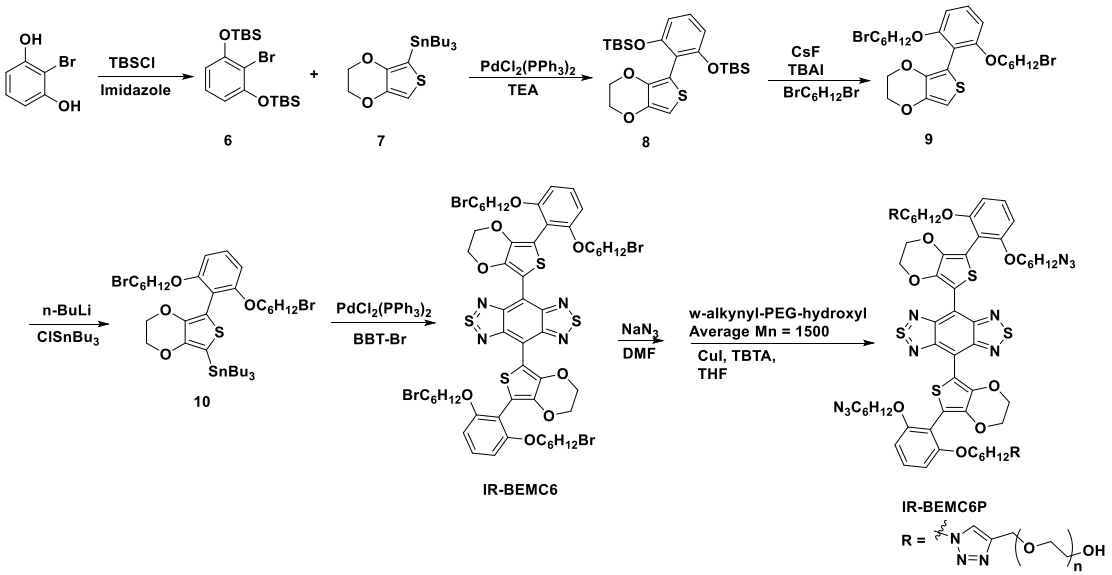
Synthesis of compound 4. A mixture of compound 3 (4 g, 8.2 mmol), 2,5-dibromothiophene (2.34g, 9.7 mmol), $Pd(PPh_3)_4$ (141 mg, 0.122mmol), and K_3PO_3 (3.46 g, 16.3 mmol) in toluene (30 mL) and water (10 mL) under N_2 was stirred at 110℃ overnight. After cooling to RT, the mixture was then poured into water and extracted twice with ethyl acetate. The combined organic phase was dried with $MgSO_4$ and evaporated *in vacuo*. The crude material was purified by silica gel column chromatography (PE/DCM=4:1) to yield compound **4** as a white solid (3.6 g, 73 %). ^{1}H NMR (400 MHz, CDCl3) δ 7.35 (d, *J* = 4.0 Hz, 1H), 7.19 (t, *J* = 8.3 Hz, 1H), 7.03 (d, *J* = 4.0 Hz, 1H), 6.61 (d, *J* = 8.4 Hz, 2H), 4.02 (t, *J* = 6.3 Hz, 4H), 3.42 (t, *J* = 6.8 Hz, 4H), 1.95 – 1.73 (m, 8H), 1.51 (ddd, *J* = 10.9, 4.7, 2.3 Hz, 8H); ^{13}C NMR (126 MHz, Chloroform-*d*) δ 158.33 , 140.82 , 138.58 , 129.72 , 110.08 , 109.12 , 105.36 , 98.74 , 68.57 , 33.90 , 32.70 , 28.96 , 27.77 , 25.14 .ESI-MS: [M+H$^+$] 595.8.

Synthesis of compound 5. At -78 ℃, n-BuLi (1 mL, 2.4 M) was added to a solution of compound

4 (1.2 g 2 mmol) in dry THF (40 mL) under N_2. After stirring for 1.5 h at -78 ℃, n-Bu_3SnCl (0.976 g, 3 mmol) was added and the mixture was stirred overnight at RT. To the solution, water (100 mL) was added and extracted by ethyl acetate. The organic phase was dried by $NaSO_4$, filtered, and removed by vacuum to yield compound **5** as a brown liquid. Compound **5** can be used without further purification.

Synthesis of IR-BTMC6. To a solution of crude compound **4** (1 g, about 1 mmol) and BBT-Br (88 mg, 0.25mmol) in toluene (15 mL) under protection gas atmosphere, $Pd(PPh_3)_2Cl_2$ (100 mg) was added. The mixture was stirred at 110 ºC for 12 h. After cooling to room temperature, the mixture was poured into water and extracted twice with ethyl acetate. The organic phase was dried with $MgSO_4$ and evaporated *in vacuum.* The crude material was purified by silica gel column chromatography (PE/DCM=5:2) to yield compound **IR-BTMC6** as a green solid (120 mg, 39 %). 1H NMR (400 MHz, CDCl3) δ 9.05 (d, *J* = 3.9 Hz, 1H), 7.79 (d, *J* = 3.9 Hz, 1H), 7.22 (d, *J* = 8.2 Hz, 1H),6.67 (d, *J* = 8.3 Hz, 2H), 4.08 (t, *J* = 6.0 Hz, 4H), 3.31 (t, *J* = 6.8 Hz, 4H), 2.02 – 1.71 (m, 8H), 1.57 – 1.41 (m, 8H); ^{13}C NMR (126 MHz, Chloroform-*d*) δ 158.26 , 152.51 , 140.41 , 138.91 , 129.96 , 113.95 , 113.34 , 110.71 , 109.70 , 105.21 , 64.46 , 33.92 , 32.70 , 28.98 , 27.87 , 25.23 . HRMS (ESI) calcd for $C_{50}H_{59}O_4N_4Br_2{}^{81}Br_2S_4$, ([M+$H^+$]) 1227.0098, Found 1227.0106.

Synthesis of IR-BTMC6P. Compound **IR-BTMC6** (80 mg, 0.052 mmol) and sodium azide (50 mg, 0.75 mmol) were dissolved in DMF (10 mL), and the mixture was stirred for 3 h at RT. Then, a large amount of water was added until all solids were dissolved. The reaction was extracted twice with ethyl acetate, and the combined organic phase was dried with $MgSO_4$ and evaporated *in vacuum*. The crude product was subjected to flash column chromatography on silica gel to yield a dark green solid (70 mg, 0.05mmol). The dark green solid was dissolved in THF (5 mL) and copper (I) iodide (CuI) (50 mg), w-alkynyl-PEG-hydroxyl PEG_{1500} (M_n = 1500) (150 mg, about 0.1 mol), and tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA) (3 mg) were added. The system was stirred at RT for 0.5 h, and then filtered with diatomite, and the solution was evaporated *in vacuum*. The crude product was purified by thin layer chromatography twice. First, ethyl acetate (EA) was used as an eluent and a small amount of impurity moved to the top of the TLC plate, but other parts of the product remained at the start point of the TLC plate. Then DCM/MeOH (10:1-5:1) was used as an eluent successively, and the PEGylation product could be separated from alkyne-PEG. **IR-BTMC6P** (190 mg, isolated yield 86%) was yielded as a light yellow solid.



Synthesis of compound 6. To a solution of compound **1** (1.89 g, 10 mmol) and imidazole (2.13 g, 30 mmol) in dry DMF (30 mL) at 0 ℃, TBSCl (4.52g, 30 mmol) was added. The mixture was stirred at room temperature for 1 h, and then the mixture was poured into water and extracted twice with petroleum ether. The organic phase was dried with $MgSO_4$ and evaporated *in vacuo.* The crude material was purified by silica gel column chromatography (PE/DCM=10:1) to yield compound **6** as a colorless liquid (3.88 g, 93 %). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.00 (t, *J* = 8.2 Hz, 1H), 6.52 (d, *J* = 8.2 Hz, 2H), 1.05 (s, 18H), 0.24 (s, 12H). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 154.26 , 127.40 , 113.19 , 109.46 , 25.93 , 18.54 , -4.07 . ESI-MS: [M+H$^+$] 417.3.

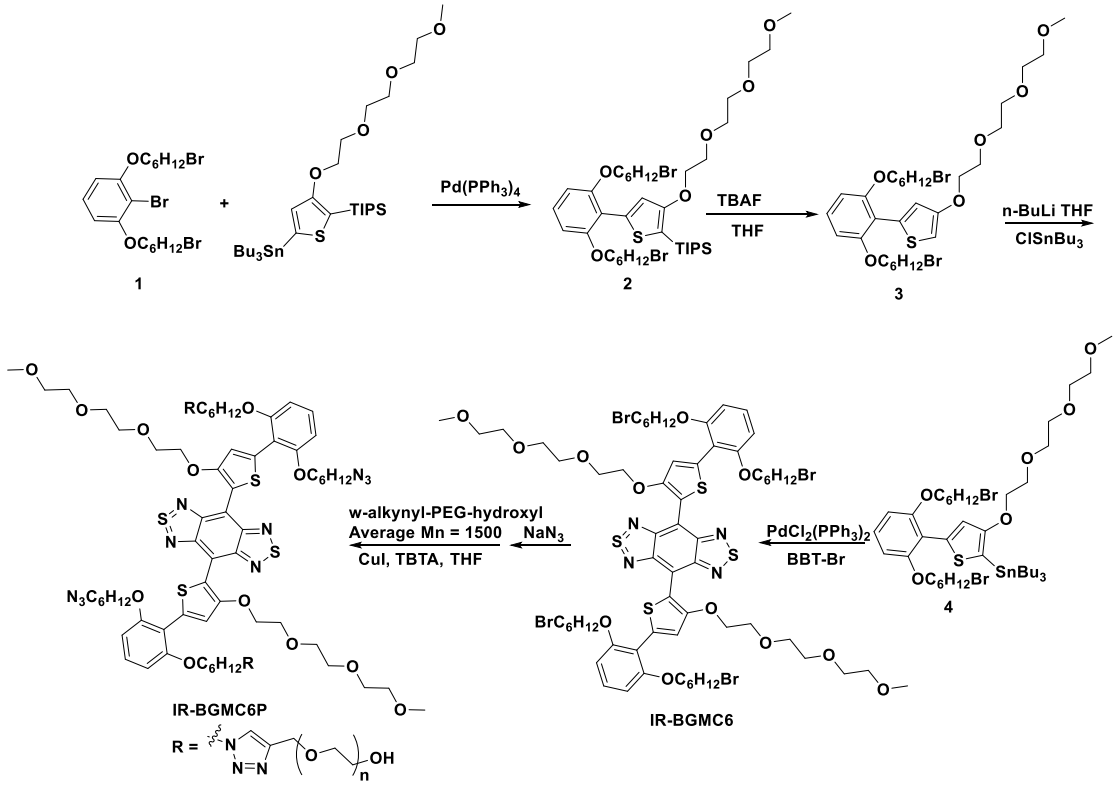
Synthesis of compound 8. To a solution of compound **6** (2 g, 4.8 mmol), TEA (812 mg, 8.02 mmol) and compound **7** (2.5 g, 5.8 mmol) in toluene (25 mL) under protection gas atmosphere, $Pd(PPh_3)_4$ (355 mg, 0.5 mmol) was added. The mixture was stirred at 120°C for 24 h. After cooling to room temperature, the solvent was evaporated *in vacuo* and the crude product was subjected to column chromatography on silica gel (PE/EA=20:1) to yield **8** as a white solid (621 mg, 27%). 1H NMR (400 MHz, CDCl3) δ 7.07 (t, *J* = 8.2 Hz, 1H), 6.54 (d, *J* = 8.2 Hz, 2H), 6.37 (s, 1H), 4.16 (s, 4 H), 0.83 (s, 18 H), 0.04 (s, 12H). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 155.81 , 141.29 , 138.79 , 129.36 , 116.13 , 113.24 , 110.40 , 98.84 , 64.73 (d, *J* = 3.6 Hz), 25.58 , 18.12 , -4.50 . ESI-MS: [M+H$^+$] 479.1.

Synthesis of compound 9. A mixture of compound 8 (530 mg, 1.11 mmol), TBAI (41 mg, 0.11 mmol), 1,6-dibromohexane (731 mg, 11.1 mmol), and CsF (422 mg, 2.8 mmol) in dry DMF (5 mL) under N_2 was stirred at room temperature (RT). After 9 h, the mixture was diluted with H_2O, and the aqueous solution was extracted with Et_2O. The organic phase was dried with $MgSO_4$ and evaporated *in vacuo.* The crude material was purified by silica gel column chromatography (PE/EA=10:1) to yield compound **9** as a white solid (620 mg, 97 %). 1H NMR (400 MHz, CDCl3) δ 7.21 (t, *J* = 8.3 Hz, 1H), 6.56 (d, *J* = 8.4 Hz, 2H), 6.40 (s, 1H), 4.18 (qd, *J* = 4.3, 2.4 Hz, 4H), 3.93 (t, *J* = 6.3 Hz, 4H), 3.37 (t, *J* = 6.8 Hz, 4H), 1.87 – 1.78 (m, 4H), 1.74 – 1.65 (m, 4H), 1.45 – 1.35 (m, 8H). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 158.48 , 140.95 , 138.71 , 129.84 , 110.23 , 109.26 , 105.51 , 98.88 , 68.71 , 64.73 , 34.00 , 32.83 , 29.08 , 27.89 , 25.26 . ESI-MS: [M+H$^+$] 611.1.

Synthesis of compound 10. The procedure of compound **10** synthesis was similar to compound **5**'s. Compound 10 was yielded as a brown oil for use without further purification.

Synthesis of IR-BEMC6. The procedure of compound **IR-BEMC6**'s synthesis was similar to compound **IR-BTMC6**'s. Compound **IR-BEMC6** was yielded as a dark green solid, and the yield was 34%. 1H NMR (500 MHz, CDCl3) δ 7.29 – 7.23 (m, 1H), 6.62 (d, *J* = 8.4 Hz, 2H), 4.32 (dd, *J* = 18.6, 4.7 Hz,4H), 4.02 (t, *J* = 6.2 Hz, 4H), 3.30 (t, *J* = 6.9 Hz, 4H), 1.80 (dq, *J* = 12.9, 6.6 Hz, 8H), 1.47 (ddd, *J* = 21.5, 11.6, 5.7 Hz, 8H). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 158.41 , 152.66 , 140.56 , 139.06 , 130.11 , 114.10 , 113.49 , 110.86 , 109.85 , 105.36 , 68.78 , 64.76 , 64.61 , 34.07 , 32.85 , 29.13 , 28.02 , 25.38 . HRMS (ESI) calcd for $C_{54}H_{63}O_8N_4Br_2{}^{81}Br_2S_4$, ([M+H$^+$]) 1343.0223, Found 1343.0216.

Synthesis of IR-BEMC6P. The procedure for compound **IR-BEMC6P**'s synthesis was similar to compound **IR-BTMC6P**'s. Compound **IR-BEMC6P** was yielded as a green solid, and the yield was 92%.



Synthesis of compound 2. To a solution of compound **1** (6.46 g, 15 mmol) and tributyl(thiophen-2-yl)stannane (7.72 g, 11.2 mmol) in toluene (25 mL) under protection gas atmosphere, $Pd(PPh_3)_4$ (130 mg) was added. The mixture was stirred at 110 °C for 24 h. After cooling to room temperature, the mixture was poured into water and extracted twice with ethyl acetate. The organic phase was dried with $MgSO_4$ and evaporated *in vacuo.* The crude product was subjected to column chromatography on silica gel (PE/EA=5:1) to yield **2** as a white solid (7.59 g, 81%). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.50 (s, 1H), 7.15 (t, *J* = 8.3 Hz, 1H), 6.60 (d,

J = 8.4 Hz, 2H), 4.13 (t, *J* = 5.2 Hz, 2H), 4.01 (t, *J* = 6.2 Hz, 4H), 3.80 (t, *J* = 5.2 Hz, 2H), 3.74 - 3.70 (m, 2H), 3.69 - 3.64 (m, 4H), 3.56 (dd, *J* = 5.7, 3.6 Hz, 2H), 3.45 - 3.32 (m, 7H), 1.91 - 1.78 (m, 8H), 1.54 - 1.38 (m, 11H), 1.12 (d, *J* = 7.4 Hz, 18H). ^{13}C NMR (101 MHz, Chloroform-d) δ 162.11 , 157.02 , 138.40 , 128.26 , 118.30 , 113.30 , 108.67 , 105.54 , 72.08 , 70.85 , 70.83 , 70.73 , 70.28 , 69.83 , 69.00 , 59.18 , 33.92 , 32.85 , 29.31 , 28.08 , 25.72 , 19.03 , 12.28 . HRMS (ESI) calcd for $C_{38}H_{65}Br^{81}BrO_6SSi^+$ ([M+H$^+$]) 837.2612, Found 837.2591.

Synthesis of compound 3. To a solution of compound **2** (3.0 g, 3.8 mmol) in 20 mL THF at -78 ^{o}C under protection gas atmosphere, Tetrabutylammonium fluoride (1.0 M in THF, 15.2 mL, 15.2 mmol) was added. After stirring at this temperature for another 1.0 h, the mixture was slowly warmed to room temperature and stirred for another 3 h. The mixture was then poured into water and extracted twice with ethyl acetate, and the combined organic phase was dried with $MgSO_4$ and evaporated *in vacuo*. The crude material was purified by silica gel column chromatography (PE/EA=5:1) to yield compound **3** as a white solid (1.89 g, 73 %). ^{1}H NMR (400 MHz, Chloroform-d) δ 7.22 (dd, J = 11.3, 1.7 Hz, 1H), 7.15 (t, J = 8.3 Hz, 1H), 6.58 (d, J = 8.4 Hz, 2H), 6.29 (d, J = 1.7 Hz, 1H), 4.24 - 4.08 (m, 2H), 3.97 (t, J = 6.3 Hz, 5H), 3.89 - 3.81 (m, 2H), 3.74 (dd, J = 5.9, 3.4 Hz, 2H), 3.71 - 3.62 (m, 5H), 3.55 (dd, J = 5.7, 3.6 Hz, 2H), 3.41 - 3.37 (m, 4H), 1.82 (ddd, J = 17.1, 10.0, 4.2 Hz, 9H), 1.61 - 1.29 (m, 10H); ^{13}C NMR (101 MHz, Chloroform-d) δ 157.35 , 156.11 , 133.25 , 128.64 , 121.22 , 113.07 , 105.46 , 97.85 , 72.08 , 70.91 , 70.81 , 70.72 , 69.94 , 69.29 , 68.97 , 59.18 , 33.99 , 32.80 , 29.07 , 27.94 , 25.46. HRMS (ESI) calcd for $C_{29}H_{45}Br^{81}BrO_6S^+$ ([M+H$^+$]) 681.1278, Found 681.1273.

Synthesis of compound 4. To a solution of compound **3** (860 mg, 1.26 mmol) in THF (25 mL) at -78 ^{o}C under protection gas atmosphere, *n*-BuLi (1.6 M in hexane, 0.95 mL, 1.5 mmol) was added dropwise. After stirring at this temperature for another 1.5 h, tributyltin chloride (0.62 g, 1.9 mmol) was added to the solution. Then the reaction was slowly warmed to room temperature and stirred overnight. After that, the mixture was poured into water and extracted twice with ethyl acetate, and the combined organic phase was dried with $MgSO_4$ and evaporated *in vacuo* without further purification.

Synthesis of IR-BGMC6. To a solution of the crude compound **4** (1.5 g, about 1.5 mmol) and BBT-Br (143 mg, 0.4 mmol) in toluene (15 mL) under protection gas atmosphere, $Pd(PPh_3)_2Cl_2$ (140 mg) was added. The mixture was stirred at 110 ^{o}C for 12 h. After cooling to room temperature, the mixture was poured into water and extracted twice with ethyl acetate. The organic phase was dried with $MgSO_4$ and evaporated *in vacuo.* The crude material was purified by silica gel column chromatography (PE/EA=2:1) to yield compound **IR-BGMC6** as a dark green solid (240 mg, 39 %). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.79 (s, 1H), 7.20 (t, *J* = 8.3 Hz, 1H), 6.65 (d, *J* = 8.4 Hz, 2H), 4.35 (t, *J* = 5.0 Hz, 2H), 4.09 (t, *J* = 6.2 Hz, 4H), 3.69 (t, *J* = 5.0 Hz, 2H), 3.60 – 3.42 (m, 8H), 3.42 – 3.18 (m, 7H), 1.97 – 1.87 (m, 4H), 1.84 (dt, *J* = 13.9, 7.0 Hz, 4H), 1.59 – 1.46 (m, 8H); ^{13}C NMR (101 MHz, Chloroform-*d*) δ 157.29 , 154.87 , 153.08 , 137.02 , 128.69 , 119.63 , 114.75 , 114.12 , 112.83 , 105.45 , 72.00 , 70.88 , 70.80 , 70.65 , 70.61 , 70.23 , 69.19 , 59.12 , 33.97 , 32.81 , 29.23 , 28.07 , 25.67. HRMS (ESI) calcd for $C_{64}H_{87}O_{12}N_4Br_2{}^{81}Br_2S_4$, ([M+H$^+$]) 1551.1890, Found 1551.1881.

Synthesis of IR-BGMC6P. Compound **IR-BGMC6** (80 mg, 0.052 mmol) and sodium azide (50 mg, 0.75 mmol) were dissolved in DMF (10 mL), and the mixture was stirred for 3 h at RT. Then a large amount of water was added until all solids were dissolved. The reaction was extracted twice with ethyl acetate, and the combined organic phase was dried with $MgSO_4$ and evaporated

in vacuo. The crude product was subjected to flash column chromatography on silica gel to yield a dark green solid (70 mg, 0.05 mmol). The dark green solid was dissolved in THF (5 mL), and copper (I) iodide (CuI) (5 mg), w-alkynyl-PEG-hydroxyl PEG_{1500} (M_n = 1500) (150 mg, about 0.1 mol), and tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA) (3 mg) were added. The system was stirred at RT for 0.5 h, and then filtered with diatomite, and the solution was evaporated *in vacuo*. The crude product was purified by thin layer chromatography twice. First, ethyl acetate (EA) was used as an eluent and a small amount of impurity (like the unreacted **IR-BGMC6**) moved to the top of the TLC plate, but other parts of the product remained at the start point of the TLC plate. Then DCM/MeOH (10:1-5:1) was used as eluent successively, and the PEGylation product could be separated from alkyne-PEG. **IR-BGMC6P** (190 mg, isolated yield 86%) was yielded as a green solid.

Bio-conjugation between IR-BEMC6P and peptide

Taking TATE as an example, the following is the bio-conjugation protocol.

1. For a typical reaction: 20 μL TATE(Dde) (50 μM) and 2 μL DBCO-PEG4-NHS (1 mM in DMSO) were added in 50 μL PBS sequentially. The mix was vortexed a little bit, and reacted on a shaker or stirred for 2 hours.
2. 10 μL IR-BEMC6P (100 μM) was added to the above reaction system. The mix was vortexed a little bit, and reacted while stirring for 3 hours.
3. Then 1-2% (w/v) of Hydrazine was added and the reaction mixture was stirred for 1 h.
4. The product was purified by HPLC and/or centrifuge-filter three times against a PBS buffer.
5. IR-BEMC6P@RGD and IR-BEMC6P@FSH were synthesized with a similar protocol.

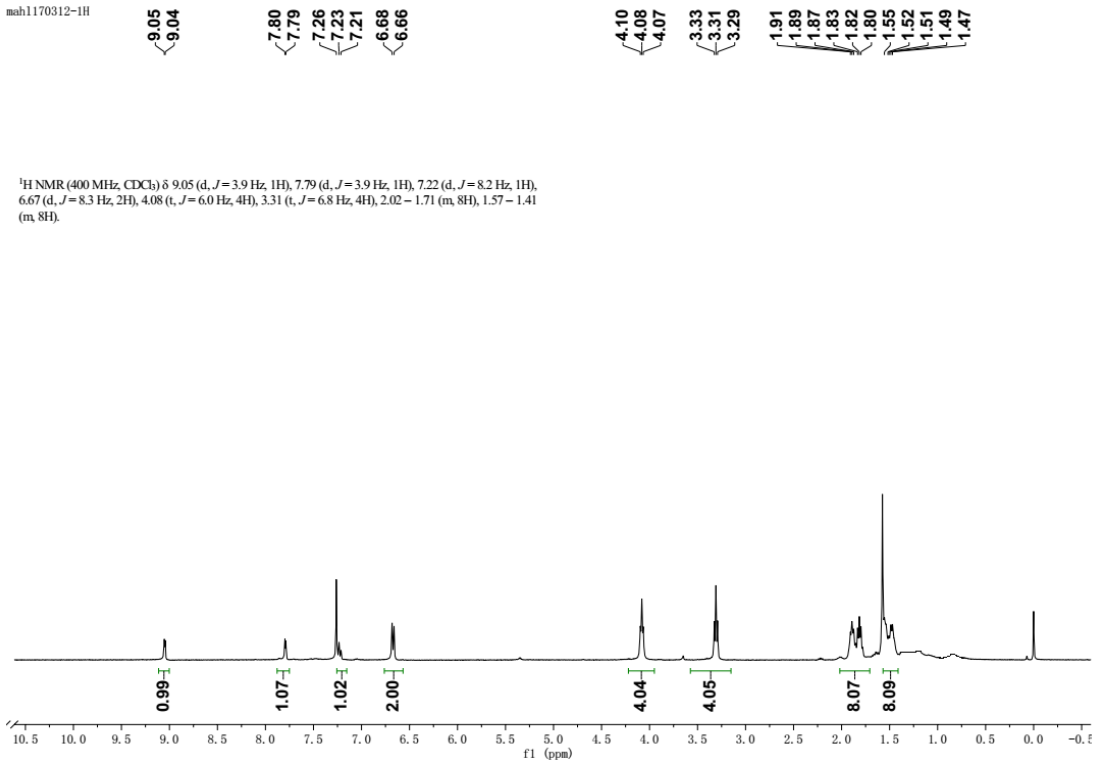


Figure S1. ^{1}H NMR (400 M) of IR-BTMC6

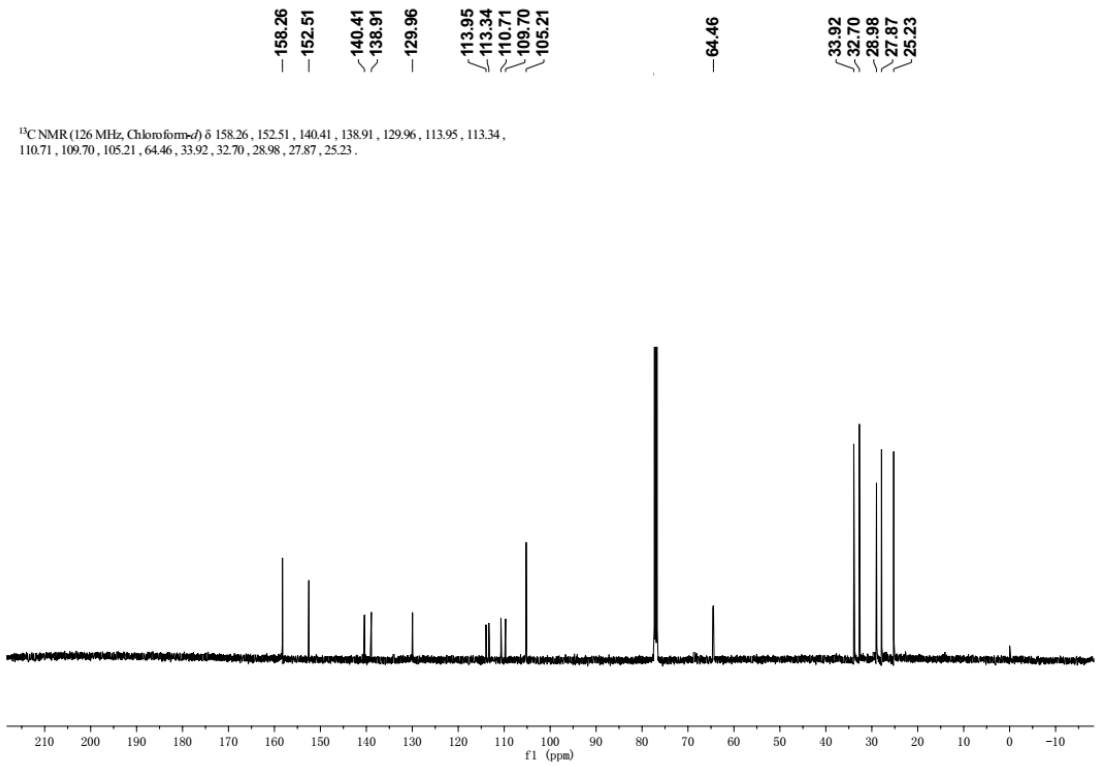


Figure S2. ^{13}C NMR of IR-BTMC6

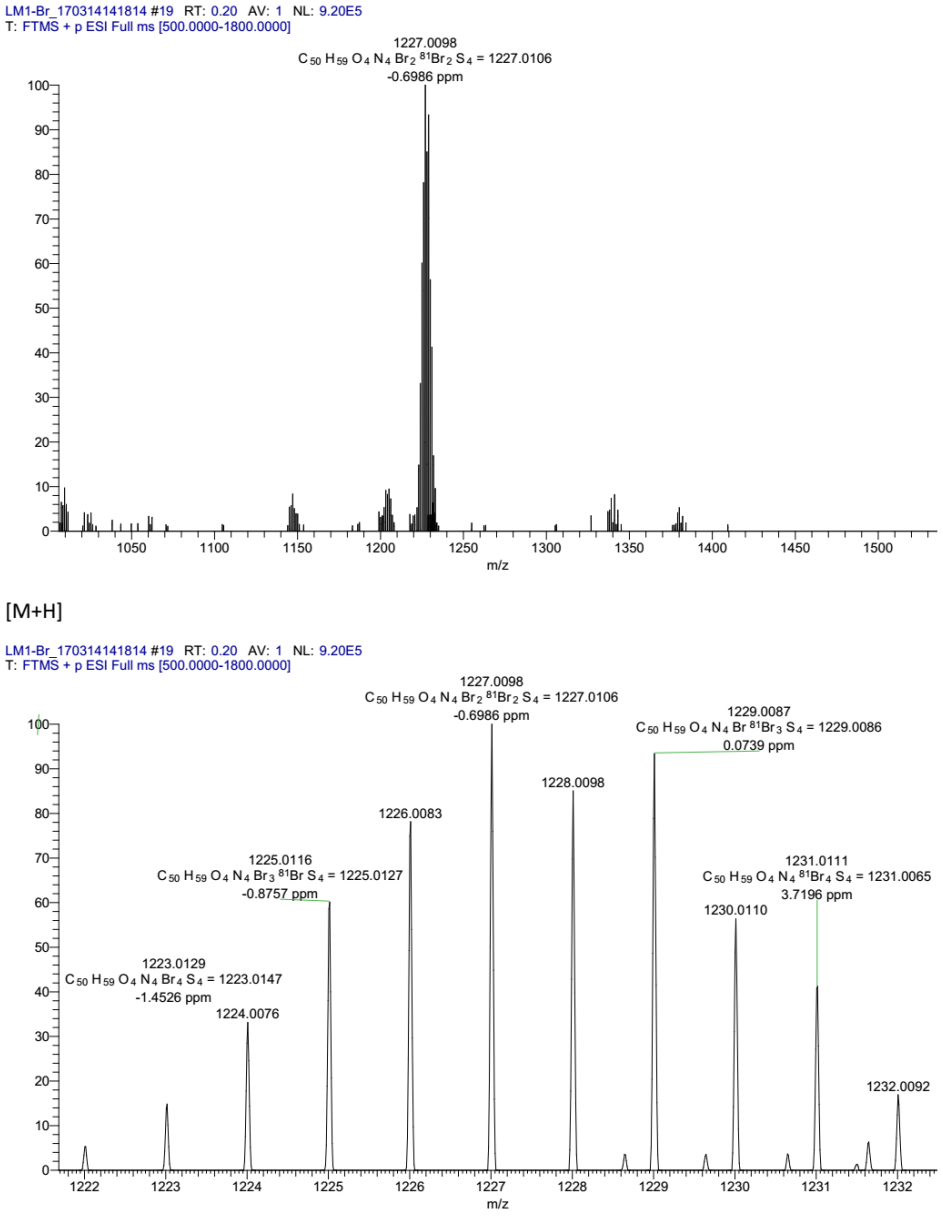


Figure S3. HRMS (ESI) of IR-BTMC6 (also named as "LM1-Br")

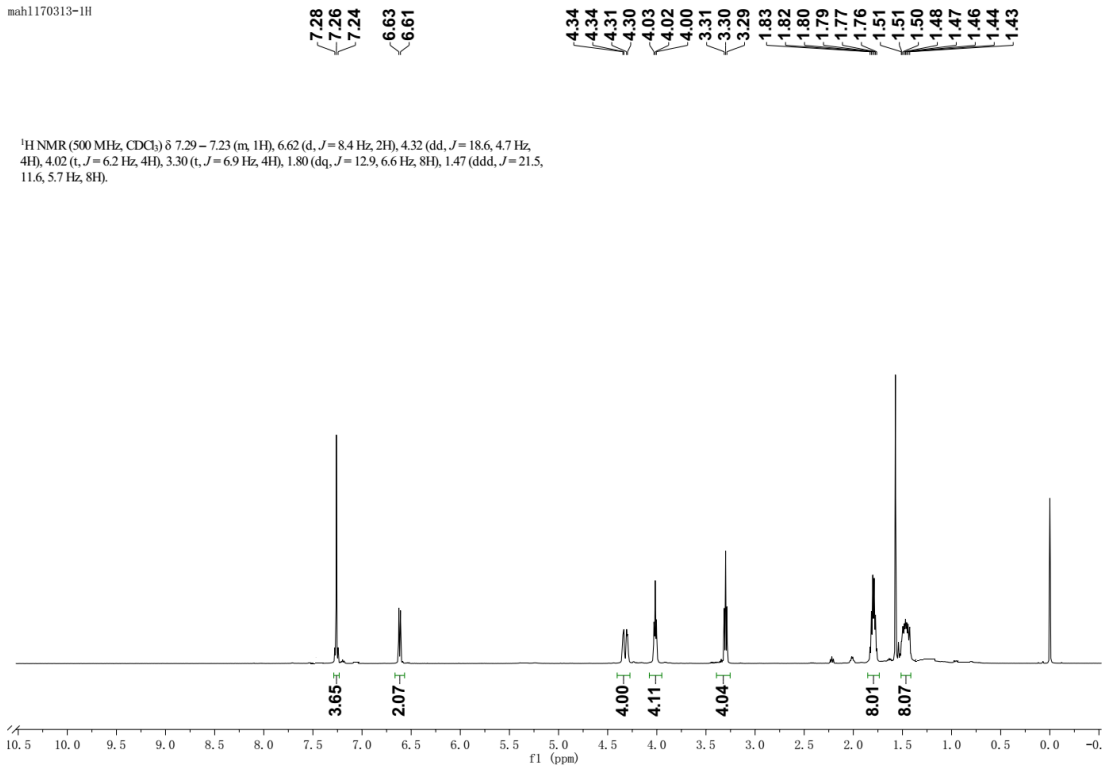


Figure S4. ^{1}H NMR (500 M) of IR-BEMC6

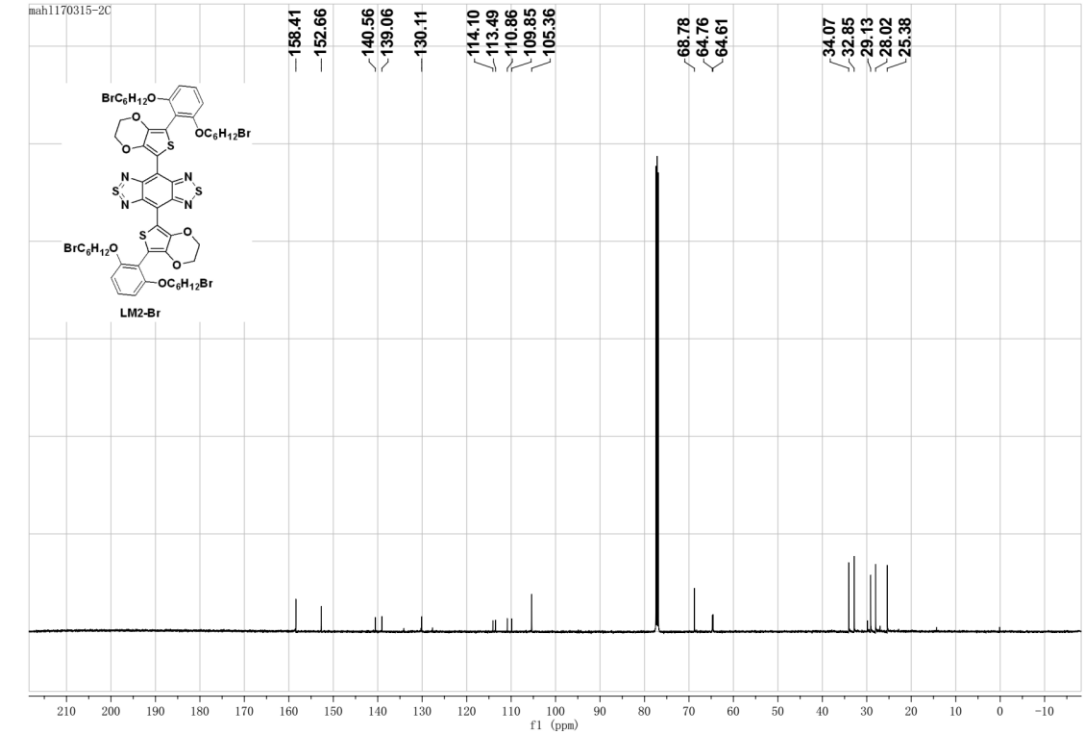
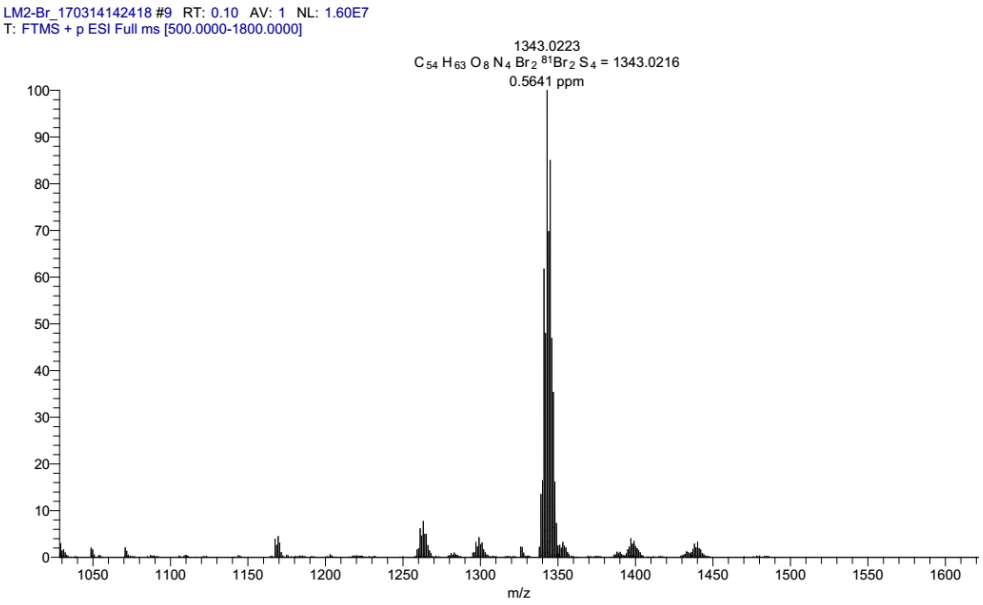


Figure S5. ^{13}C NMR of IR-BEMC6



[M+H]

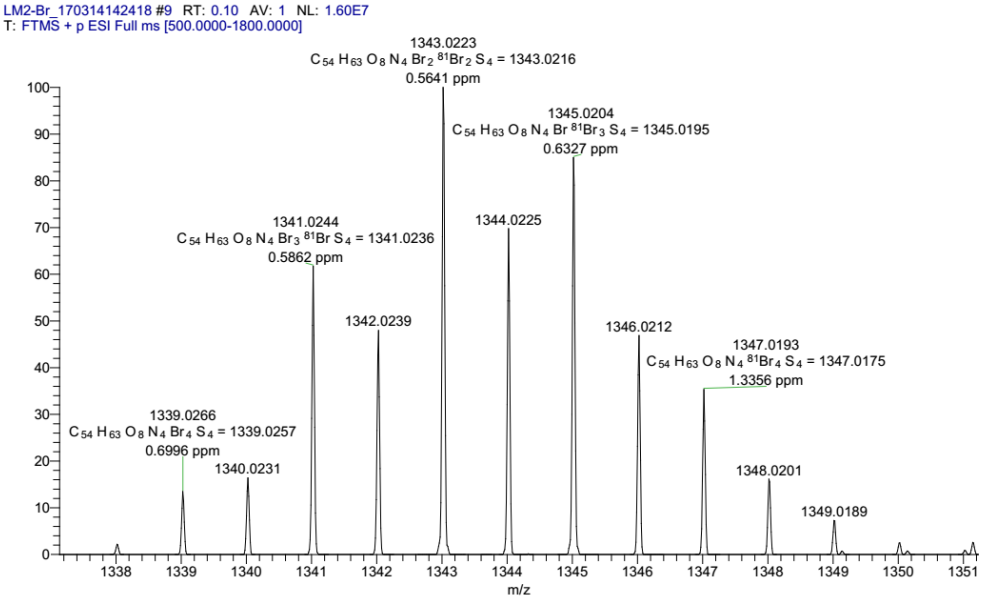


Figure S6. HRMS (ESI) of IR-BEMC6 (also named as "LM2-Br")

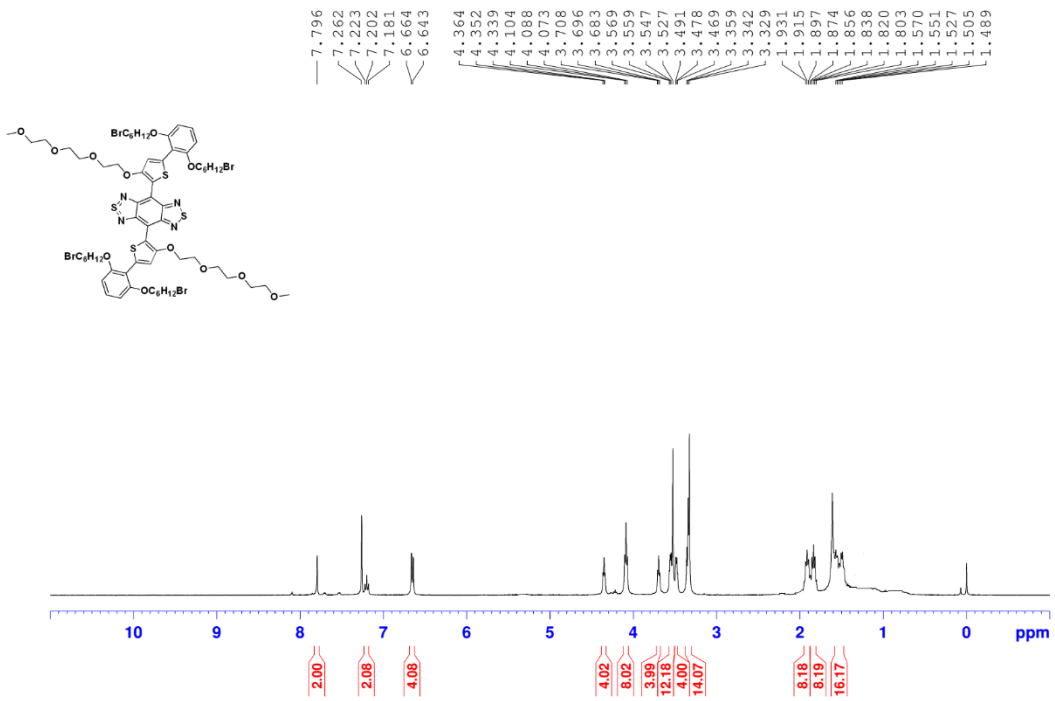


Figure S7. ^{1}H NMR (400 M) of IR-BGMC6

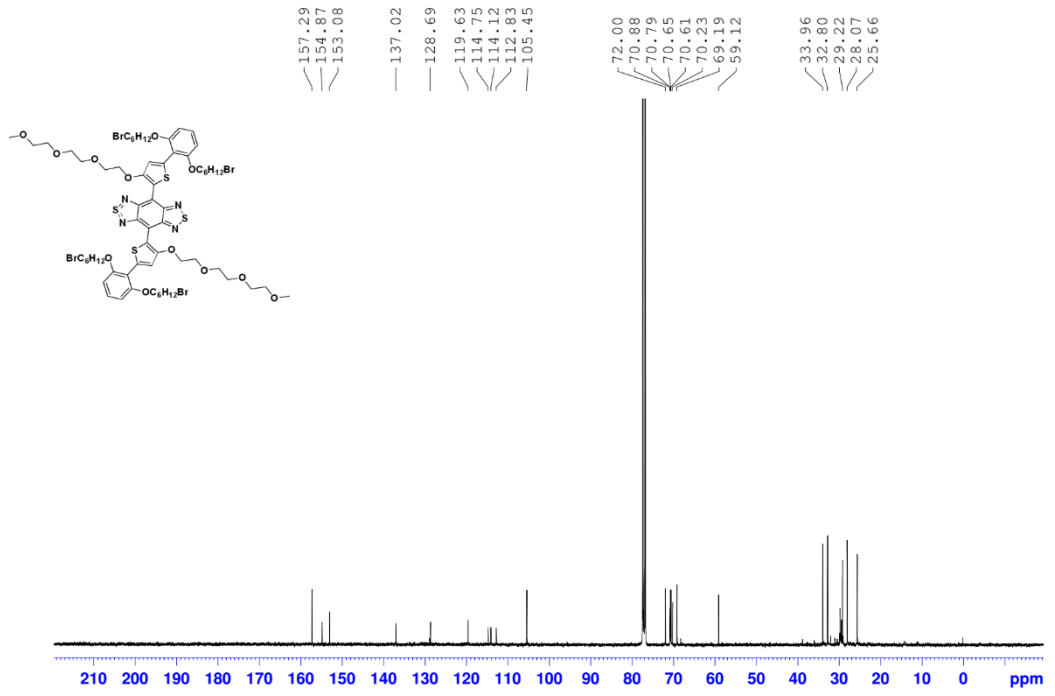


Figure S8. ^{13}C NMR of IR-BGMC6

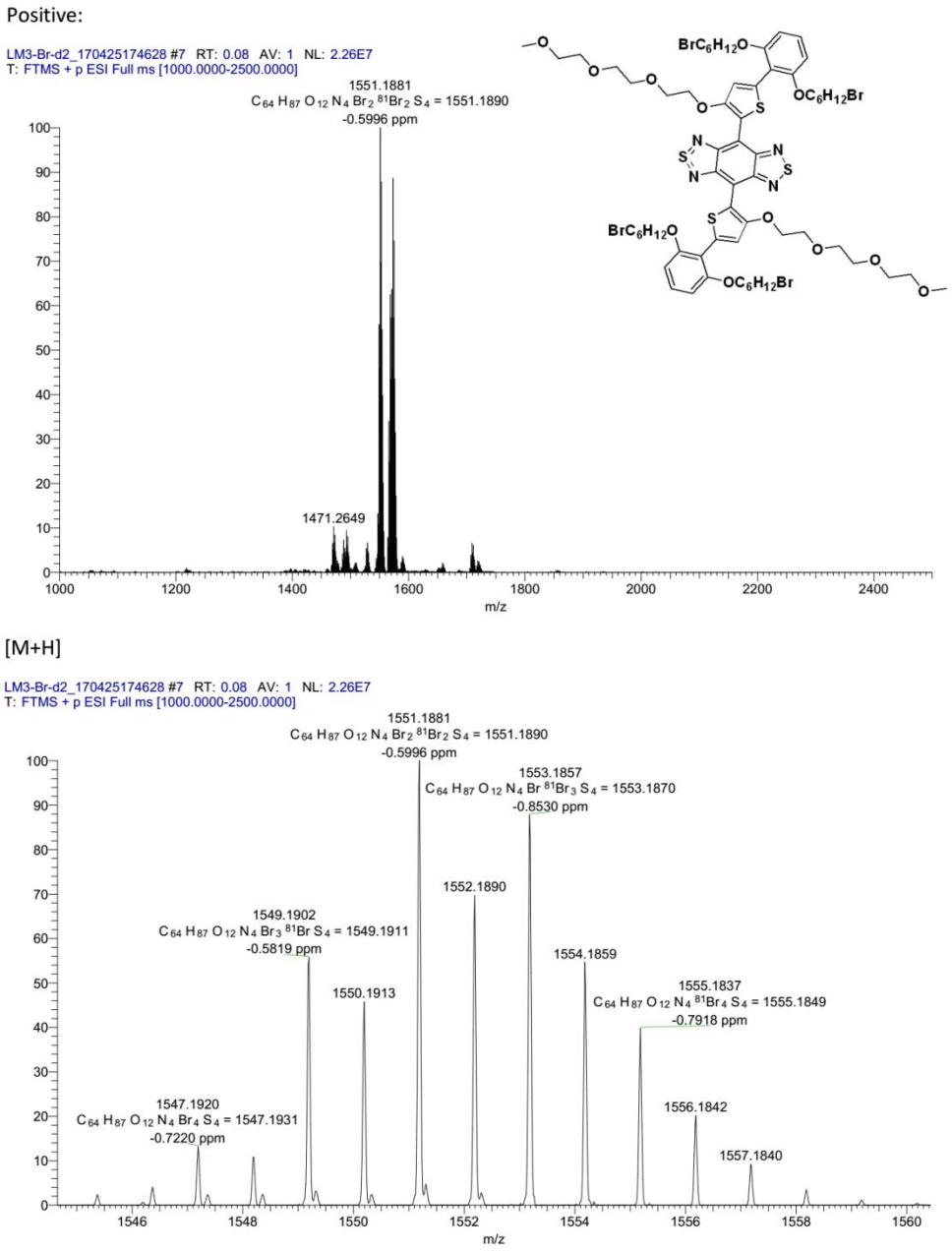


Figure S9. HRMS (ESI) of IR-BGMC6 (also named as "LM3-Br")

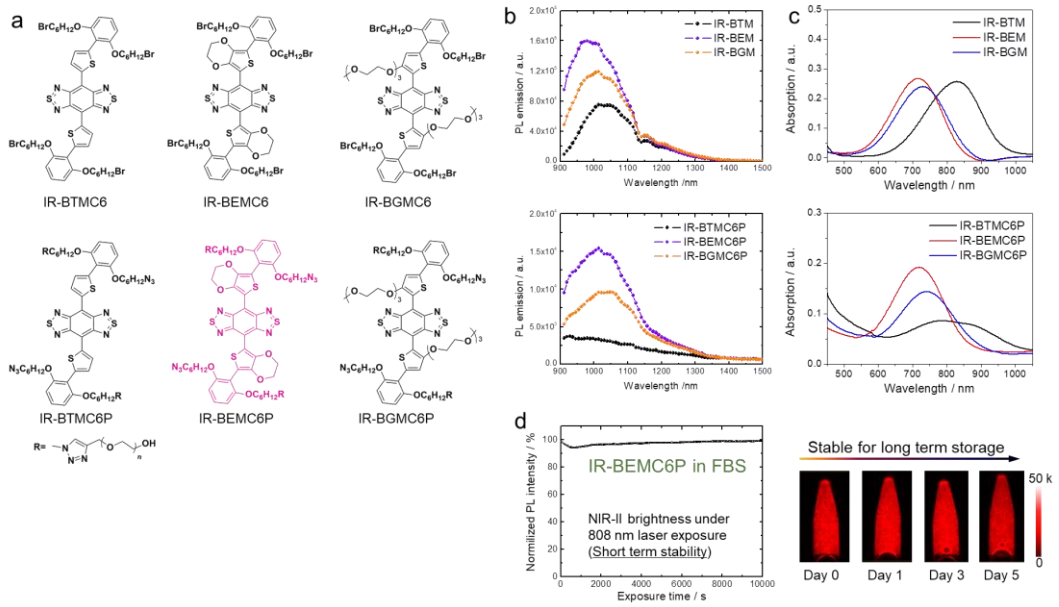


Figure S10. The chemical structure of designed renal-excretion NIR-II dyes. a) Chemical structure of IR-BTMC6P, IR-BEMC6P and IR-BGMC6P with S-D-A-D-S architecture contained a PEG for water solubility, and azide groups for functionalization. b) NIR-II emission spectra of IR-BTM, IR-BEM, IR-BGM, and their PEGylation derivatives. c) Absorption spectra of IR-BTM, IR-BEM, IR-BGM, and their PEGylation derivatives. d) The probe (IR-BEMC6P) was very stable under both short-term excitation and long-term storage.

Table S1. Optical property and quantum yield summary of IR-BTM, IR-BEM, IR-BGM and their PEGylation derivatives (quantum yield was determined by HiPCO SWCNT (0.4%) as the reference fluorophore).

name	spacer	terminal group	ε_{max} ($M^{-1}*cm^{-1}$) $\times 10^3$	λ_{abs}(nm)	λ_{ex} (nm)	Stokes shift (nm)	Φ_f%
IR-BTM	Thiophene	C8Br	15	829	1034	205	4.8
IR-BEM	EDOT	C8Br	10.2	721	989	268	10.2
IR-BGM	Thiophene TEG	C8Br	9.2	728	1020	292	7.6
IR-BTMC6P	Thiophene	2PEG 1500, 2azide	0.23	848	1005	157	0.5
IR-BEMC6P	EDOT	2PEG 1500, 2azide	1.31	725	1028	303	1.8
IR-BGMC6P	Thiophene TEG	2PEG 1500, 2azide	2.42	737	1036	299	1.5

Note: The quantum yield value of HiPCO SWCNT (0.4%) is confirmed by comparing with the QY of IR26 as 0.5%. Discrepancies in the reported quantum yield of IR26 (QY = 0.05%, 0.1%, 0.5%) and the dearth of well characterized reference fluorophores spanning 1000-1700 nm make precise NIR-II dye quantum yield characterization difficult.[4] Although our review paper has re-scaled the reported quantum yields of the entire NIR-II fluorophores based on the QY of IR26 as 0.05%,[4] we still used the 0.5% value here as the reference to keep consistent with previous results.

As result, the absolute QY of IR-BEMC6P should be between 0.18 to 1.8%.

Table S2. Calculated first vertical S0-S1 excitation energies (E01), first vertical S1-S0 emission energies (E10), electronic configurations determined at the TD-ωB97XD*/6-31G (d) level of theory in water.

Mol	ω*	E_{01} (λ_{01}) eV (nm)	f_{01}	Electronic configuration	E_{10} (λ_{10}) eV (nm)	f_{10}	$Ex\lambda_{10}$
IR-BTM	0.1256	1.44(862)	0.57	HOMO → LUMO 99%	0.85(1463)	0.67	1.50 (826)
IR-BEM	0.1239	1.66(746)	0.44	HOMO → LUMO 98%	0.99(1246)	0.50	1.72 (719)
IR-BGM	0.1182	1.68(736)	0.38	HOMO → LUMO 99%	0.91(1369)	0.55	1.71 (724.5)

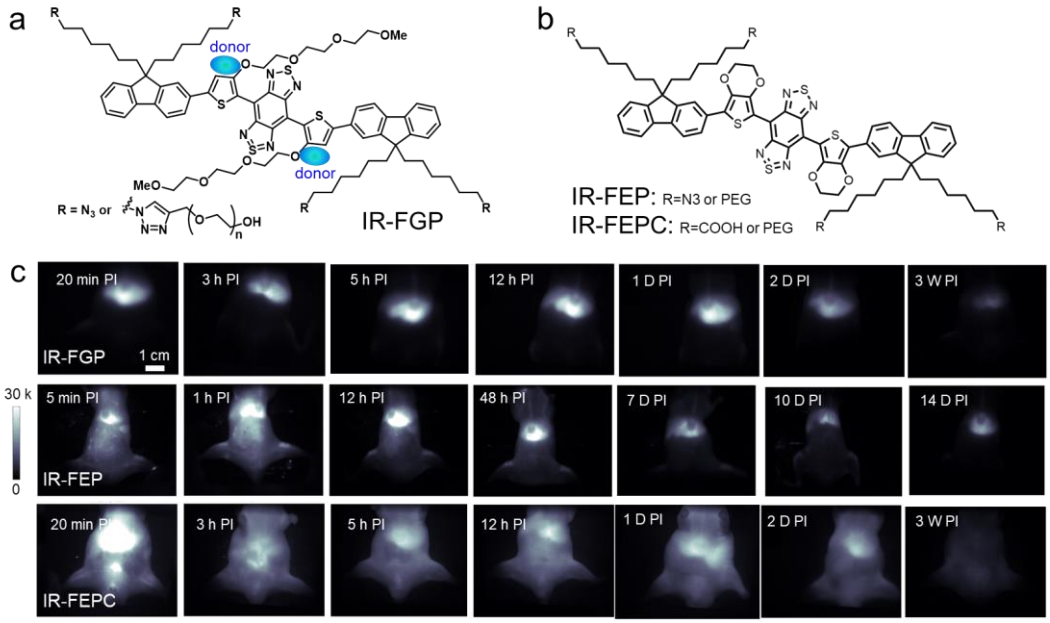


Figure S11. Chemical structure and whole body imaging of fluorene modified S-D-A-D-S dyes: a) IR-FGP; b) IR-FEP and IR-FEPC. c) Selected time points from NIR-II imaging of the mice in the

supine position after an intravenous injection of IR-FGP, IR-FEP, and IR-FEPC, respectively.
Note: IR-FEP can partly excrete from urine.

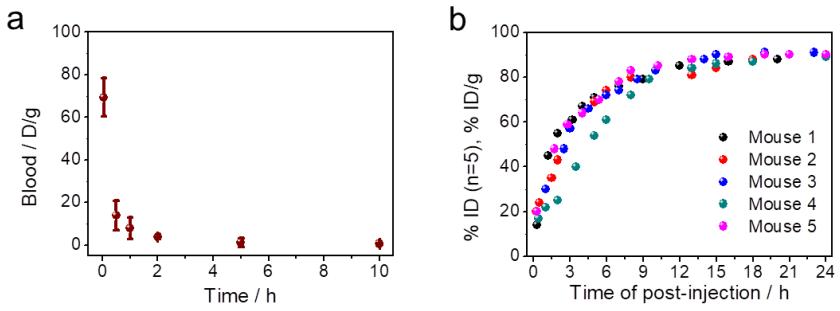


Figure S12. Evaluation of pharmacokinetics and biocompatibility of IR-BEMC6P demonstrates fast renal excretion in the NIR-II emission region (n = 5 mice/group). a) Time-course of blood concentration in 10 h. Fluorescence signal decreased from ~70 % to <1%. b) The cumulative signal curve for urine excretion of IR-BEMC6P in five mice.

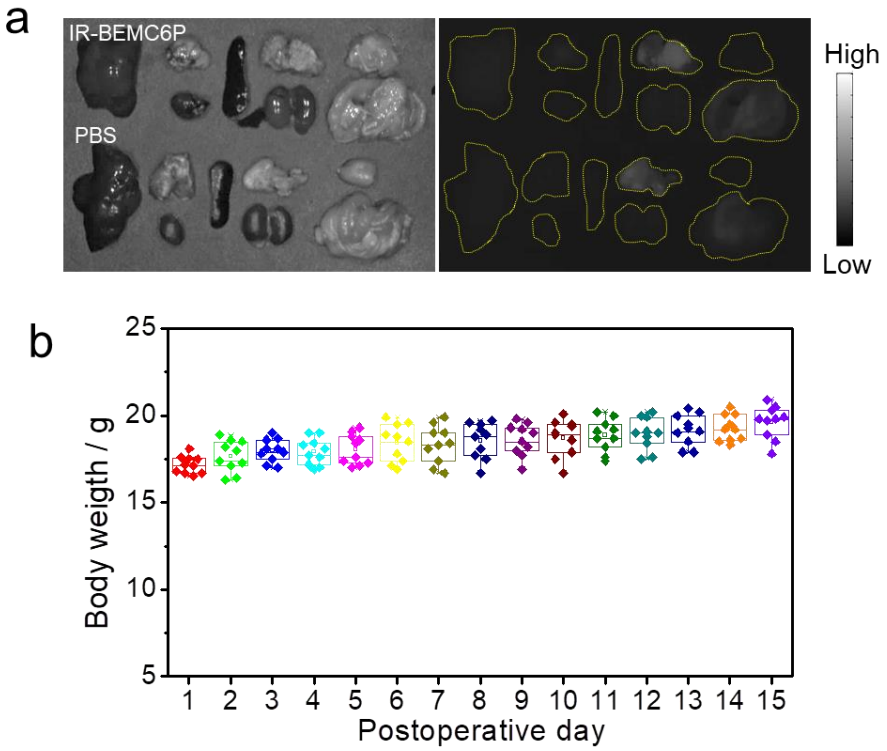


Figure S13. a) Biodistribution of main organs of IR-BEMC6P and PBS buffer treated mice after 24 h p.i.: liver, lung, heart, spleen, stomach, kidney, muscle and intestine. b) Body weight of IR-BEMC6P treated mice (n=10) over a period of 15 days.

Table S3. Animal hematology (complete blood counts) and serum biochemistry test from mice injected with IR-BEMC6P at 24 h post-injection.

			IR-BEMC6P		PBS		
	Test Name	Test ID	Test Result average	Test Result SD	Test Result average	Test Result SD	Unit
1	Bilirubin, Total, animal	TBLA5	<0.3		<0.3		mg/dL
2	Bilirubin, Direct, animal	DBLA5	<0.3		<0.3		mg/dL
3	Alkaline Phosphatase, animal	ALKA5	121.69	5.05	95.78097	34.44026	U/L
4	ALT/GPT(Alanine Trans.), animal	ALTA5	104.25	94.84	32.90709	2.074197	U/L
5	AST/GOT(Aspartate Trans.), animal	ASTA5	156.98	82.79	110.9476	7.859276	U/L
6	Cholesterol, animal	CHOA5	99.53	7.73	88.80856	2.867521	mg/dL
7	Triglycerides, animal	TGA5	132.57	25.97	180.4668	14.62912	mg/dL
8	Sodium, animal	NAA5	308.26	37.37	324.4555	41.72919	mmol/L
9	Potassium, animal	KAA5	5.57	0.46	5.34223	0.528228	mmol/L
10	Chloride, animal	CLAA5	266.13	35.09	280.4257	41.52243	mmol/L
11	Calcium, animal	CALA5	2.34	0.13	2.152224	0.272725	mmol/L
12	Magnesium, animal	MGA5	0.88	0.06	0.807562	0.184687	mmol/L
13	Phosphorus, Inorganic, animal	PHOA5	7.27	1.16	5.467173	0.419848	mg/dL
14	BUN, animal	BUNA5	19.05	0.90	24.68131	1.313614	mg/dL
15	Creatinine, animal	CRAA5	-	-	0.212444	0.02765	mg/dL
16	Albumin, animal	ALBA5	3.32	0.26	3.253266	0.074988	g/dL
17	Amylase, animal	AMYA5	2422.20	234.22	2239.724	155.1286	U/L
18	CK, Total, animal	CKA5	1910.55	1195.35	1109.551	356.9534	U/L
19	LD(Lactate Dehydrogen.), animal	LDHA5	758.14	459.64	517.5704	114.4292	U/L
20	Glucose, animal	GLUA5	170.51	24.59	194.3288	31.30476	mg/dL
21	Protein, Total, animal	PROA5	5.02	0.33	4.900788	0.251547	g/dL
22	Uric Acid, animal	URIA5	3.06	0.62	5.040315	0.39776	mg/dL
1	WBC Count	WBCA	3.25	0.04	6.455	0.886397	K/uL
2	RBC Count	RBCA	9.55	0.41	9.44	0.74355	M/uL
3	Hemoglobin	HGBA	14.00	0.57	13.25	0.718795	g/dL
4	Hematocrit	HCTA	43.85	2.19	42.1	2.477902	%
5	MCV	MCVA	45.95	0.35	44.6	1.469694	fL
6	Platelets	PLTA	511.50	103.94	1119.5	150.9845	K/uL
7	Polys	POLYA	21.40	1.27	16.05	5.961264	%
8	Lymphocytes	LYMPA	72.70	1.56	79.275	5.495074	%
9	Monocytes	MONOA	2.50	0.14	1.475	0.386221	%
10	Eosinophils	EOSA	2.60	0.57	2.5	0.804156	%
11	Basophils	BASOA	0.20	0.14	0.175	0.125831	%
12	Polys Absolute	POLAA	0.69	0.03	1.05875	0.526884	K/uL
13	Lymphocytes Absolute	LYMAA	2.36	0.08	5.095	0.546469	K/uL
14	Monocytes Absolute	MONAA 09	0.08	0.00	0.097	0.032537	K/uL
15	Eosinophils Absolute	EOAA	0.08	0.02	0.1575	0.041065	K/uL
16	Basophils Absolute	BASAA	0.01	0.00	0.01225	0.00943	K/uL

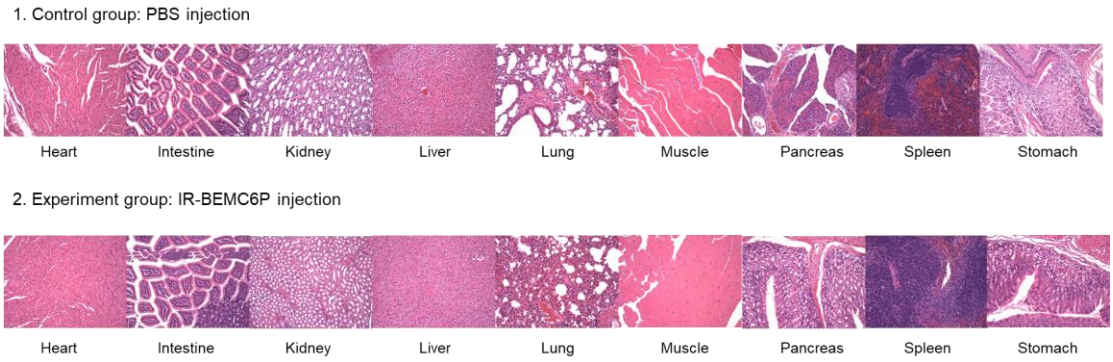
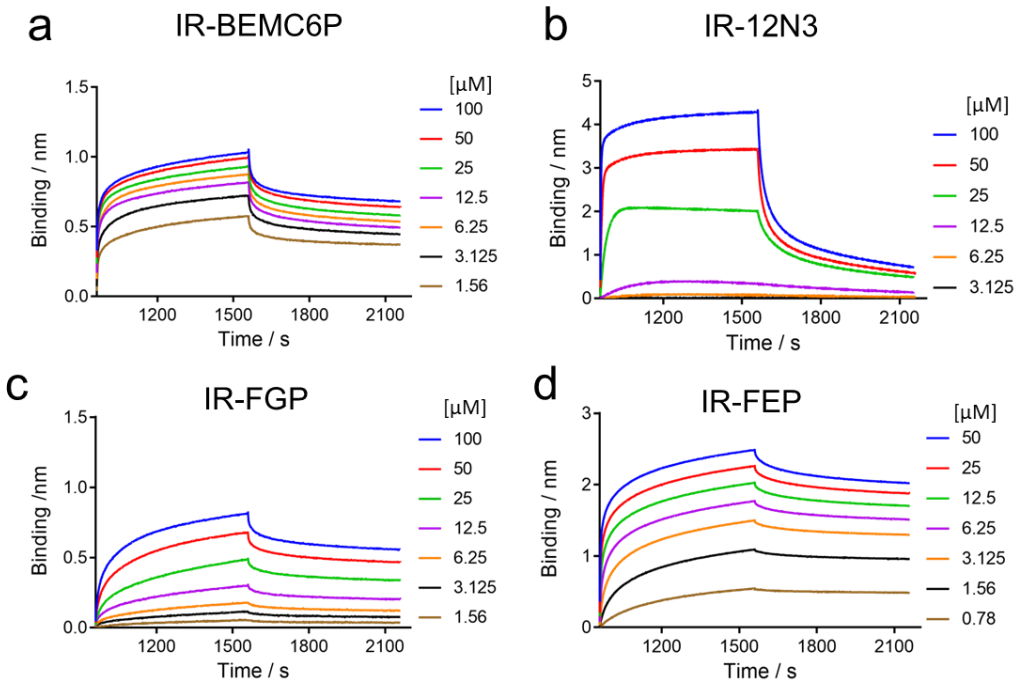


Figure S14. Pathology images of PBS and IR-BEMC6P treated mice at 24 h post-injection. All the main organs were processed according to a routine H&E staining procedure.

Table S4. DLS Size of IR-BTMC6P, IR-BGMC6P, IR-BEMC6P, IR-FGP and IR-FEP.

Dyes	DLS	
	hydrodynamic diameter (nm)	S.D. / nm
IR-BTMC6P	4.7	0.3
IR-BGMC6P	3.5	0.4
IR-BEMC6P	4.0	0.4
IR-FGP	90.1	7.9
IR-FEP	4.9	0.1

Note: All renal-excretion dyes were found to be smaller than 5 nm in DLS testing. Although IR-FEP has the size smaller than 5 nm, it cannot be mainly renal excreted from the body, indicating that other parameters are also very important for renal-excretion ability.



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Concentration (μM)	IR-BEMC6P		IR-FGP		IR-FEP	
	kon(1/M*s)	kobs(1/s)	kon(1/M*s)	kobs(1/s)	kon(1/Ms)	kobs(1/s)
100 (50 for FEP)	9.93E+02	9.99E-02	1.74E+02	1.80E-02	9.21E+02	4.63E-02
50	1.89E+03	9.51E-02	2.84E+02	1.47E-02	1.36E+03	3.42E-02
25	3.67E+03	9.26E-02	3.58E+02	9.46E-03	2.03E+03	2.56E-02
12.5	6.09E+03	7.70E-02	7.34E+02	9.71E-03	2.92E+03	1.84E-02
6.25	1.13E+04	7.15E-02	1.57E+03	1.02E-02	3.89E+03	1.23E-02
3.13	1.44E+04	4.59E-02	2.00E+03	6.87E-03	5.20E+03	8.26E-03
1.56	2.64E+04	4.19E-02	1.01E+04	1.60E-02	6.37E+03	5.12E-03

Figure S15. Kinetic binding assay of IR-BEMC6P, IR-12N3, IR-FGP and IR-FEP to albumin measured by biolayer interferometry. Long liver uptake dyes (IR-FGP, IR-FEP) have slower de-binding speeds than short liver uptake dye (IR-12N3) and renal excretion dye (IR-BEMC6P).

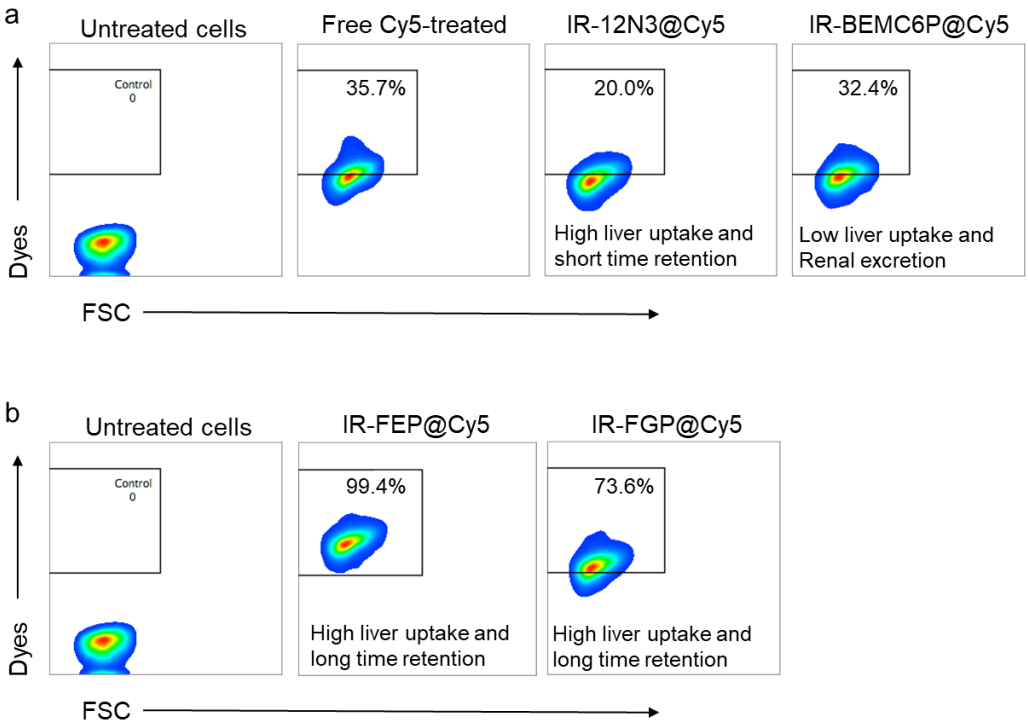

Figure S16. Flow Cytometry result of Cy5 labeled IR-BEMC6P, IR-12N3, IR-FEP and IR-FGP uptake by macrophage cell. IR-12N3 and IR-BEMC6P (a) have much lower macrophage uptake than IR-FEP and IR-FGP (b).

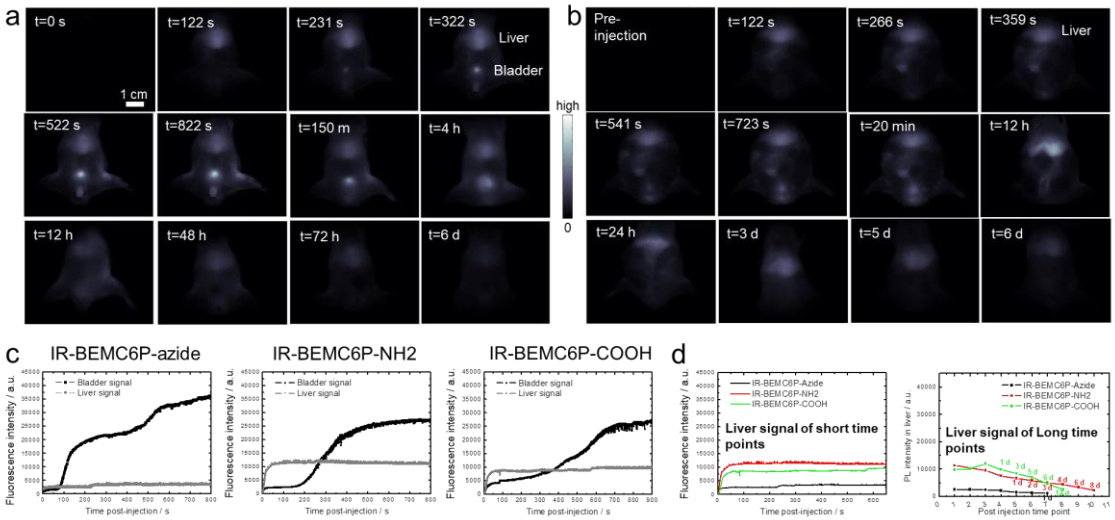

Figure S17. Selected time points from NIR-II imaging of a mouse in the supine position after an intravenous injection of IR-BEMC6P-NH2 (a) and IR-BEMC6P-COOH (b). c) Bladder and liver signal of a mouse with an injection of IR-BEMC6P-Azide, IR-BEMC6P-NH2, and IR-BEMC6P-COOH, respectively. There was 92%, 55%, and 20% excretion in 12 hours for IR-BEMC6P-Azide, IR-BEMC6P-NH2, and IR-BEMC6P-COOH, respectively. d) The liver signals of IR-BEMC6P-azide, IR-BEMC6P-NH2, and IR-BEMC6P-COOH are <2000, 11500, and 9500 counts, respectively. It takes 1 d for IR-BEMC6P-Azide, 8 d for IR-BEMC6P-NH2, and 12 d

for IR-BEMC6P-COOH to excrete most of the injected dye.

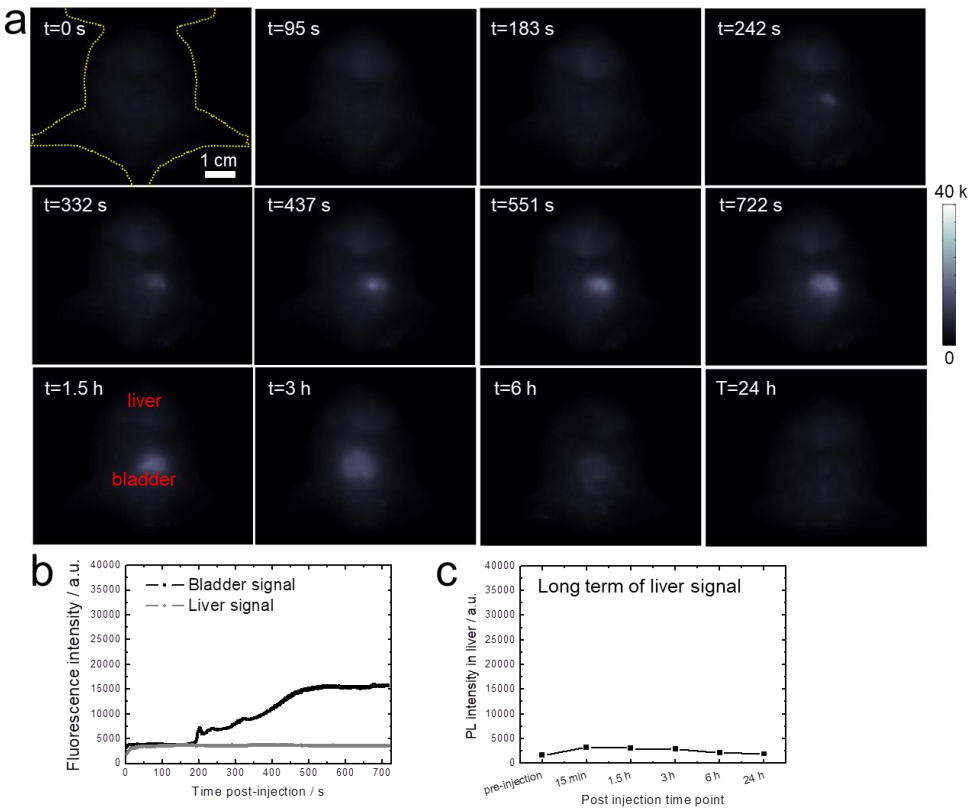

Figure S18. Excretion test of IR-BEMC6P@RGD. a) Selected time points from NIR-II imaging of a mouse in the supine position after an intravenous injection of IR-BEMC6P@RGD. b) Bladder and liver signal of the mouse with an injection of IR-BEMC6P@RGD. c) Long-term liver signal of the mouse with an injection of IR-BEMC6P@RGD.

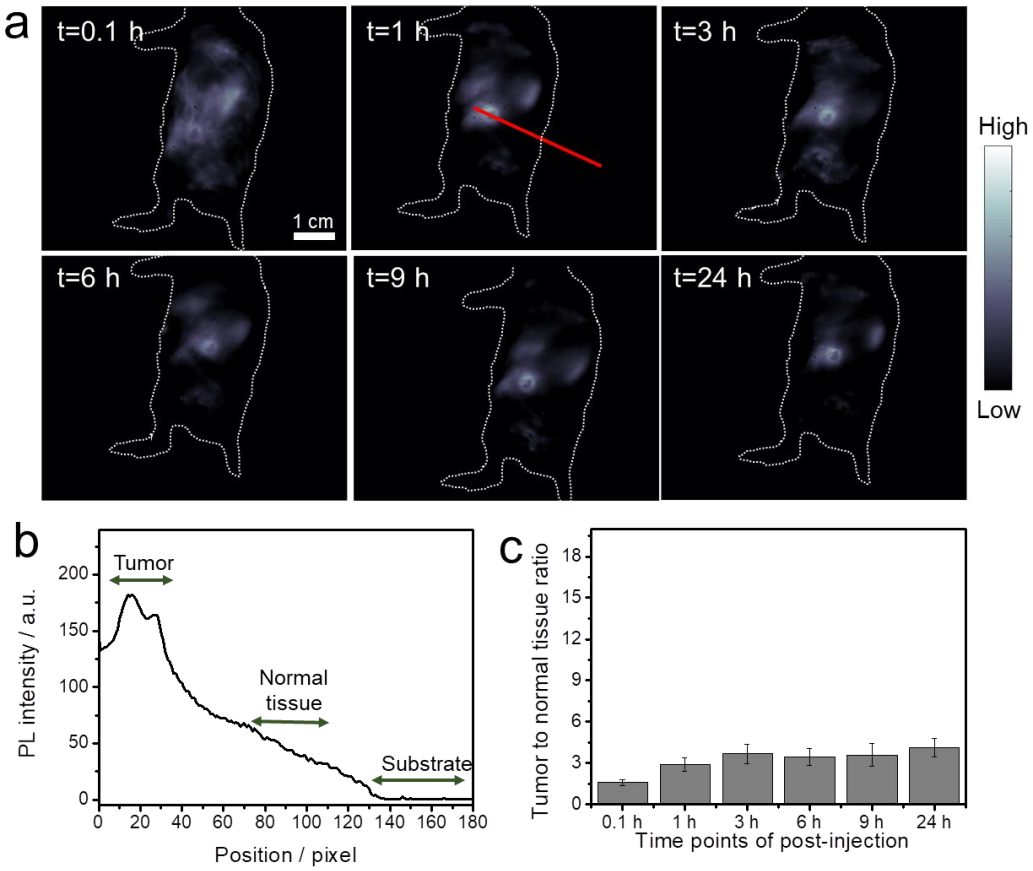

Figure S19. Tumor (U87) uptake of free IR-BEMC6P. After injection of free IR-BEMC6P, the U87 tumor-bearing mouse was subjected to NIR-II imaging, and the tumor to normal tissue ratio was found to be less than 4. a) Selected time points from NIR-II imaging of a U87 tumor-bearing mouse after intravenous injection of IR-BEMC6P. b) Profile position to calculate tumor to normal tissue ratio. c) Tumor to normal tissue ratio of U87 bearing tumor mouse after intravenous injection of IR-BEMC6P.

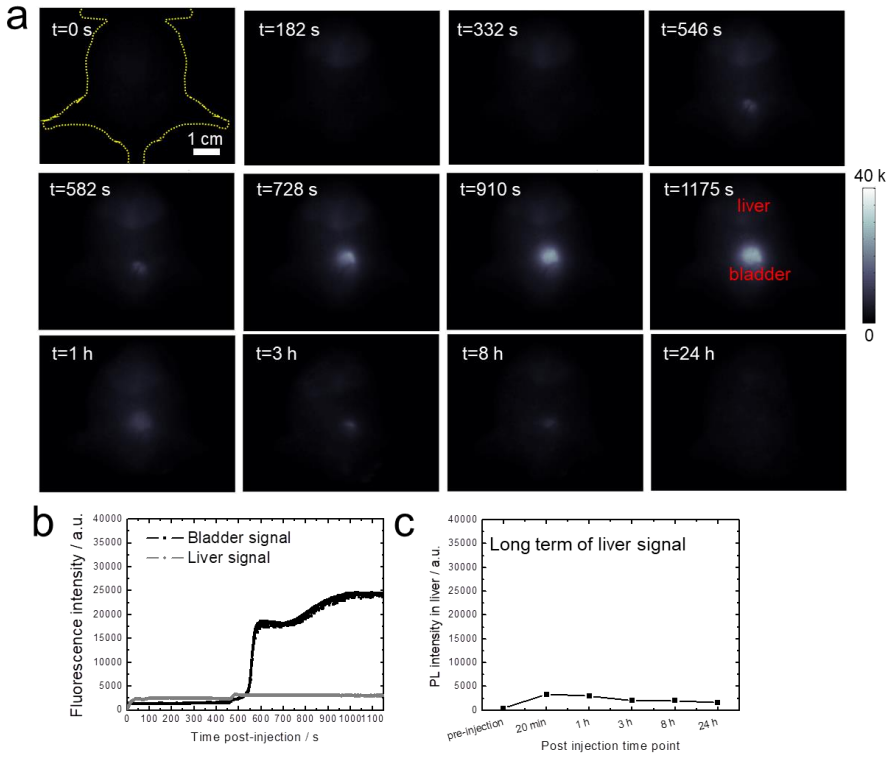

Figure S20. Excretion test of IR-BEMC6P@TATE. a) Selected time points from NIR-II imaging of a mouse in the supine position after an intravenous injection of IR-BEMC6P@TATE. b) Bladder and liver signal of a mouse with an injection of IR-BEMC6P@TATE. c) Long-term liver signal of a mouse with an injection of IR-BEMC6P@TATE.

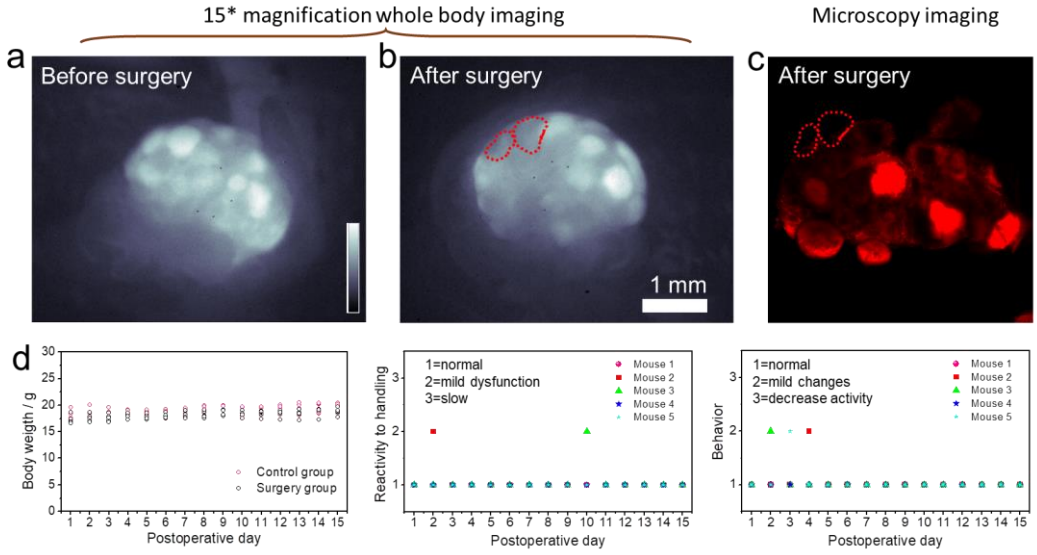

Figure S21. NIR-II imaging-guided micro-surgery (IR-BEMC6P@FSH). For the imaging of FSH receptors, adult female mice were prepared for NIR-II imaging-guided micro-surgery. Briefly, mice were intraperitoneally injected with 5-10IU of equine gonadotropin (eCG) and underwent a live scan 48 h later. eCG treated mice formed large follicles. 3 hours post-injection of IR-BEMC6P@FSH, we conducted the NIR-II imaging-guided micro-surgery. As shown in a, the ovary was taken out of the body, and two of the mature follicles were cut under the guidance of

both visible and NIR-II light. After the micro-surgery, the ovary was monitored by the NIR-II set-up (b). Two days later, the ovary from one of the mice was sliced for microscopy imaging, and the position of cut follicles was obviously recognized (c). d) Validation of local and systemic effects of the imaging-guided micro-surgery. Systemic effects: three metrics of health were measured over 15 days for ovary surgery mice (n = 5). These metrics include measures of the reactivity to handling, physical appearance, and body weight.

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[4] S. Zhu, B. C. Yung, S. Chandra, G. Niu, A. L. Antaris, X. Chen, *Theranostics* **2018**, *8*, 4141-4151.