Electronic Supplementary Information

"Turn-On" NIR-II Fluorescence of a Dually Quenched Probe for Sensitive Imaging of Cathepsin B In Vivo

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Contents:

1. Experimental Procedures

Materials: Unless otherwise stated, all chemicals and reagents were purchased from Shanghai Macklin Biochemical Technology Co. Ltd. (China). All amino acids were purchased from Glbiochem Co., Ltd (Shanghai, China). Isobutyl chloroformate (IBCF), 4-methylmorpholine (MMP), 2-(7-Azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HATU) and N,N-Diisopropylethylamine (DIPEA) was purchased from Meryer Chemical Technology Co., Ltd. (Shanghai, China). 3H-[1,2,3]-Triazolo[4,5-b]pyridin-3-ol (HOAt) was purchased from Jiangsu Aikang Biopharmaceutical (Jiangsu, China). The CTSB inhibitor (CA-074-Me) was ordered from Abcam (Cambridge, MA, USA). Dulbecco's modified eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA). RIPA lysate were obtained from KeyGen Biotech Co., Ltd. (Jiangsu, China). Phosphate buffered saline (PBS) was purchased from Grand Island Biological Company. All chemicals were analytical grade or better. Deionized water was obtained by a Milli-Q water purification system (Billerica, MA, USA).

Instrumentation: High-performance liquid chromatography (HPLC) analyses were performed on an Agilent 1260 Infinity II Prime system equipped with a G1322A pump and an in-line diode array UV detector using an Agilent Zorbax 300SB-C18 RP column, with acetonitrile (0.1% of TFA) and water (0.1% of TFA) as the eluents (Agilent, USA). HPLC purification was performed on a 1290 Infinity system equipped with two LC-20AP pumps and an SPD-20A UV-Vis detector using a Shimazu PRC-ODS column (Agilent, USA). NIR-II fluorescence spectra were obtained on an NIR17S

InGaAs spectrometer (Ideaoptics, China) equipped with an 808 nm C.W. laser (Changchun New Industries Optoelectronics Tech Co., Lid., China). Transmission electron micrograph (TEM) images were obtained on a JEM-2100 transmission electron microscope (JEOL, Japan). ^{1}H-NMR spectra were measured on an Avance III HD 500 MHz spectrometer (Bruker, Germany). ^{13}C-NMR spectra were measured on an Avance III HD 600 MHz spectrometer (Bruker, Germany). Mass spectra (MS) were measured with Infini Lab LC/MSD (Agilent, USA). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF-MS) spectra were recorded on a Ultraflextreme (Bruker). In vivo NIR-II images were taken on an In Vivo NIR-II Imaging System (Series III 900/1700, Suzhou NIR-Optics Technologies Co., Ltd., China).

TEM Sample Preparation

For **AF3-NPs**, 10 μL **AF3-NPs** (5 μM) dispersion was dropped onto the copper grid. For cell lysate-treated **AF3-NPs**, a 5 μM **AF3-NPs** solution was incubated with cell lysate at 37 °C for 8 h in PBS (0.01 M, pH 7.4) buffer, subsequently, 10 μL of the reaction mixture was dropped onto the copper grid. The copper grids were then allowed to dry naturally at room temperature and were immediately observed under TEM.

Cell culture protocol

Human breast cancer MDA-MB-231 cells were grown in high glucose Dulbecco's modified Eagle's medium (DMEM) (Gibco) containing 10% fetal bovine serum (FBS)

(Gibco) and 1% penicillin/streptomycin (Gibco). All cells were cultured in a humidified incubator with 5% CO_2 at 37 ºC.

Preparation of MDA-MB-231 cells lysates

MDA-MB-231 cells (approximately 3×10^6 in total), pretreated with or without 400 μM CA-074-Me (CTSB inhibitor) for 2 h, were cultured to confluence in T25 flasks. Then the medium was removed, and the cells were washed three times with 1× PBS buffer (pH 7.4). Subsequently, 50 μL of RIPA lysis buffer mixed with 2 μL of phenylmethylsulfonyl fluoride (PMSF) solution was added to the cells collected from the bottom of the flask, and the cell suspension was collected and placed on ice for further lysis for 30 min. The samples were centrifuged at 12000 g for 5 min, and the supernatant was collected. The lysates were prepared and stored at -80 ºC in a short-term use.

Cytotoxicity Assay

Cell toxicity was assessed using the standard (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) assay. Cells were seeded in 96-well microplates (Nunc, Denmark) at a density of 50,000 cells/mL in 100 μL of DMEM containing 10% FBS. After 24 h of cell attachment, the plates were washed with 100 μL of PBS per well. Then, cells were cultured in a medium containing 0-160 μM of **AF3-NPs** for 24 h. Cells in a culture medium without **AF3-NPs** were used as the blank control. Five replicate wells were used for each control and test concentration. Subsequently, 10 μL

of MTT (5 mg/mL) was added to each well, and the plates were incubated at 37 ºC for an additional 4 h in a 5% CO_2 humidified incubator. The medium was then carefully removed, and 150 μL of DMSO was added to dissolve the purple formazan crystals. The optical density of the solutions was determined on a microplate reader (Thermo Fisher Scientific) at 570 nm. Cell viability was expressed as a percentage of the control culture value and was calculated using the following equation:

$$\text{Cell viability (\%)} = (OD_{sample} - OD_{blank})/(OD_{control} - OD_{blank}) \times 100$$

NIR-II imaging of CTSB activity in cells

MDA-MB-231 cells were divided into two groups. The experimental group (**AF3-NPs**) involved cells incubated with 5 μM **AF3-NPs**. The control group (Inhibitor + **AF3-NPs**) involved cells pretreated with 400 μM CA-074-Me (CTSB inhibitor) for 2 h, followed by incubation with 5 μM **AF3-NPs**. After incubation for a specific time, cells were washed three times with PBS. These cells were then imaged on an Olympus-IX71 epifluorescence microscope under 808 nm excitation (exposure time: 1000 ms per frame). A 1000 nm long-pass (1000LP) filter was used to collect NIR-II fluorescence images.

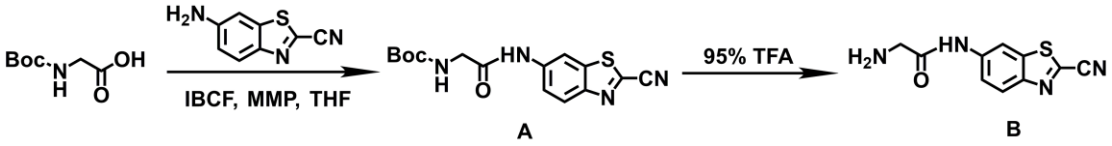
In Vivo Imaging Experiments

All the animal experiments were operated in strict accordance with the Guidelines for Care and Use of Laboratory Animals of Southeast University, and procedures were approved by Southeast University Animal Care and Use Committee. A total of 8×10^6

MDA-MB-231 breast cancer cells were subcutaneously implanted into the right thigh of each 4-week-old BALB/c nude mouse. After the tumors grew to 5-10 mm in diameter, the mice were randomly divided into two groups (n = 3 per group). Mice in the experimental group were injected intratumorally (i.t.) with **AF3-NPs** (0.31 mg/kg). Mice in the control group were pre-injected i.t. with CA-074-Me (40 mM, 20 μL), and after 30 min, **AF3-NPs** (0.31 mg/kg) were injected i.t. NIR-II imaging was performed at 0, 1, 2, 4, 8, 12 and 24 h post-injection under 808 nm excitation. A 1000LP filter was used to collect NIR-II fluorescence images.

2. Syntheses and Characterizations

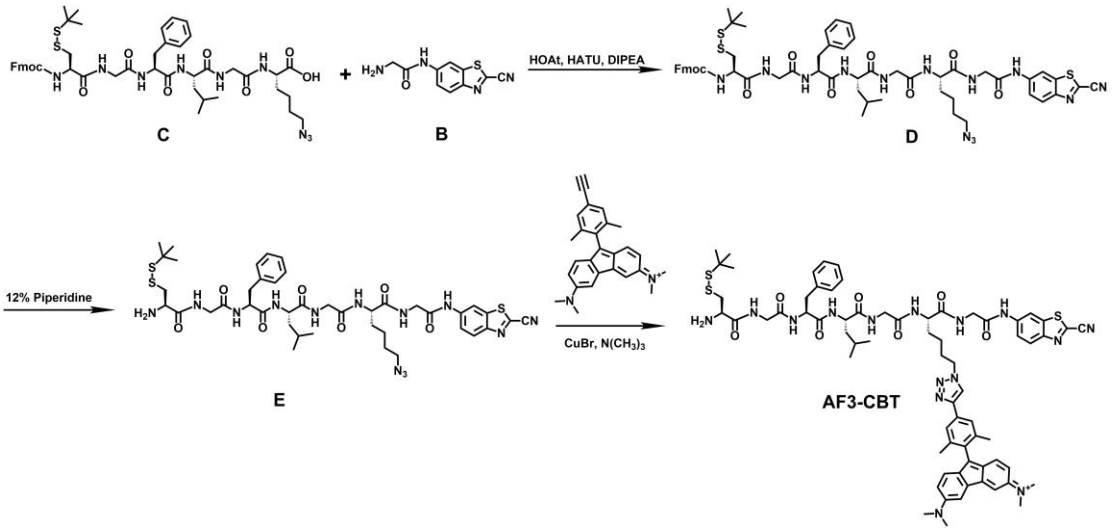
Scheme S1. The synthetic route for **G-CBT (B)**.



*Synthesis of **A***: IBCF (121.8 μL, 0.90 mmol) was added to a mixture of Boc-Gly-OH (157.7 mg, 0.90 mmol) and MMP (202.2 μL, 1.80 mmol) in dry tetrahydrofuran (THF, 15.00 mL) at 0 °C under N_2 gas. After reaction for 30 min, 2-cyano-6-aminobenzothiazole (CBT, 173.4 mg, 0.96 mmol) was added to the reaction mixture and stirred at 0 °C for 1 h. Then the mixture was stirred at room temperature overnight. Compound **A** was purified by HPLC. MS: calculated for A [$(M+H)^+$]: 333.0; obsvd. ESI-MS [$(M+H)^+$]: *m*/*z* 333.1 (**Fig. S1**).

*Synthesis of **B***: The Boc protecting groups of compound **A** (270 mg, 0.8 mmol) were removed using 95.0% of TFA with 5.0% dichloromethane (DCM) for 3 h. MS: calculated for **G-CBT** (**B**) [$(M+H)^+$]: 233.0; obsvd. ESI-MS [$(M+H)^+$]: *m*/*z* 233.0 (**Fig. S2**).

Scheme S2. The synthetic route for **AF3-CBT**.



*Synthesis of **C***: Solid phase peptide synthesis (SPPS, 1 g 2-chlorotrityl chloride resin) was used to prepare the peptide Fmoc-Cys(StBu)-Gly-Phe-Leu-Gly-Lys(N_3)-OH (C). Compound **C** was purified by HPLC using water-acetonitrile with 0.1% TFA as the eluent from 5:5 to 0:10. MS: calculated for C [$(M+H)^+$]: 960.4; obsvd. ESI-MS [$(M+H)^+$]: *m*/*z* 960.4 (**Fig. S3**).

*Synthesis of **D***: **B** (23.2 mg, 0.1 mmol) was added to a mixture of compound **C** (96.0 mg, 0.1 mmol), HATU (57 mg, 0.15 mmol), HOAt (20.42 mg, 0.1 mmol) and DIPEA (52.25 μL, 0.3 mmol) in N,N-Dimethylformamide (DMF, 2 mL). The mixture was stirred for 3 h at 40 °C. Compound **D** was purified by HPLC using water-acetonitrile with 0.1% TFA as the eluent from 7:3 to 0:10. MS: calculated for D [$(M+H)^+$]: 1174.4; obsvd. ESI-MS [$(M+H)^+$]: *m*/*z* 1174.4 (**Fig. S4**).

*Synthesis of **E***: The Fmoc protecting group of compound **D** was cleaved with 12% piperidine in DMF (800 μL) at 0 ºC for 7 min, then 116 μL TFA was added to terminate the reaction. The compound **E** was purified by HPLC using a water/acetonitrile mixed solvent (volume ratio from 6:4 to 1:9) with 0.1% TFA as the eluent. MS: calculated for

E [$(M+H)^+$]: 952.4; obsvd. ESI-MS [$(M+H)^+$]: *m*/*z* 952.3 (**Fig. S5**).

Synthesis of ***AF3-CBT***: The NIR-II FL small-molecule dye, AF3-Alkyne, was synthesized according to a recent publication[1]. CuBr (2.87 mg, 0.02 mmol) in 100 μL trimethylamine ($N(CH_3)_3$) was added to a mixture of compound E (17.14 mg, 0.018 mmol) and AF3-Alkyne (3.8 mg, 0.01 mmol) in 800 μL DMSO. The mixture was stirred overnight at 37 ºC. Then, the AF3-CBT was purified by HPLC using a water/acetonitrile mixed solvent (volume ratio from 5:5 to 1:9) with 0.1% TFA as the eluent. MS: calculated for AF3-CBT [$(M+H)^{2+}/2$]: 666.4; obsvd. ESI-MS [$(M+H)^{2+}/2$]: *m/z* 666.2 (**Fig. S6**). ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 10.35 (s, 1 H), 8.75 (d, *J* = 10.8 Hz, 1 H), 8.63 (s, 1 H), 8.50 – 8.03 (m, 8 H), 7.87 – 7.55 (m, 4 H), 7.21 (dd, *J* = 35.0, 7.9 Hz, 6 H), 6.69 (d, *J* = 8.9 Hz, 2 H), 6.39 (d, *J* = 8.8 Hz, 1 H), 4.38 (d, *J* = 52.1 Hz, 6 H), 3.97 (s, 2 H), 3.84 – 3.67 (m, 3 H), 3.37 (d, *J* = 17.1 Hz, 7 H), 3.20 – 3.01 (m, 5 H), 2.90 (s, 1 H), 2.75 (s, 1 H), 2.52 (s, 6 H), 2.22 (s, 5 H), 1.95 – 1.90 (m, 1 H), 1.68 – 1.47 (m, 5 H), 1.41 – 1.24 (m, 13 H), 0.88 – 0.81 (m, 6 H) (**Fig. S7**). ^{13}C NMR (151 MHz, DMSO-d_6) δ (ppm): 172.69, 170.33, 169.30, 168.07, 167.37, 166.60, 165.95, 165.21, 156.97, 156.73, 156.49, 156.25, 155.72, 145.95, 144.73, 144.07, 137.65, 136.02, 135.05, 134.00, 133.36, 130.63, 129.98, 128.41, 127.98, 127.53, 126.86, 126.35, 124.59, 123.19, 122.80, 120.06, 119.01, 114.99, 114.96, 113.05, 111.92, 111.03, 110.57, 109.51, 52.17, 50.83, 49.81, 49.49, 47.75, 46.39, 41.09, 40.29, 39.91, 39.25, 39.18, 35.98, 33.44, 29.62, 27.73, 27.38, 27.03, 26.92, 24.90, 23.45, 22.45, 21.33, 20.44, 19.94, 18.10 (**Fig. S8**).

3. Supplementary Figures and Tables

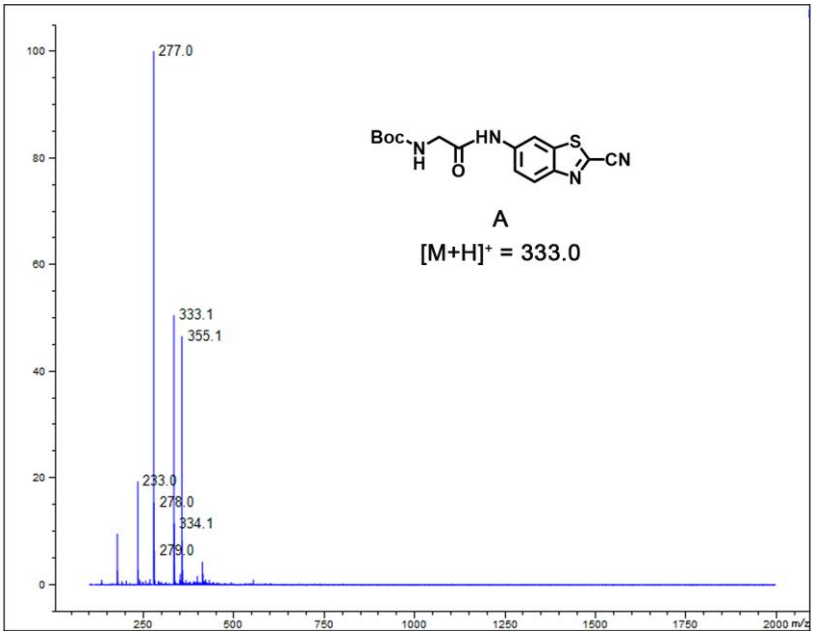


Fig. S1. ESI-MS spectrum of compound **A**.

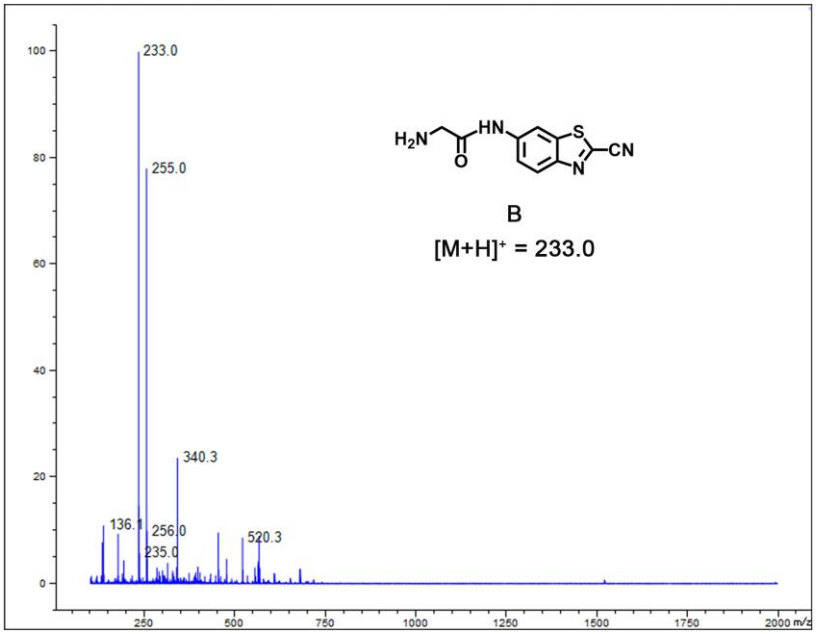


Fig. S2. ESI-MS spectrum of compound **B**.

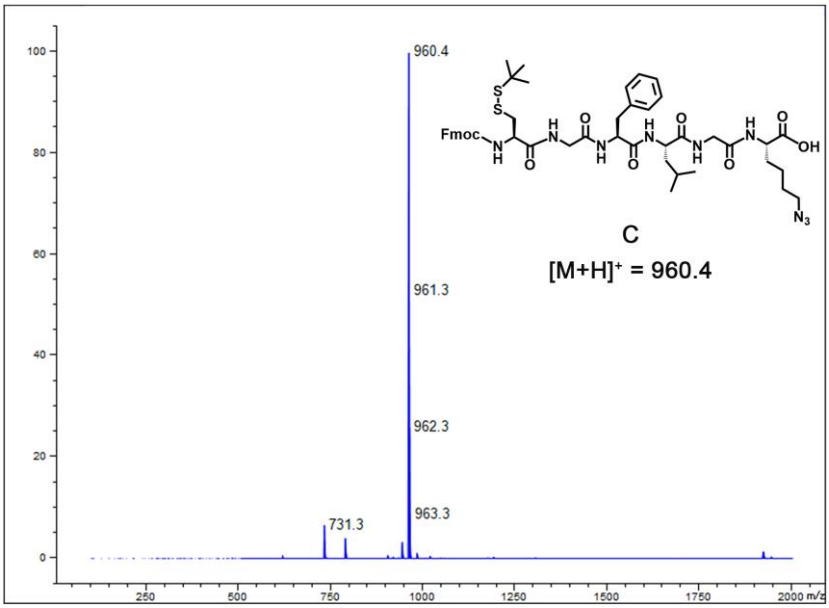


Fig. S3. ESI-MS spectrum of compound **C**.

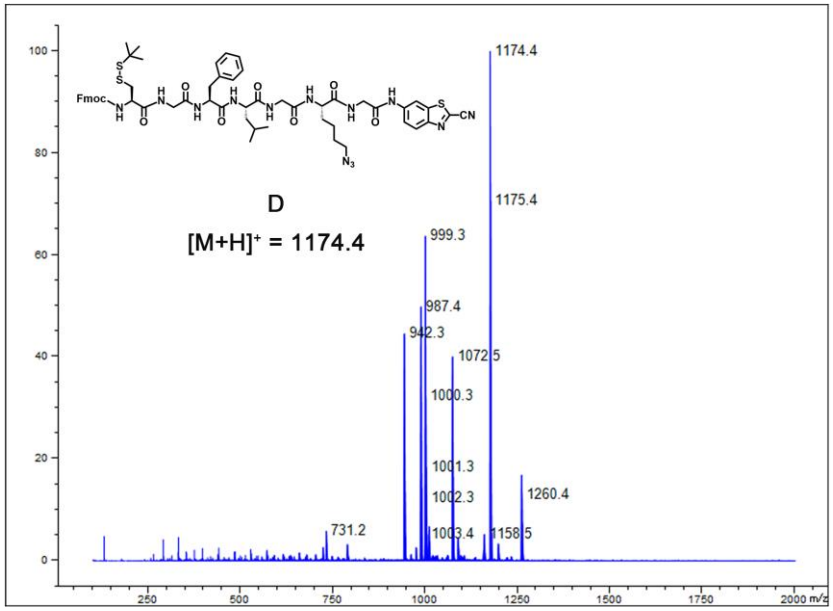


Fig. S4. ESI-MS spectrum of compound **D**.

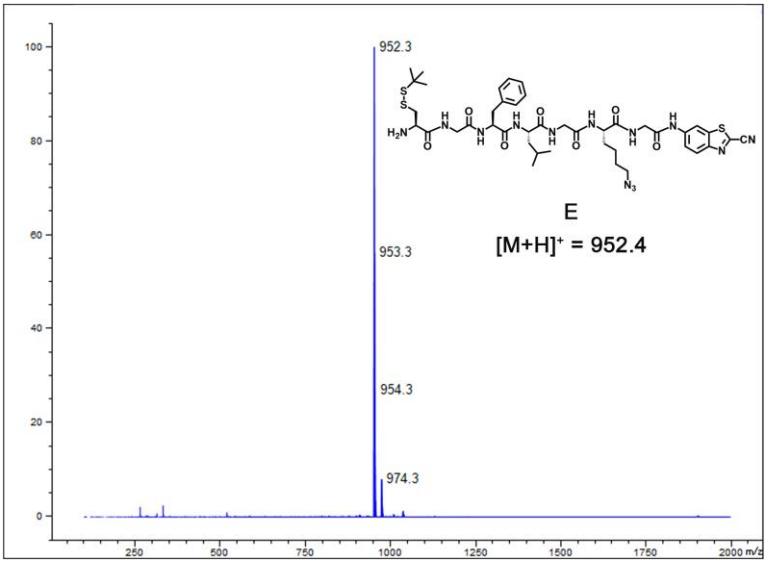


Fig. S5. ESI-MS spectrum of compound **E**.

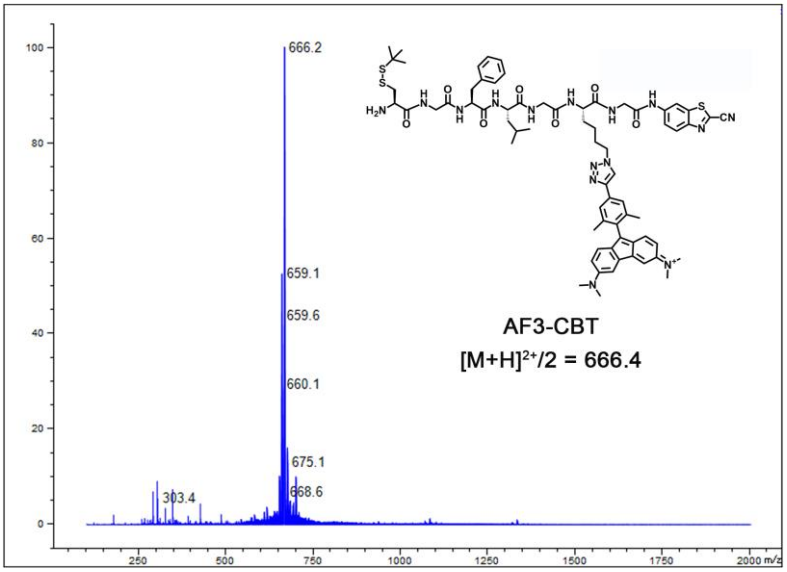


Fig. S6. ESI-MS spectrum of compound **AF3-CBT**.

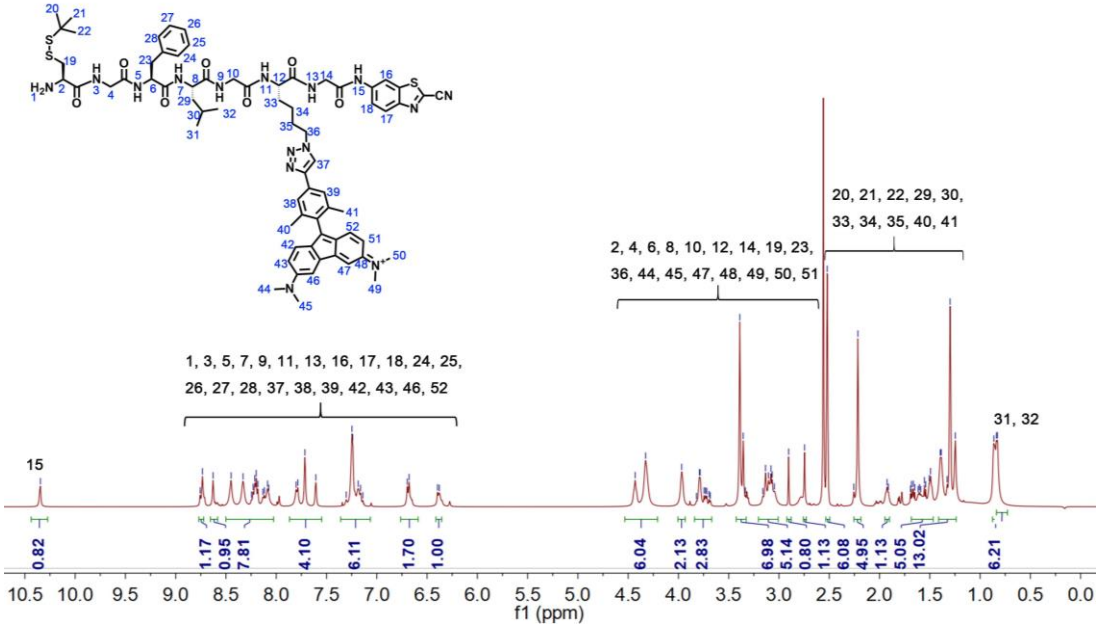


Fig. S7. ^{1}H NMR spectrum of compound **AF3-CBT** in DMSO-d_6.

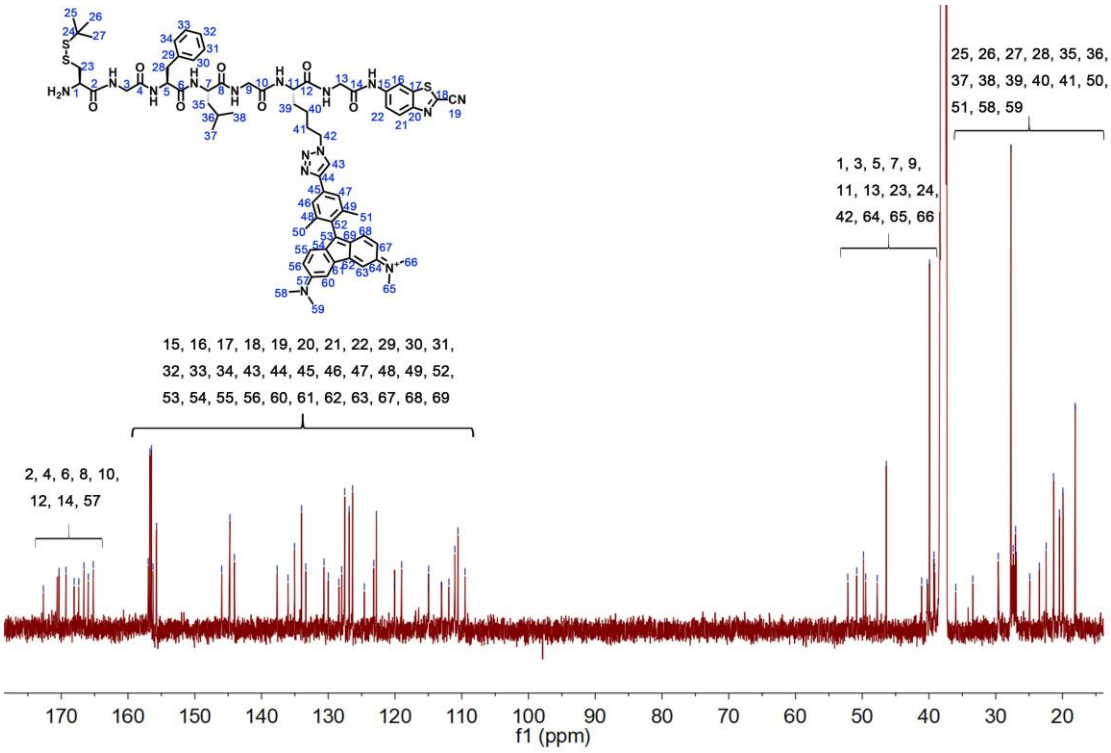


Fig. S8. ^{13}C NMR spectrum of compound **AF3-CBT** in DMSO-d_6.

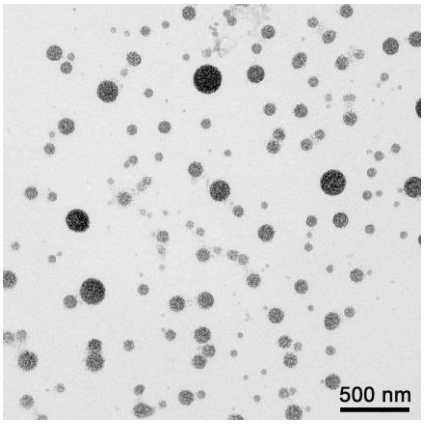


Fig. S9. TEM image of 5 μM **AF3-NPs** in PBS (pH 7.4).

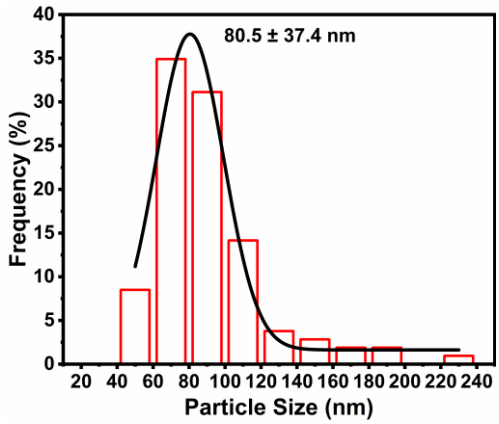


Fig. S10. Size distribution histogram of **AF3-NPs** in Fig. S9.

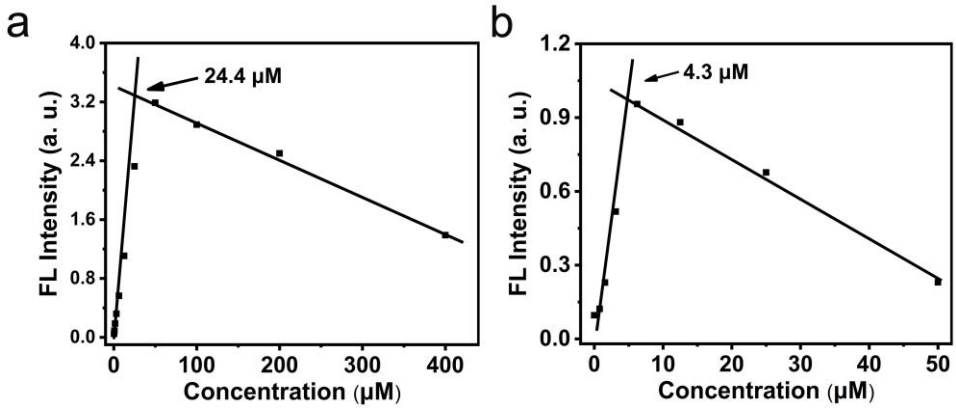


Figure S11. Critical aggregation concentration (CAC) analyses for (a) **AF3-CBT** and (b) **AF3-Dimer** in PBS (0.01 M, pH 7.4) buffer.

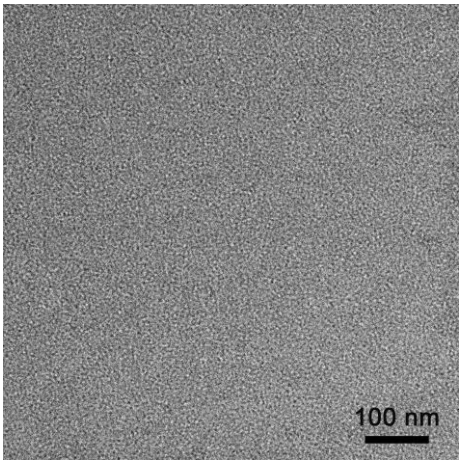


Figure S12. TEM image of 10 μM **AF3-CBT** in PBS (0.01 M, pH 7.4) buffer.

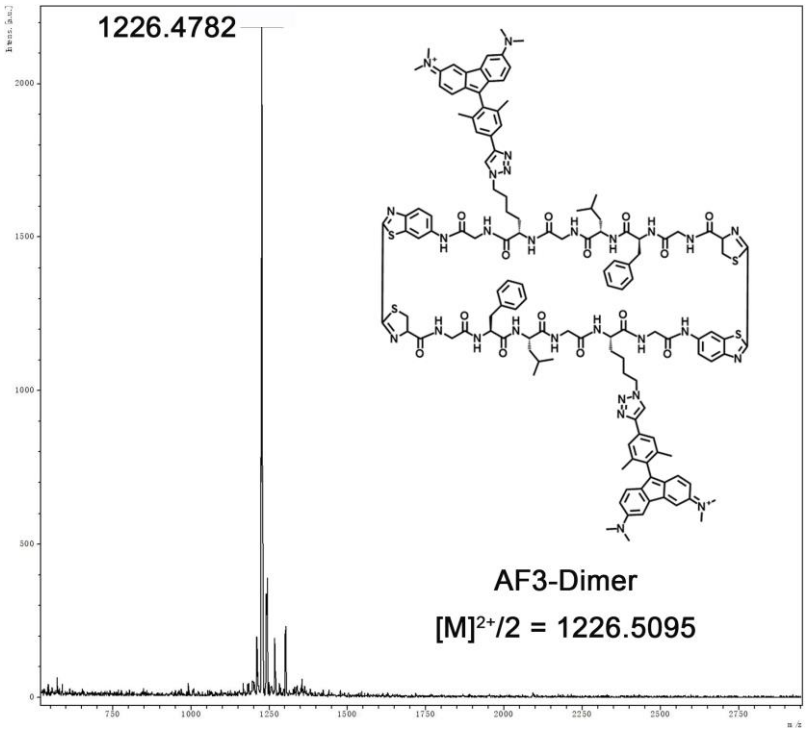


Fig. S13. MALDI-TOF-MS spectrum of the HPLC peak at 17.0 min in Fig. 2c.

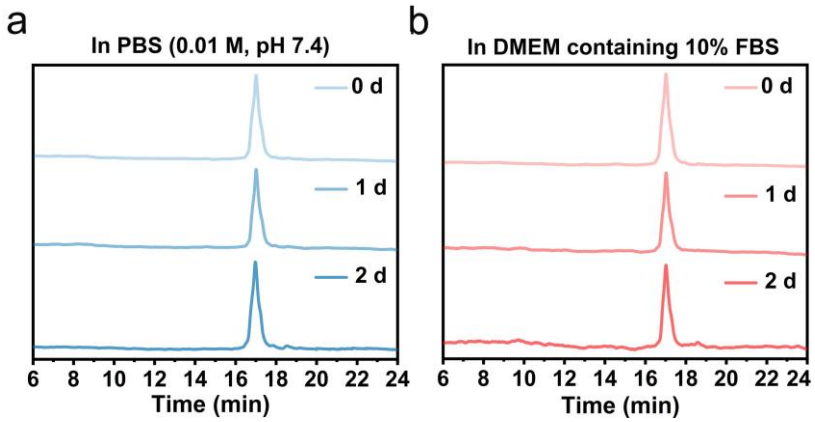


Fig. S14. HPLC traces of 5 μM **AF3-NP** incubated in (a) PBS (0.01M, pH 7.4) or (b) DMEM containing 10% FBS at 37 ℃ for different times.

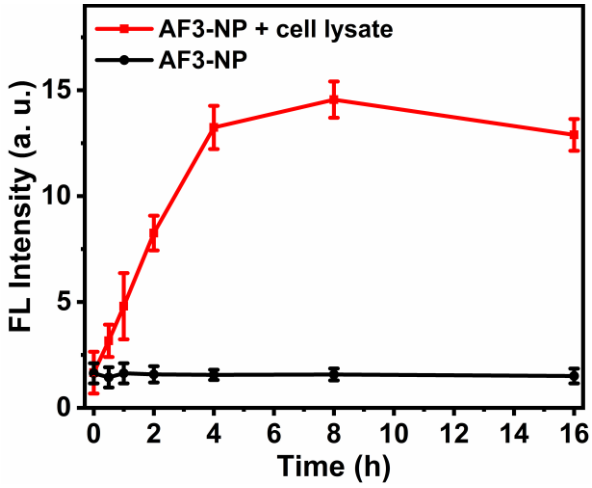


Fig. S15. Time-course NIR-II fluorescence intensities of 5 μM **AF3-NP** incubated with (or w/o) cell lysates in PBS (0.01 M, pH 7.4) buffer at 37 °C. λ_{ex} = 808 nm; λ_{em} = 1030 nm.

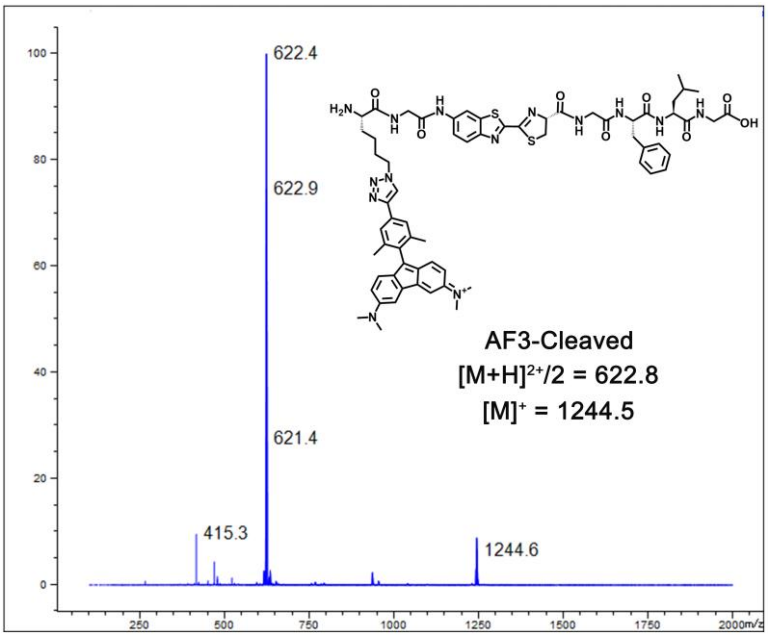


Fig. S16. ESI-MS spectrum of the HPLC peak at 14.3 min in Fig. 2c.

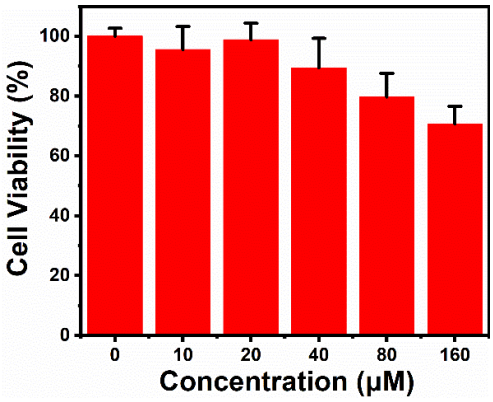


Fig. S17. Cell viability of MDA-MB-231 cells after being incubated with different concentrations of **AF3-NPs** for 24 h.

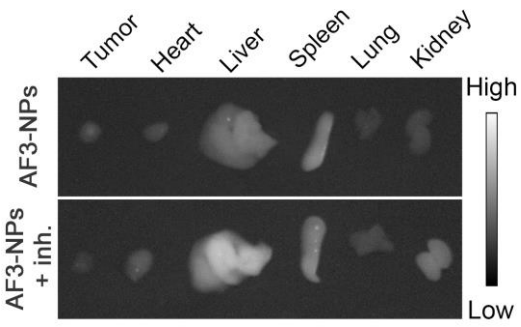


Fig. S18. NIR-II FL images of main organs and tumors harvested from the mice 24 h post-injection of **AF3-NPs**.

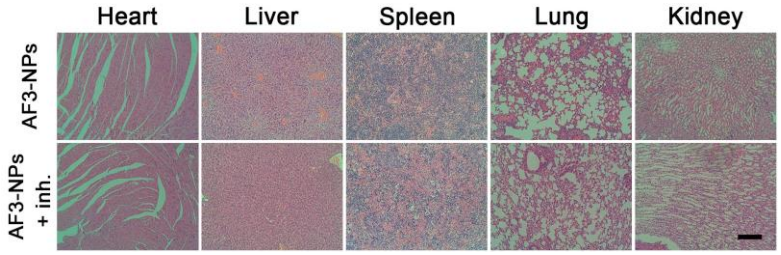


Fig. S19. Hematoxylin and eosin (H&E)-stained tissue slices of the main organs (heart, liver, spleen, lung, and kidney) harvested from mice 24 h post-injection of **AF3-NPs**. Scale bar: 100 μm.

Tables S1. HPLC conditions for the purification of intermediate compounds and **AF3-CBT**.

Compound	Mobile phase (A:B, 0.1%TFA)	Method	Flow (mL/min)	Total running Time (min)
A	H_2O:CH_3CN	From 50:50 to 0:100	10	25
B	H_2O:CH_3CN	From 80:20 to 10:90	10	25
C	H_2O:CH_3CN	From 20:80 to 0:100	10	25
D	H_2O:CH_3CN	From 20:80 to 0:100	10	25
E	H_2O:CH_3CN	From 50:50 to 0:100	10	25
AF3-CBT	H_2O:CH_3CN	From 50:50 to 0:100	3	30

Tables S2. HPLC condition for Fig. 2c.

Time (min)	Flow (mL/min)	H_2O %	CH_3CN %
0	3.0	70	30
5	3.0	70	30
25	3.0	10	90
26	3.0	0	100
29	3.0	0	100
30	3.0	70	30

4. Reference

1 K. Yan, Z. Hu, P. Yu, Z. He, Y. Chen, J. Chen, H. Sun, S. Wang, F. Zhang, *Nat. Commun*. 2024, **15**, 1, 2593.