Supporting information

A Simple Strategy for Efficient Design of Mitochondria Targeting NIR-II Phototheranostics

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Experimental Section

Materials

MitoTracker green, MTT Assay Kit and Reactive Oxygen Species Assay Kit were purchased from Beyotime Company (China). IR780 was purchased from Shanghai Macklin Biochemical Co., Ltd (China). All of the aqueous solutions were prepared using purified deionized (DI) water purified with a purification system (Direct-Q3, Millipore, USA). The other solvents used in this work were purchased from Sinopharm Chemical Reagent (China) and Aladdin-Reagent (China).

Cell culture and animal models

RAW 264.7, U937 and 4T1 mouse breast cancer cell line was obtained from the Cell Bank of the Chinese Academy of Sciences. 4T1 (1×10^6) in 100 μL of PBS in suspension was injected into all mouse bodies in the form of a subcutaneous injection of cells to complete the effective construction of tumor models. When the diameter of the tumor volume reached 100 mm^3, the tumor-bearing mice were used for the next stage of the experiment. All procedures were approved by the Institutional Ethics Committee for Animal Experimentation and were conducted in accordance with the Shenzhen People's Hospital (The Second Clinical Medical College, Jinan University; The First Affiliated Hospital, Southern University of Science and Technology).

Photothermal property of $TDTN^+$

AIEgens were dispersed in PBS solution at different concentration (50 μg/ml-200 μg/ml, 1% DMSO fraction) and pure PBS was used as a control. An 808 nm NIR laser (Changchun New industries Optoelectronics Technology, China) was applied to the solutions with energy density of 0.3 W/cm^2 or 0.6 W/cm^2. During irradiation, an IR camera thermo graphic system (HBT-2A, Hao Bo Technology, and China) was utilized to monitor the temperature change. Temperature variations of AIEgens solution (200 ug/mL, 1% DMSO fraction) under dark was used as control. Temperature variations of AIEgens solution (200 ug/mL, 1% DMSO fraction) over five cycles of heating and natural cooling was also measured by the IR camera.

Resistance to photobleaching

$TDTN^+$ were dispersed in PBS solution at concentration of 10 μg/mL (Containing 1% DMSO). Absorbance of $TDTN^+$ before and after laser irradiation (808 nm, 0.6 W/cm^2) for 5min were measured by UV-Vis absorption spectrum. IR780 was used as a control group.

ESR measurements

$^1O_2$ generation was evaluated by TEMP. 3 μL TEMP was mixed with 100 μL AIEgens or IR780 at 10 μg/mL and irradiated by 808 nm NIR laser (0.3W/cm^2 ,1 min). The signals of $^1O_2$ can be shown by the ESR spectrometer. As a comparison, the PBS group was detected too.

$^1O_2$-Generation Detection

ABDA was used as the $^1O_2$ - monitoring agent. In the experiments, 10 μL of ABDA stock solution (7.5 mM) was added to 2 mL of $TDTN^+$ suspension at different concentration (50 μg/ml-200 μg/ml, 1% DMSO fraction) and treated with 808 nm NIR laser (0.3W/cm^2 ,3 min) or dark. The absorption of ABDA at 378 nm was recorded at various irradiation times to obtain the decay rate.

***In vivo* real-time NIR-II fluorescence microscopic cerebrovascular imaging**

In vivo real-time NIR-II fluorescence microscopic cerebrovascular imaging was operated on the NIR-II fluorescence microscopic imaging optical system (NIRII-MS, Sunnyoptical). A 793 nm laser and InGaAs camera (SW640, TEKWIN SYSTEM, China) was equipped as excitation source and signal detector respectively. A 1200 nm long-pass filter was placed before InGaAs camera to get NIR-II signal beyond 1200 nm. The mice with a cranial window were injected with $TDTN^+$ @F127 nanoparticles (1.5 mg/mL, 0.2 mL) and immediately imaged with a can lens (LSM03, Thorlabs) to get a wide-field image. Then the objective was replaced by a 25× objective lens (XLPLN25XWMP2, 25×, NA = 1.05, Olympus) to record cerebrovascular with high magnification.

Visible-NIR-II colocalization imaging mitochondria in vitro

To testify colocalization performance of NIR+ Materials with mitochondria, Fluorescence imaging at visible-NIR-II spectral region was carried out on home-built visible-NIR-II fluorescent microscopic imaging optical system. Fluorescence was record by a wide-spectrum sensitive camera (GA1280, TEKWIN SYSTEM, China). Before imaging, HEK293T cells were incubated in pore plate filled with HBSS (0.5 mL) and Mito-tracker green dye (200 nM) for 0.5 hour and then, in another pore plate filled with HBSS (0.5 mL) mixed with $TDTN^+$ in DMSO solution (4 mg/mL, 0.5 μL) and F127 solution (20%, 1 μL) for 2 hour. Mercury lamp was illuminated was filtered out blue light and reflected by a 488 nm long-pass dichroic mirror (DMLP). Green fluorescence (510-550 nm) of Mito-tracker green was recorded by the GA1280 camera after getting through 488 nm DMLP and a band-pass filter (510-550 nm). A 793 nm laser was reflected by a 900 nm DMLP to excite fluorescence of $TDTN^+$ Materials. Then, NIR-II (900-1200 nm)

fluorescence signal was recorded by the GA1280 camera after passing through 900 nm DMLP and a 900 nm long-pass filter. A 60× oil objective lens (UPLSAPO60XO, 60×, NA = 1.35, Olympus) was used to get high magnification images.

In vitro anticancer effect of AIEgens

The phototoxicity was measured by MTT assay. 4T1 cells were seeded in 96-well plates at a density of 5×10^3 cells per well and incubated for 12 h. Afterwards, cells were incubated with 4 different groups: (1) PBS; (2) 808 nm NIR laser (L, 0.3W/cm^2 ,5 min); (3) $TDTN^+$; and (4) $TDTN^+$ +L. The AIEgens concentration was 50 μg/mL in group 3 and 4. Then, cells in group 2, and 4 were exposed to 808 nm NIR laser for 5 min. At the end of the incubation, 5 mg/mL MTT PBS solution was added, and the plate was incubated for another 4 h. Finally, the absorbance values of the cells were determined by using a microplate reader (Emax Precision, USA) at 570 nm. The background absorbance of the well plate was measured and subtracted. The cytotoxicity was calculated by dividing the optical density (OD) values of treated groups (T) by the OD values of the control (C) (T/C × 100%). We then used the same method to verify the in vitro phototoxicity of AIEgens at different concentration (0, 100 and 150 μg/mL).

The phototoxicity and dark toxicity of $TDTN^+$ towards U937 and RAW 264.7 cells was measured by MTT assay. U937 and RAW 264.7 cells were seeded in 96-well plates respectively at a density of 5×10^3 cells per well and incubated for 12 h. Afterwards, cells were incubated with different concentration of $TDTN^+$ and then exposed to 808 nm NIR laser for 5 min (At the same time, the group without laser irradiation was set as the dark toxicity group). At the end of the incubation, 5 mg/mL MTT PBS solution was added, and the plate was incubated for another 4 h. Finally, the absorbance values of the cells were determined by using a microplate reader (Emax Precision, USA) at 570 nm. The background absorbance of the well plate was measured and subtracted. The cytotoxicity was calculated by dividing the optical density (OD) values of treated groups (T) by the OD values of the control (C) (T/C × 100%).

Intracellular reactive oxygen species (ROS) generation

For determination of ROS levels *via* fluorescent imaging, 4T1 cells were seeded in 96-well plates at a density of 5×10^3 cells per well and incubated for 12 h. Afterwards, cells were incubated for 4 different groups: (1) PBS; (2) 808 nm NIR laser (L, 0.3W/cm^2, 5 min); (3) $TDTN^+$; and (4) $TDTN^+$ +L. The AIEgens concentration was 50 μg/mL in group 3 and 4. Then, the fluorescent dye, DCF (10 μM), was added and co-incubated for 20 min at 37 °C. Then, cells in group 2, and 4 were

exposed to 808 nm NIR laser for 1 min. ROS level was determined by confocal laser scanning microscope (CLSM; IX81, Olympus, Japan).

***In vivo* antitumor study**

When tumor size reached approximately 100 mm^3, the mice were divided randomly into 4 groups (each group included 5 mice): (1) PBS; (2) 808 nm NIR laser (L, 0.6W/cm^2 ,5 min); (3) $TDTN^+$; and (4) $TDTN^+$ +L. The $TDTN^+$ dose was 5 mg/kg in group 3 and 4. The NIR light was performed 0.5 h after intratumor injection. The treatment was conducted every 2 days for 14 days. Mice body weight and tumor volume in all groups were monitored every 2 days. A caliper was employed to measure the tumor length and tumor width and the tumor volume was calculated according to following formula. Tumor volume = tumor length × tumor width ^{2} / 2. After 14 days treatment, all the mice were sacrificed. The tumors of all mice were harvested, washed with PBS, and fixed with paraformaldehyde for histology analysis. And the tumor tissues were weighed, and fixed in 4% neutral buffered formalin, processed routinely into paraffin, and sectioned at 4 μm. Then the sections were stained with hematoxylin and eosin (H&E) and finally examined by using an optical microscope (BX51, Olympus, Japan).

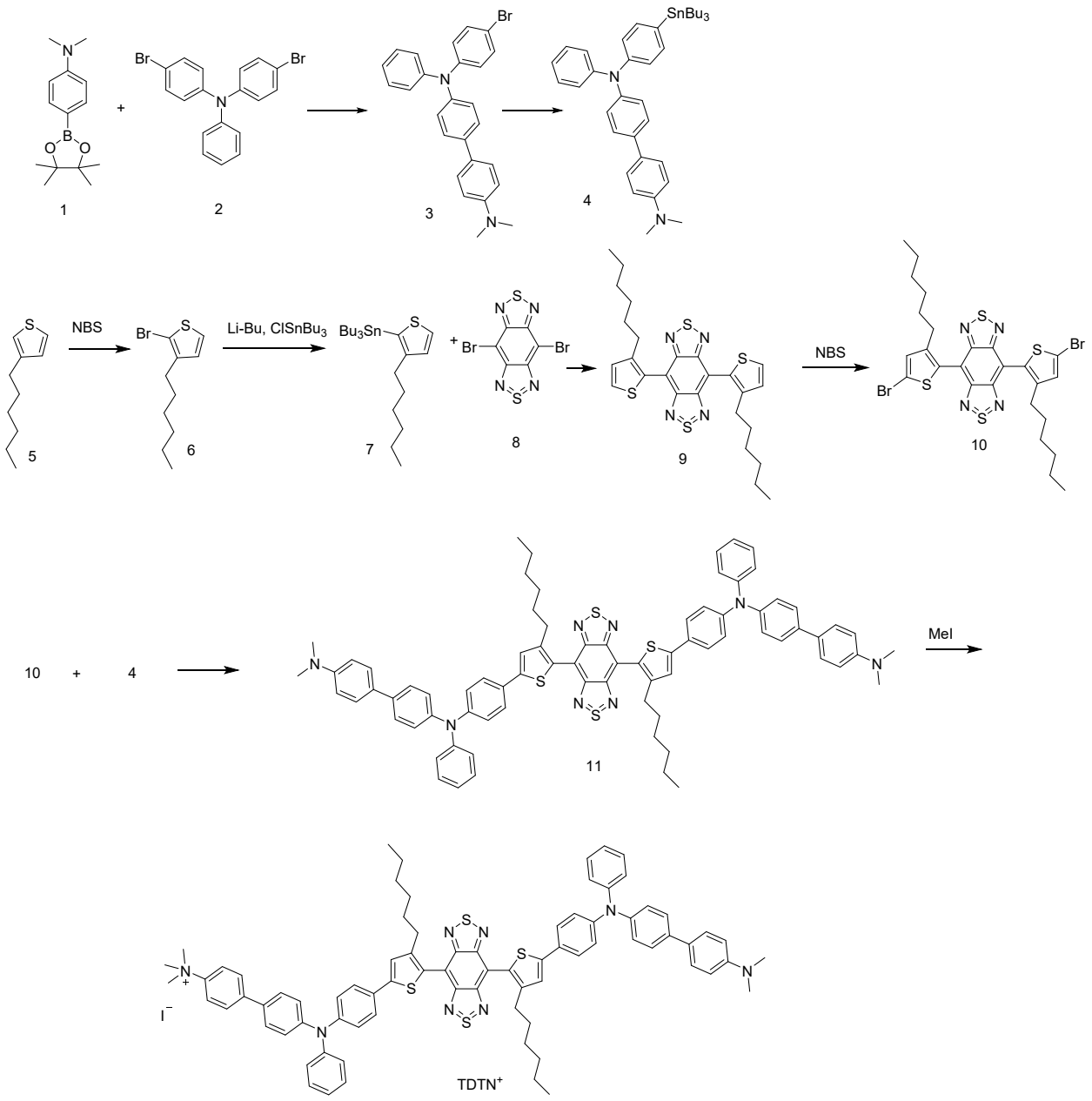
Evaluation of intratumoral oxidative stress

When tumor size reached approximately 100 mm^3, the mice were divided randomly into 4 groups (each group included 5 mice): (1) PBS; (2) 808 nm NIR laser (L, 0.6W/cm^2 ,5 min); (3) $TDTN^+$; and (4) $TDTN^+$ +L. The AIEgens dose was 5 mg/kg in group 3 and 4. Then the fluorescent dye, DCFHDA (10 μmol/L, 50 μL) was injected intratumorally 10min after injection in all groups. Next, The NIR light was performed 0.5 h after intratumor injection. Subsequently, tumors from each group were dissected. The cryosections were observed by a confocal laser scanning microscope (CLSM; IX81, Olympus, Japan).

Statistical analysis

Data analyses were conducted using the GraphPad Prism 5.0 software. Significance between every two groups was calculated by the Student's t-test. *$P < 0.01$, **$P < 0.005$, ***$P < 0.001$.

Synthetic Section



Scheme S1. Synthetic route to **TDTN$^+$.**

Synthetic route of **3**.

Into a 100 mL round-bottle flask, **1** (0.1 g, 0.4 mmol), **2** (1.6 g, 0.4 mmol), $Pd(PPh_3)_4$ (40 mg, 0.035 mmol) and K_2CO_3 (0.77 g, 5.6 mmol) were added. The flask was vacuumed and purged with dry nitrogen three times. Then 10 mL THF and 2 mL H_2O were added, and the system was stirred overnight at 80 °C. After cooling down to room temperature, water was added, and the mixture

was extracted with DCM. The organic phase was combined and dried over Na_2SO_4. After evaporation of the solvent, the residue was purified by column chromatography on silica gel (48% yield).

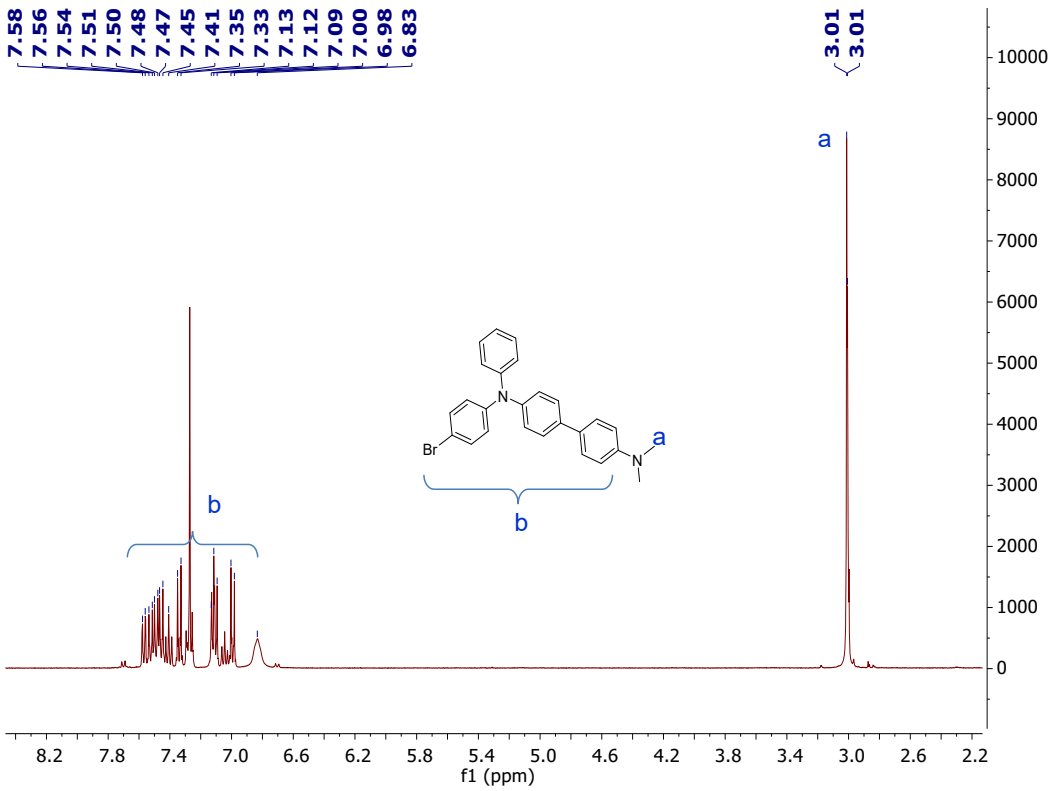


Figure S1. The ^1H NMR of molecule 3 in $CDCl_3$.

Synthetic route of **4**.

Add nBuLi (2.3 mL, 5.8 mmol, 2.4 M in hexane) dropwise to a solution of **3** (2.3 g, 5.2mmol) in THF (30 mL) at -78 °C. Stirring the reaction mixture 1 h at -78 °C. Then tributyltin chloride (1.8 g, 5.8 mmol) was added into the reaction at one portion. After stirring the mixture for 12 h at room temperature, KF solution was added to quench the reaction. The mixture was extracted with hexane three times, the combined organic phase was dried with Na_2SO_4. After removing the solvent, the product was used directly without further purification.

Synthetic route of **6**.

3-Hexylthiophene, **5** (2.6 g, 0.016 mol) was dissolved in a mixture of chloroform and acetic acid (1:1, 50 mL), and N-bromosuccinimide (2.6 g, 0.016 mol) was added slowly at room temperature. The compound was extracted with hexane (3×50 mL), the combined organic layer was washed with water (3×100 mL), dried over anhydrous Na_2SO_4 and concentrated to

yield a yellow oil which was purified by column chromatography on silica gel using hexane as the eluent to obtain a clear oil (yield: 96%).

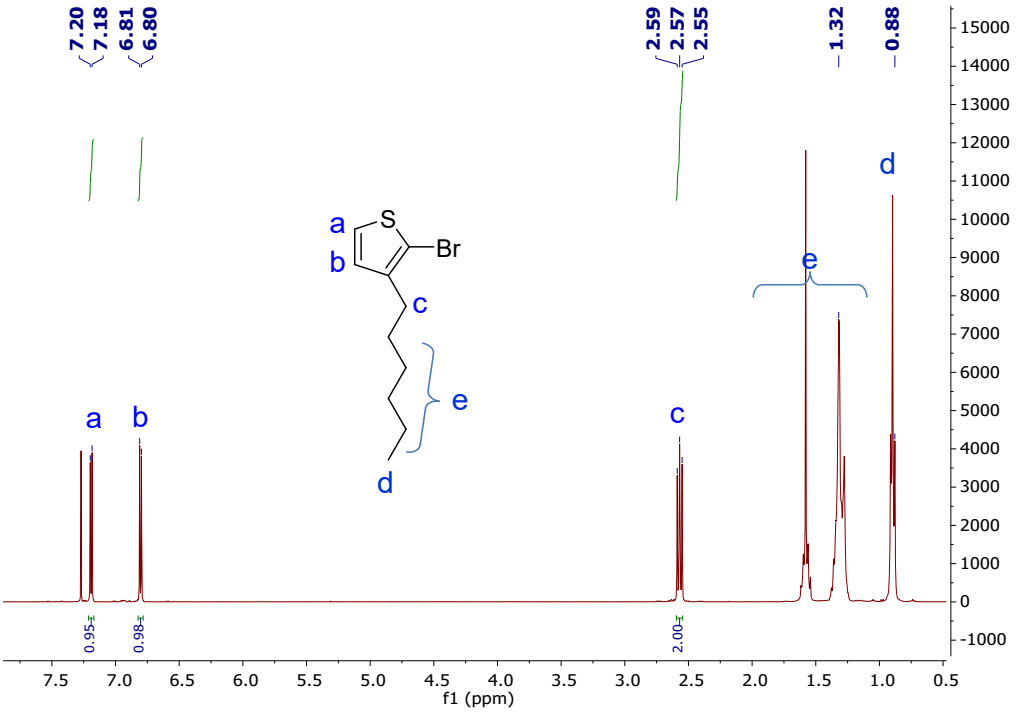


Figure S2. The ^{1}H NMR spectrum of molecule 6 in $CDCl_3$.

Synthetic route of **7**.

Add nBuLi (2.3 mL, 5.8 mmol, 2.4 M in hexane) dropwise to a solution of **6** (1.3 g, 5.2 mmol) in THF (30 mL) at -78 °C. Stirring the reaction mixture 0.5 h at -78 °C. Then tributyltin chloride (1.8 g, 5.8 mmol) was added into the reaction at one portion. After stirring the mixture for 12 h at room temperature, KF solution was added to quench the reaction. The mixture was extracted with hexane for three times, the combined organic phase was dried with Na_2SO_4. After removing the solvent, the product was used directly without further purification.

Synthetic route of **9**.

A 5 mL tube was charged with **7** (0.5 g, 1 mmol), **8** (87 mg, 0.25 mmol), $Pd_2(dba)_3$ (22 mg, 0.025 mmol), P(*o*-tol)$_3$ (66 mg, 0.21 mmol), and degassed dry toluene (1.5 mL), and sealed with a Teflon cap. The reaction mixture was heated with stirring to 130 °C overnight. Upon cooling, the crude product was quenched with KF solution and extracted with DCM. The combined organic phase was dried with Na_2SO_4. After removing the solvent, the product was purified with a silica column to obtain a purple solid (yield: 35%).

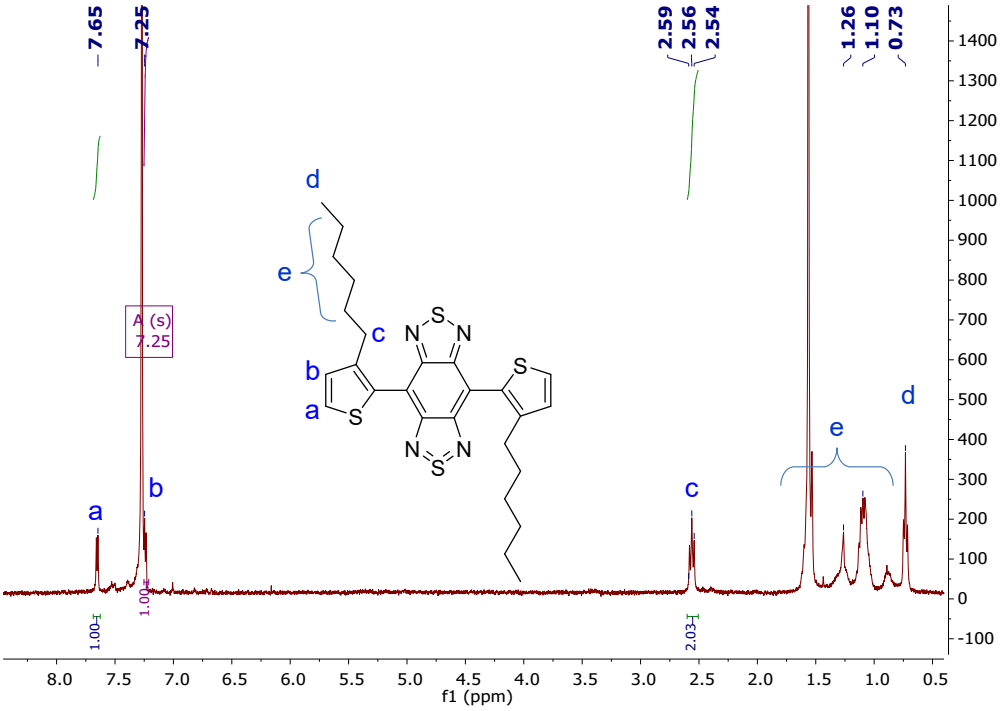


Figure S3. The ^{1}H NMR spectrum of molecule 9 in $CDCl_3$.

Synthetic route of **10**.

Compound **9** (0.3 g, 0.57 mmol) was dissolved in a mixture of 10 mL $CHCl_3$ and 10 mL acetic acid under argon atmosphere, 0.22 g NBS (1.25 mmol) was added slowly over the course of 30 mins in a mixture of 5 mL $CHCl_3$ and 5 mL acetic acid at room temperature under the exclusion of light. The mixture was stirred overnight and was then dried by condensed air. The crude product was purified by silica gel column (hexane) to get the product (yield= 80%).

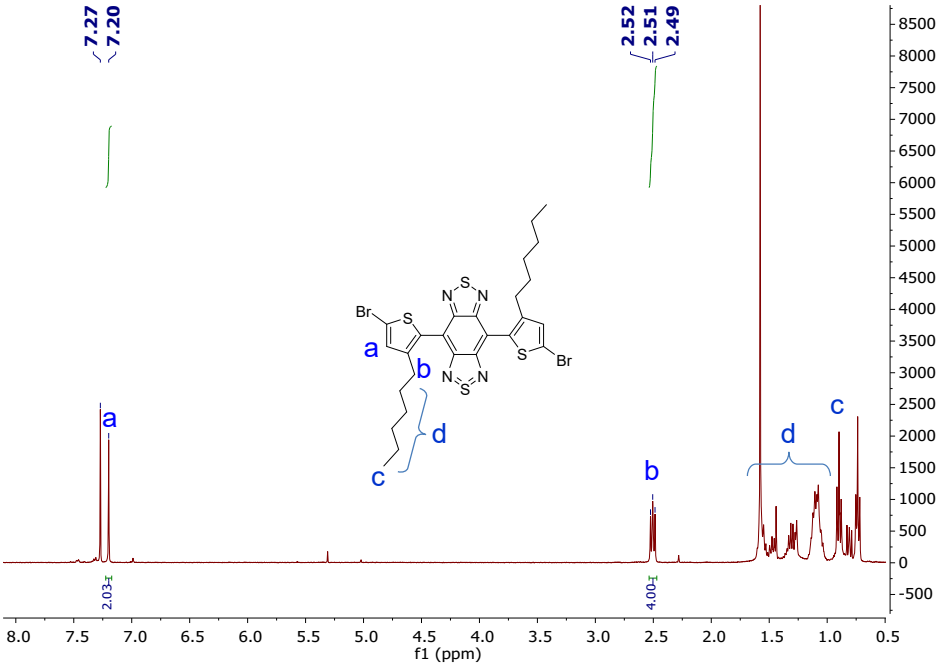


Figure S4. The ^{1}H NMR spectrum of molecule 10 in $CDCl_3$.

Synthetic route of **TDTN**.

To a solution of compound **4** (294 mg, 0.45 mmol) and **10** (101 mg, 0.15 mmol) in toluene (10 mL) was added $Pd(PPh_3)_4$ (4 mg). The mixture was stirred for 12 h at 100 °C. After cooling down to room temperature, the mixture was poured into water and extracted with DCM. The organic layer was washed with saturated KF and brine before being dried over MgSO4. After evaporation of the solvent, the residue was purified by column chromatography on silica gel to afford product (yield: 30%).

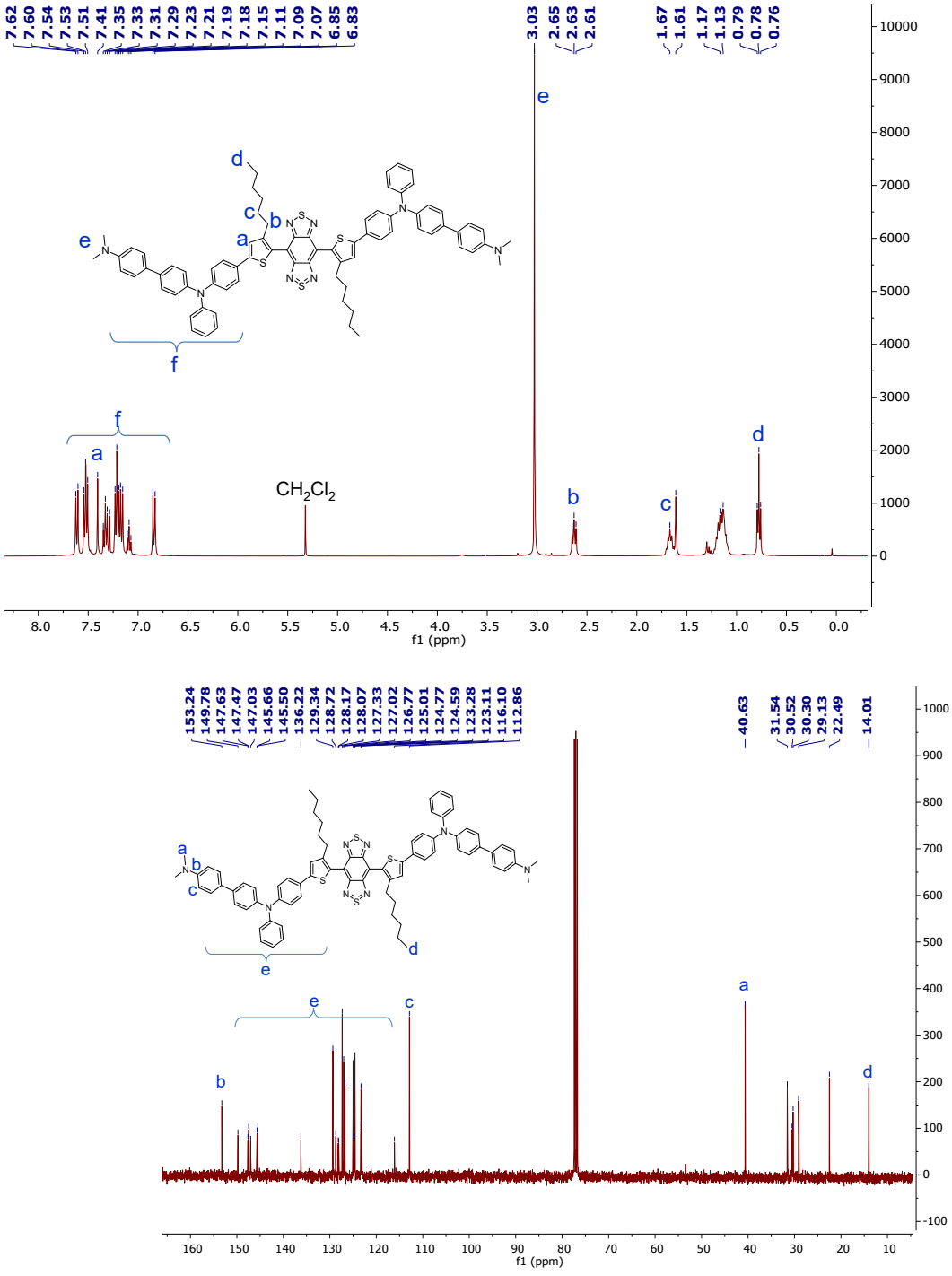


Figure S5. The ^{1}H NMR and ^{13}C NMR spectrum of molecule **TDTN** in $CDCl_3$.

Synthetic route of **TDTN**$^{+}$.

To a solution of compound **TDTN** (100 mg) in acetone (5 mL) was added MeI (1 mL). The mixture was refluxed overnight. After cooling down to room temperature, the mixture was subjected to rotary evaporation. The crude product was purified by DCM washing for several times.

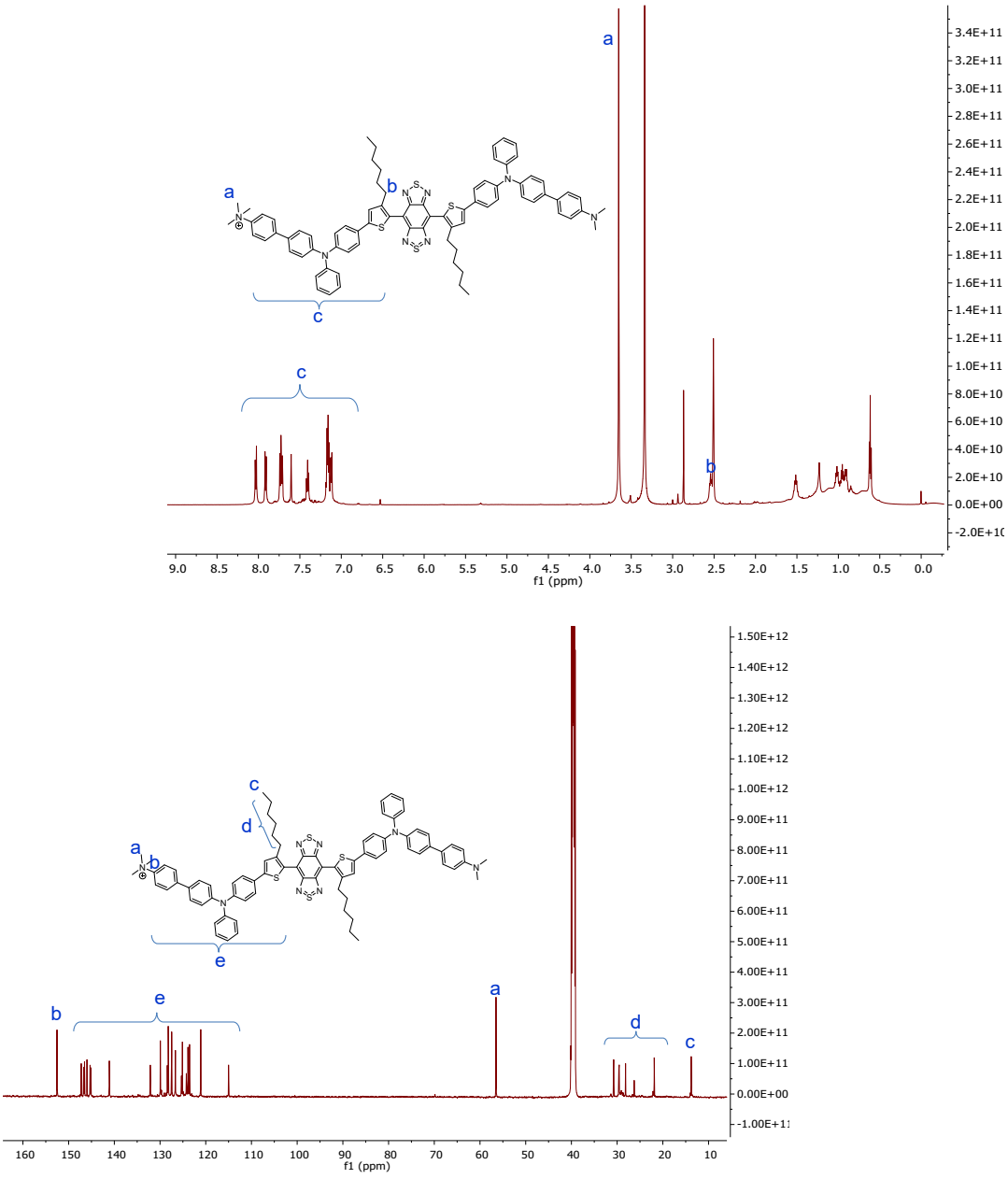


Figure S6. The ^{1}H NMR and ^{13}C NMR spectrum of molecule TDTN^{+} in DMSO-d6.

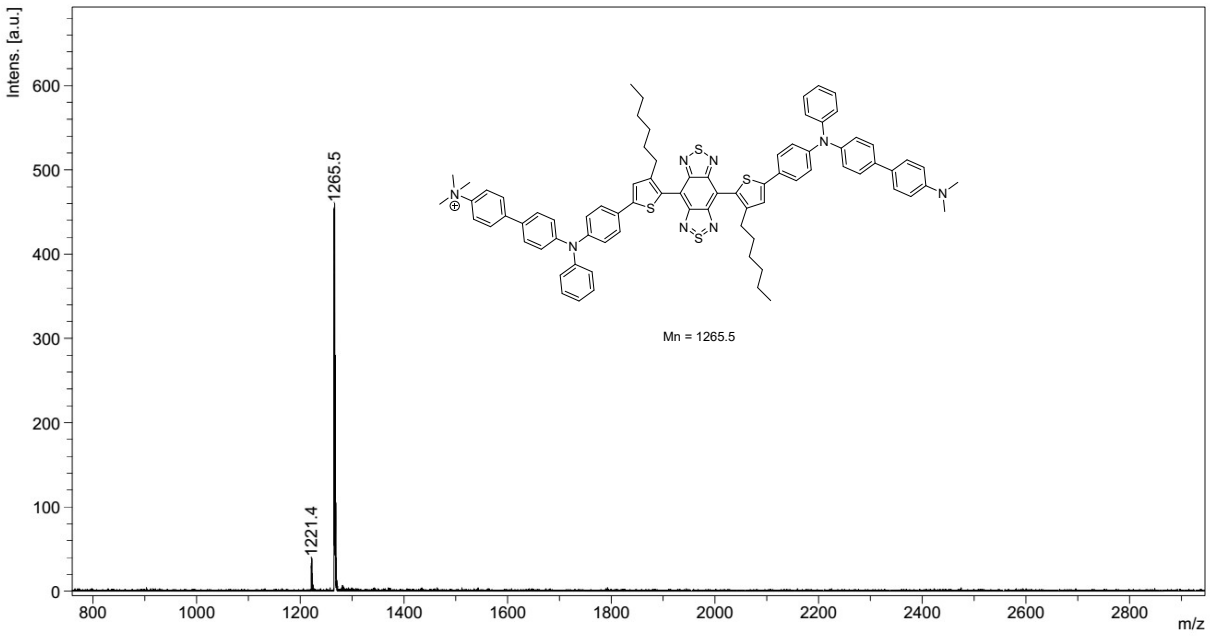


Figure S7. The mass spectrum of molecule $TDTN^+$.

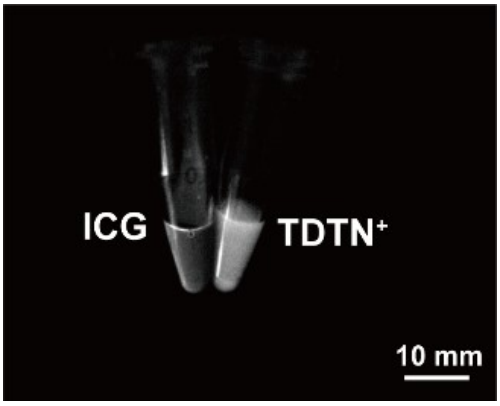


Figure. S8. NIR-II fluorescence of $TDTN^+$ NPs (0.5 mg/mL) and ICG (0.5 mg/mL) in water under 793 nm excitation (30 mW/cm^2). The 1200 nm long-pass filter was applied to collect the photoluminescence.

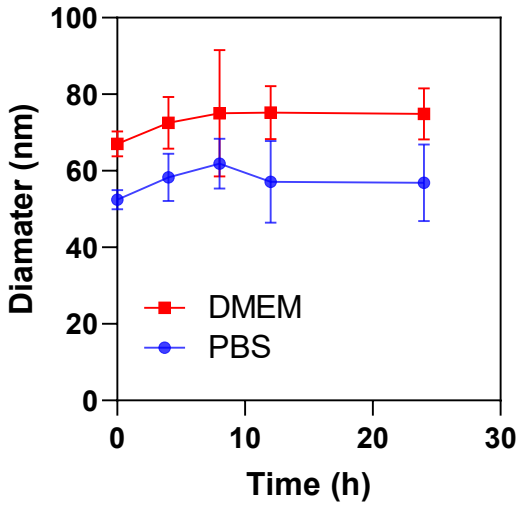


Figure S9. The diameter changes of $TDTN^+$ NPs in PBS and DMEM solution.

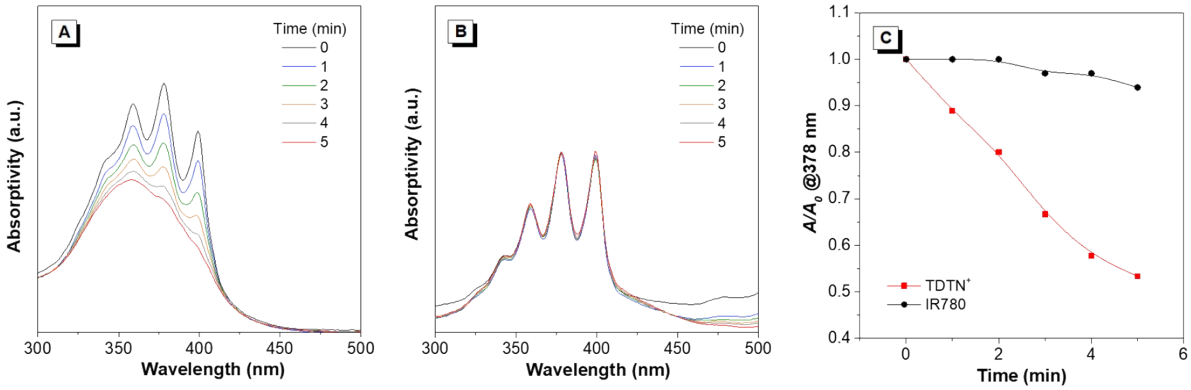


Figure S10. The UV-vis spectrum of ABDA in the absence of $TDTN^+$ (A)and IR780 (B) under laser irradiation in aqueous solution. (C) Plot of relative UV intensity (A/A_0) of ABDA change under laser irradiation.

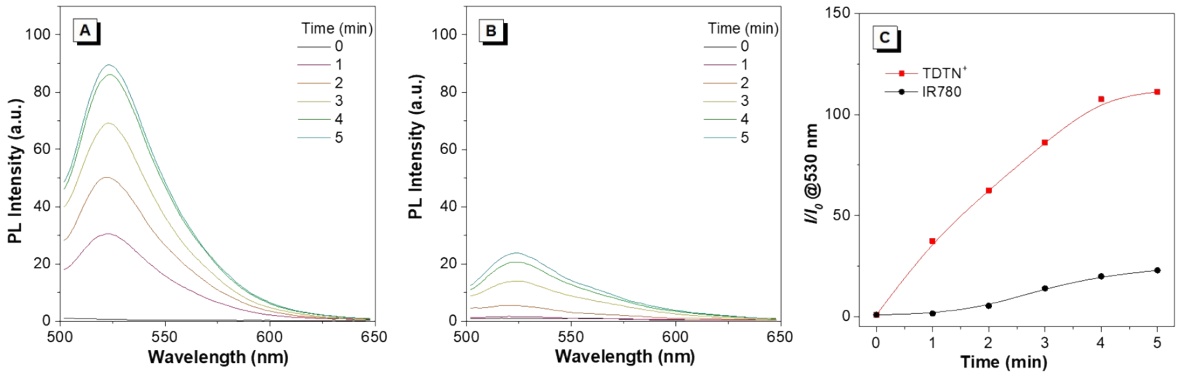


Figure S11. The PL spectrum of HPF in the absence of $TDTN^+$ (A) and IR780 (B) under laser irradiation in aqueous solution. (C) Plot of relative PL intensity (I/I_0) of ABDA change under laser irradiation.

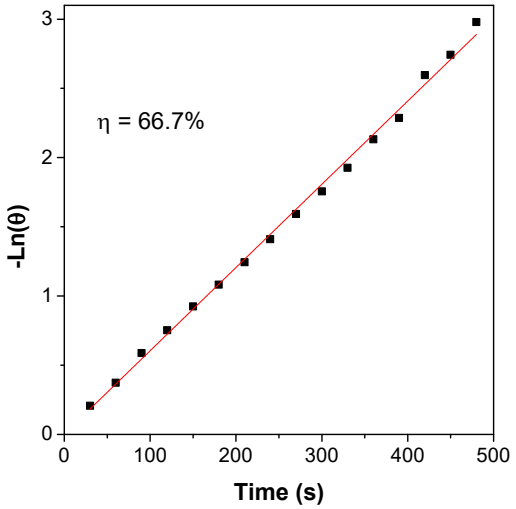


Figure S12. The photothermal conversion efficiency of $TDTN^+$ under 808 nm irradiation.

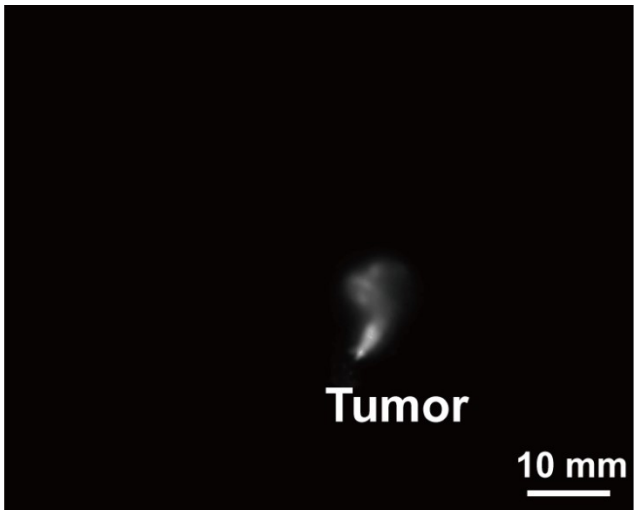


Figure S13. In vivo NIR-II wide-field fluorescence tumor vascular imaging of tumor-bearing mice under 793 nm (30 mW/cm^2) excitation. The 1200 nm long-pass filter was applied to collect the photoluminescence.

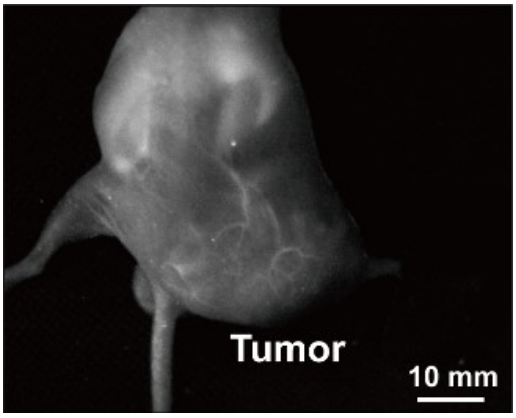


Figure S14. In vivo NIR-II wide-field fluorescence tumor vascular imaging of tumor-bearing mice under 793 nm excitation (50 mW/cm^2). The 1200 nm long-pass filter was applied to collect the photoluminescence.

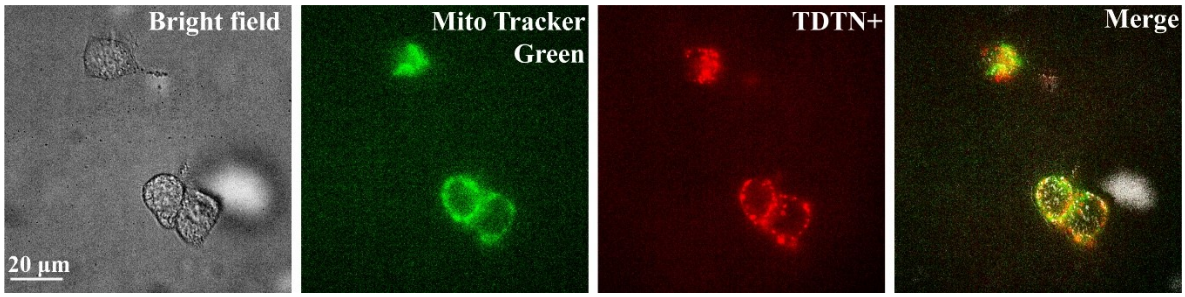


Figure S15. Mitochondrial co-localization of another field of view. Bright-field and fluorescent imaging of HEK293T cells incubated with Mito tracker green (green channel) and $TDTN^+$ solution (NIR-II channel). The merging image confirmed the co-localization effect of $TDTN^+$ on mitochondria.