Supporting Information

Conjugated polymer nanoparticles with absorption beyond 1000 nm for NIR-II fluorescence imaging system guided NIR-II photothermal therapy

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EXPERIMENTAL SECTION

Materials. 6,7-Bis(4-(hexyloxy)phenyl)-4,9-di(thiophen-2-yl)-[1,2,5]thiadiazolo[3,4-g]quinoxaline(TTQ, 97%), (4,4'-di-n-dodecyl-2,2'-bithiophene-5,5'-diyl)bis(trimethylstannane) (2TC, 97%) were purchased from SunaTech Inc. Tris-(dibenzylideneacetone)dipalladium(0) and triphenylphosphine were purchased from J&K Scientific Ltd. PS-PEG was obtained from Xi'an Ruixi Biological Technology Co, Ltd (Xi'an, China). Unless indicated otherwise, all synthetic procedures were performed in an anhydrous and oxygen-free environment, and all reagents were received from commercial sources. These regents were used without further purification, except toluene,

which was dried and distilled with N_2 before use. MDA-MB-231 cells and Annexin V-FITC/propidium iodide (PI) cell apoptosis kit was obtained from KeyGen Biological Technology Co., Ltd (Nanjing, China). Dulbecco's Modified Eagle's Medium (DMEM, Gibco, U.S.) was obtained from Gene Tech Co. (Shanghai, China).

Characterization. The ^{1}H NMR spectra were recorded with a Bruker Ultra Shield Plus 400 MHz spectrometer. $CDCl_3$ was chosen as the solvent, and tetramethylsilane (TMS) was selected as the internal standard and used at 298 K. Gel permeation chromatography (GPC) was performed with THF as the eluent using Shim-pack GPC-80 X columns to determine the number-average molecular weight (M_n) and polydispersity (PDI) of the polymers. The morphology of nanoparticles was observed using a transmission electron microscope (TEM, Hitachi HT7700) with an acceleration voltage of 100 KV. Dynamic light scattering (DLS) analysis was conducted on a commercial laser light scattering spectrometer (ALV-7004; ALV-GmbH, Langen, Germany) equipped with a multi-τ digital time correlator and a He-Ne laser (λ = 632.8 nm). The Hydrodynamic diameters (D_h) data were extracted through a CONTIN analysis. All samples we used for the test were optically cleared with Millipore filters (0.45 μm). The tests were conducted at 90°

scattering angle and under room temperature. A Shimadzu UV-3600 spectrophotometer was utilized to record the absorption spectra of our samples at room temperature. NIR-II fluorescence spectra were measured using an NIR-II spectrophotometer (Fluorolog 3, Horiba). NIR InGaAs was selected as the detector, with an excitation wavelength of 808 nm obtained from a diode laser operating at 25.0 ± 0.5 °C. After the raw emission data were collected, the fluorescence signal was further confirmed and corrected for the sensitivity of InGaAs detector profile and output through the T1c channel. The laser was purchased from Changchun New Industries Optoelectronics Technology Co., Ltd. The *in vitro* and *in vivo* NIR-II FI experiments were conducted on an NIR-II imaging system (Wuhan Grand-imaging Technology Co., Ltd) with different filters and two types of lenses (50 or 100 mm) under the 808 nm laser irradiation. A 640 × 512 pixel two-dimensional InGaAs array from Princeton Instruments in NIR-II fluorescence windows was equipped in this NIR-II FI system. All photothermal tests were detected using a Fotric 225 instrument (IR thermal camera, ± 2 °C) purchased from Fotric Co., Ltd (Shanghai, China). The methyl thiazolyl tetrazolium (MTT) analysis was conducted using a PowerWave XS/XS2 microplate spectrophotometer (BioTek, Winooski, VT). The flow cytometry experiments were

performed using a Flow Sight Imaging Flow Cytometer (Merck Millipore, Darmstadt, Germany).

Synthesis of TTQ-2TC. For the synthesis of the conjugated copolymer, a solution of monomer 6,7-Bis(4-(hexyloxy)phenyl)-4,9-di(thiophen-2-yl)-[1,2,5]thiadiazolo[3,4-g]quinoxaline (TTQ) (43.15 mg, 0.05 mmol), (4,4'-di-n-dodecyl-2,2'-bithiophene-5,5'-diyl)bis(trimethylstannane) (2TC) (41.40 mg, 0.05mmol), dried toluene (2 mL), tris(dibenzylideneacetone)dipalladium(0) (1.38 mg, 1.5 μmol), triphenylphosphine (1.58 mg, 6 μmol), and a magnetic stirring bar were added into a schlenk tube. The solution was deoxygenated after flushing with nitrogen for 20 min. The reaction was conducted at 100°C under N_2 atmosphere in dark for 30 min with magnetic stirring, and stopped by rapidly cooled down to room temperature and precipitated in methanol. The polymer was collected by filtration and dried under vacuum to yield TTQ-2TC (30.6 mg, 71%) as a dark red solid.

Preparation of conjugated polymer nanoparticles. The preparation of PEG encapsulated conjugated polymer nanoparticles was used as previously reported.[1] Briefly, to prepare nanoparticles through nanoprecipitation, TTQ-2TC (0.5 mg) and PS-PEG (5

mg) were dissolved in THF (2 mL) by bath sonication, and the resulted THF solution containing the conjugated polymers (0.25 mg mL^{-1}) and PS-PEG (2.5 mg mL^{-1}) was then quickly injected into 10 mL water under continuous ultrasonication for 5 min. After nanoprecipitation occurred, THF was removed by rotary evaporation under vacuum at 50°C. The aqueous solutions were filtered through a polyethersulfone (PES) syringe driven filter (0.8 μm) (Millipore) to remove impurities and big particles. The filtered aqueous solution was washed three times using a 100 K centrifugal filter units (Millipore) under centrifugation at 2,800 rpm for 10 min. The final TTQ-2TC NPs solution was stored in dark at 4°C。

Photostability. TTQ-2TC NPs was dissolved in PBS, DMEM and FBS at a concentration of 0.1 mg mL^{-1}. The NIR-II fluorescence signal of TTQ-2TC NPs was measured with the NIR-II spectrophotometer mentioned above under laser illumination (808 nm, 0.5 w cm^{-2}) for more than 30 min. The fluorescence intensity of the region of interest was plotted as a function of time.

Quantum yield test. The fluorescence quantum yield (QY) of TTQ-2TC-3T NPs was measured using the method described in a previous study. The QYs were determined

according to the reference fluorophore IR1061, which has a QY value of 1.7 ± 0.5% in THF. The parameter n is the refractive index of solvent. Five different concentrations around or less than an OD of 0.1 (approximately 0.1, 0.08, 0.06, 0.04 and 0.02) were measured, and all samples were analyzed at 25 °C. After comparing the slopes of the integrated fluorescence, which was plotted against the absorbance for both the reference and samples, the QY was calculated using the following equation:

$$\mathbf{QY_{(sample)}} = \mathbf{QY_{(ref)}} \times \frac{\mathbf{slope_{(sample)}}}{\mathbf{slope_{(ref)}}} \times \frac{\mathbf{n^2_{(sample)}}}{\mathbf{n^2_{(ref)}}}$$

Cell lines and cell culture. MDA-MB-231 cells were bought from Key-gen Biotech Co. Ltd. (Nanjing, China), and incubated in complete Dulbecco's Modified Eagle Medium (DMEM) cell culture medium containing 10% fetal bovine serum (FBS) under the conventional environment (37°C, 5% CO_2).

Cytotoxicity studies for TTQ-2TC NPs alone. The MTT assay was used to determine the *in vitro* cytotoxicity of TTQ-2TC NPs in MDA-MB-231 cell lines. Cells were seeded in 96-well plates (Costar, IL, U.S.A.) at a density of 2×10^4 cells mL^{-1}, respectively. After incubating with complete DMEM (containing 10% FBS) for 24 h under standard condition

(37℃, 5% CO_2) in dark, different concentrations of TTQ-2TC NPs (100 μL) were diluted in DMEM and added to the wells. The cells were cultured in a dark environment for another 24 h. After the cells adhered, TTQ-2TC NPs solution diluted with DMEM solution was added. After that, TTQ-2TC NPs suspensions were replaced by fresh DMEM and then washed with PBS buffer; 100 μL of freshly prepared MTT solution was added into each well. After incubation at 37 °C for 3 h, the supernatant was removed and 200 μL of dimethyl sulfoxide (DMSO) was added, and the plate was gently shaken for 10 min at room temperature to dissolve all the precipitates formed. A PowerWave XS/XS2 microplate spectrophotometer was used to record the absorbance intensity at 490 nm.

In vitro cell uptake ability of TTQ-2TC NPs. Six-well plates were used to incubate MDA-MB-231 cells (3×10^5 cells $well^{-1}$) in DMEM (containing 10% FBS). After 24 h, the cells were divided into two groups that were subjected to various treatments: (a) cells with fresh complete DMEM (containing no FBS); (b) cells with fresh DMEM (containing no FBS) for one day and then incubation with a 50 μg mL^{-1} solution of TTQ-2TC NPs for 4 h; After the different treatments, the cells were washed 3 times with PBS, and the harvested cells were added in a 96-wells plate. NIR-II images of the two groups of cells were obtained

with a commercial NIR-II fluorescence imaging system. The NIR-II signal intensity of cells indicated the in vitro cellular uptake capability of TTQ-2TC NPs.

Assessment of NIR-II PTT *in vitro* using the MTT assay. To determine the *in vitro* therapic efficiency of TTQ-2TC NPs, the MDA-MB-231 cells were seeded in 96-wells (Costar, IL, U.S.A.) plates at a concentration of 2×10^4 cells mL^{-1}, respectively. After 24 h, cells were transferred to a mixed medium containing different doses (0.0625, 0.125, 0.25, 0.5 and 0.1 mg mL^{-1}) of materials from different groups (TTQ-2TC NPs, TTQ-2TC NPs + Laser) and incubated in the dark. Thereafter, selected wells from the different groups were illuminated with or without an 1064 nm laser at a power of 1.0 W cm^{-2} for 5 min. After another incubation for 24 h, fresh DMEM containing MTT was added to each well and incubated for 3 h. Then, DMEM was removed and DMSO (200 μL) was added. Thereafter, the 96-well plates were shaken for 10 min. Finally, the absorbance of each well was measured at 490 nm using a PowerWave XS/XS2 microplate spectrophotometer.

Assessment of the NIR-II photothermal effect *in vitro* by confocal imaging. MDA-MB-231 cells were cultured with DMEM in CLSM culture dishes (Costar) until the cell density increased to 1×10^5 cells mL^{-1} per well. Materials in different groups (PBS, PBS + laser,

only TTQ-2TC NPs, and TTQ-2TC NPs + laser) were added to fresh DMEM to obtain the mixed medium (0.1 mg mL^{-1}). After the MDA-MB-231 cells had been co-incubated with this mixed medium for 4 h in the dark, the media were removed, the cells were washed by PBS and replaced with fresh DMEM. Next, the selected wells from different groups were illuminated with or without a 1064 nm laser at a power of 1 W cm^{-2} for 5 min. After a 24 h incubation to allow cells to undergo apoptosis, the MDA-MB-231 cells that had detached from the CLSM culture dishes (Costar) were washed several times with PBS and then incubated with Calcein AM/propidium iodide (PI) solution for 5 min. Then, the cells were imaged by CLSM (Olympus Fluoview FV1000).

Animal Experiments. All experiments using animals were performed according to the specifications of The National Regulation of China for Care and Use of Laboratory Animals, and the protocol was approved by the Jiangsu Administration of Experimental Animals. Five- to six-week-old BALB/c mice were obtained from the Shanghai Laboratory Animal Center, Chinese Academy of Science (SLACCAS). MDA-MB-231 tumors were established in female BALB/c nude mice (aged 5-6 weeks) by injecting MDA-MB-231 cells mixed with 50 μL of PBS under the skin beside the left armpit. The tumor volume was

measured using the equation $V = 0.5\ L\ W^2$, where L refers to the longitudinal diameter and W indicates the transverse diameters of the tumors.

***In vivo* NIR-II fluorescence imaging.** When the tumors reached a volume of 90-120 mm^3, MDA-MB-231 tumor bearing nude mice (n = 3 per group) were intravenously injected with TTQ-2TC (100 μL, 1.0 mg mL^{-1}). The tumor bearing mice were imaged alive by anesthetizing them with isoflurane during the test time (just about 10 minutes) in the case of the effect of respiration. The real-time *in vivo* NIR-II fluorescence imaging was performed at different post-injection times by using an *in vivo* NIR-II fluorescence imaging system with 1064 nm LP filters. The excitation wavelength was 808 nm produced by a semiconductor laser. The analysis of the signal intensity of NIR-II image was performed using the NIR-II *in vivo* imaging system software.

***In vivo* NIR-II photothermal therapy.** When the tumor volume reached 90-120 mm^3, the MDA-MB-231 tumor-bearing mice were weighed, randomly divided into 4 groups (n = 6 per group), and given the following treatments: (a) mice treated with PBS (100 μL) without laser irradiation, (b) mice treated with PBS and 1064 nm laser irradiation, (c) mice treated with TTQ-2TC (1.0 mg mL^{-1}, 100 μL) without laser irradiation, and (d) mice treated with TTQ-2TC

(1.0 mg mL^{-1}, 100 μL) and then exposed to the 1064 nm laser irradiation. About 12 h after the intravenous injection, the tumor regions of the abovementioned group b (PBS + laser group) and group d (TTQ-2TC NPs + laser group) were irradiated under 1064 nm continuous laser irradiation for 10 min at 1.0 W cm^{-2} power density. Tumor volume and mice weight were assessed every other day in the next 14 days. After 14 days, these mice were sacrificed, and the tumors and their major organs were stained with hematoxylin-eosin (H&E) staining. The histological tumor sections were observed using an optical microscope. The weight of the tumors were also measured.

Statistical Analysis. All data were reported as mean ± standard deviation and all experiments were repeated at least three times. The results were statistically analyzed by using one-way analysis of variance in GraphPad Prism software (version 7), and p value ≤ 0.05 was considered to be statistically significant.

REFERENCES

(1) Zhang, W.; Sun, X.; Huang, T.; Pan, X.; Sun, P.; Li, J.; Zhang, H.; Lu, X.; Fan, Q.; Huang, W. 1300 nm absorption two-acceptor semiconducting polymer nanoparticles for NIR-II photoacoustic imaging system guided NIR-II photothermal therapy. *Chem. Commun.* **2019**, 55 (64), 9487-9490.

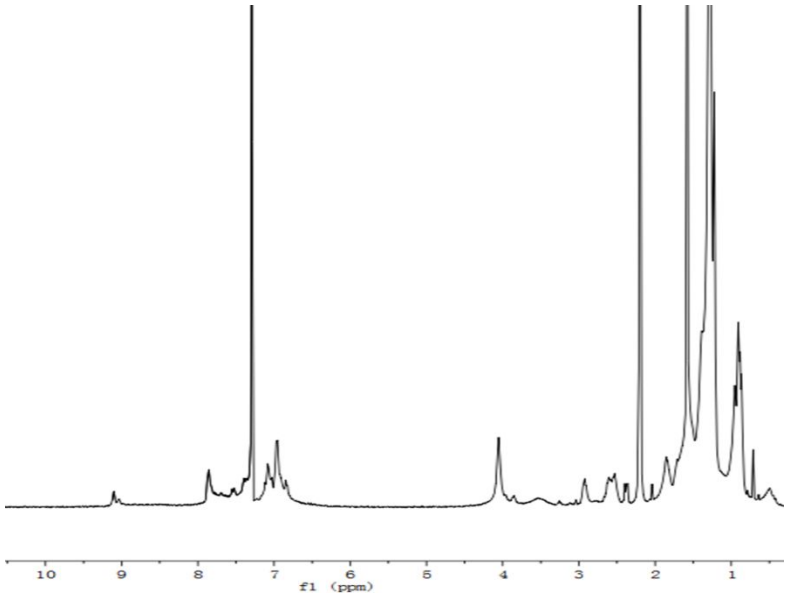

Figure S1. ^{1}H NMR spectrum of TTQ-2TC in $CDCl_3$.

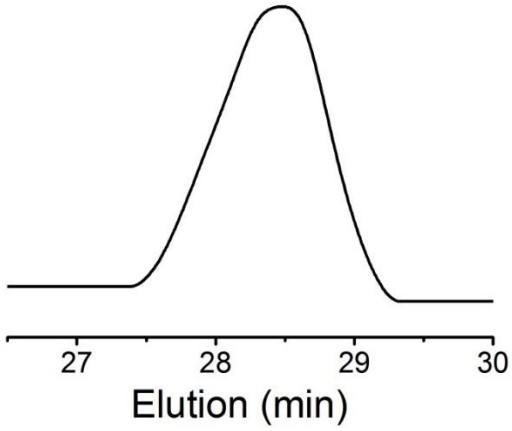

Figure S2. The GPC curve of TTQ-2TC.

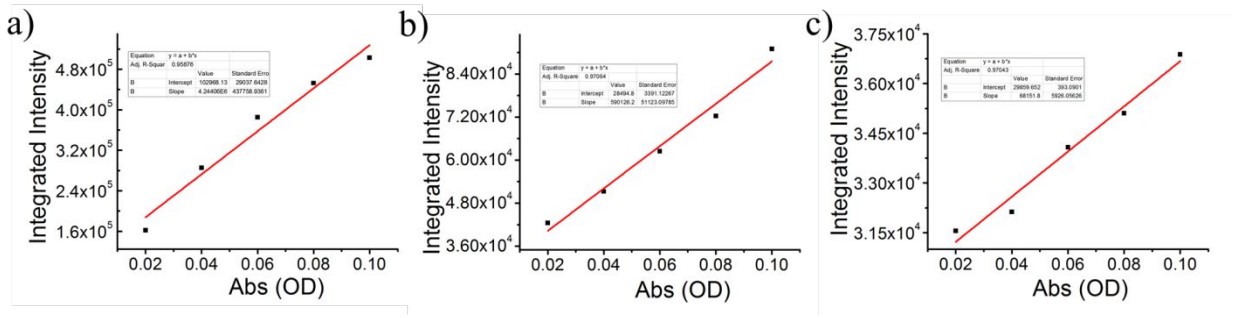

Figure S3. Quantum yield measurement of TTQ-2TC NPs with IR 1061 as the reference sample. Pot of the integrated fluorescence intensity of (a) IR1061 in THF, (b) TTQ-2TC in THF, (c) TTQ-2TC NPs in water.

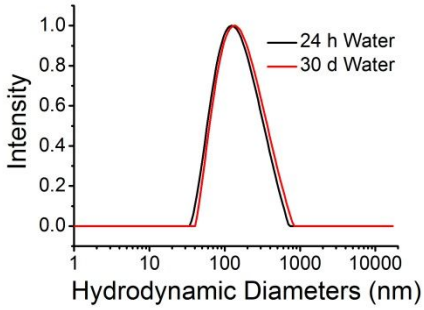

Figure S4. Hydrodynamic diameters (D_h) of TTQ-2TC NPs dispersed in water after storage for 24 hours and 30 days, respectively.

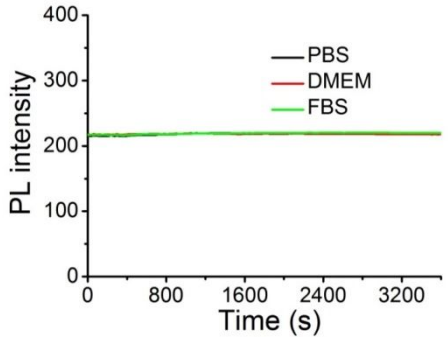

Figure S5. Photostability test curves of TTQ-2TC NPs in PBS, DMEM and FBS under continuous 0.5 w cm^{-2} 808 nm laser radiation.

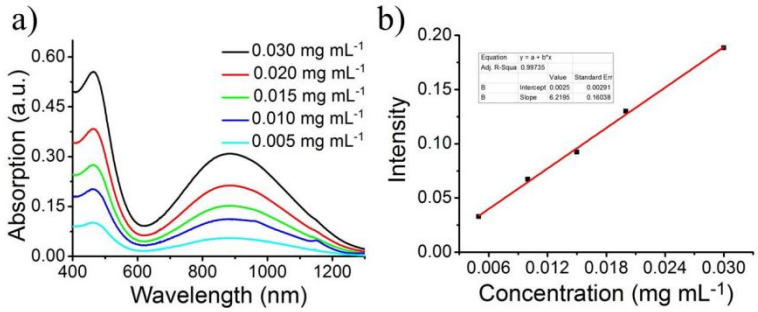

Figure S6. a) UV-vis-NIR spectra of TTQ-2TC NPs in water at different concentrations. b) The mole extinction coefficient of TTQ-2TC NPs at 1064 nm.

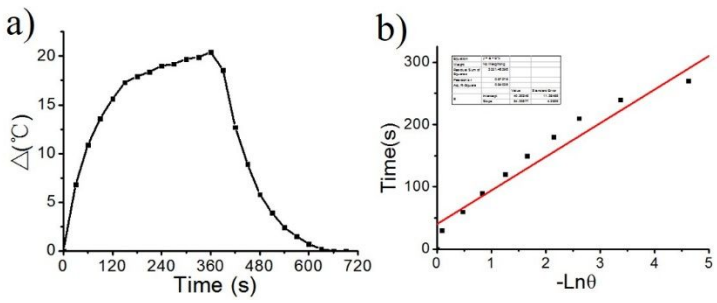

Figure S7. a) Temperature change (ΔT) of TTQ-2TC NPs with 1064 nm laser (1.0 W cm^{-2}). b) Linear time data versus negative natural logarithm was obtained from the cooling period.

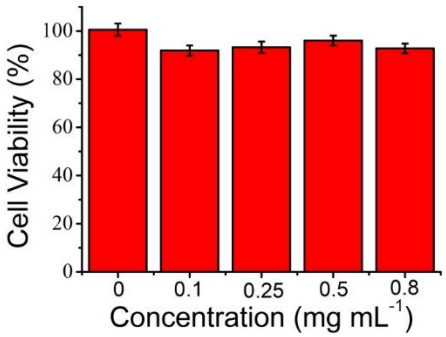

Figure S8. Cell viability of MDA-MB-231 cells after incubation in dark with TTQ-2TC NPs at various concentrations for 24 h.

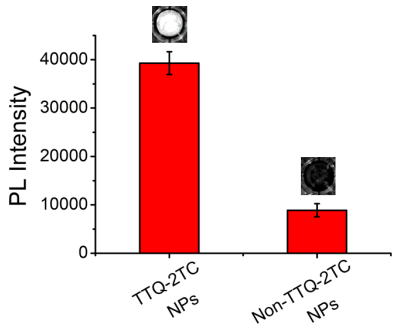

Figure S9. In vitro cell uptake ability of TTQ-2TC NPs to MDA-MB-231 cells. NIR-II fluorescence signals intensity of pure MDA-MB-231 (Non-TTQ-2TC NPs), and MDA-MB-231 cells incubated with TTQ-2TC NPs (TTQ-2TC NPs).

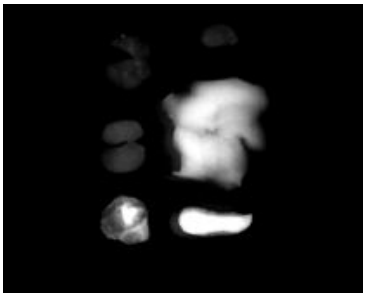

Figure S10. *Ex vivo* fluorescence imaging of major organs (heart, liver, spleen, lung, and kidney).

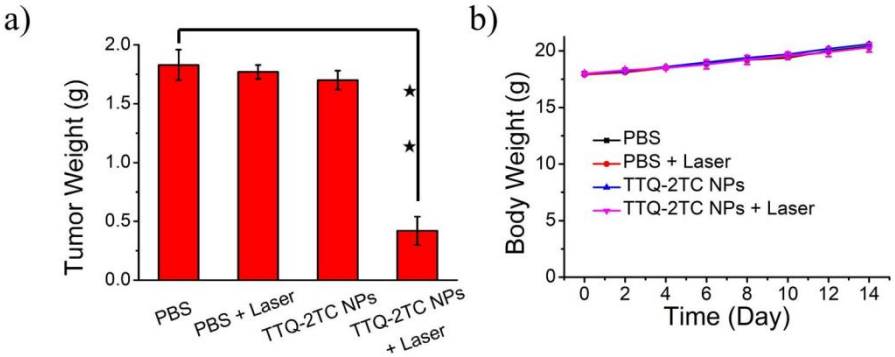

Figure S11. (a) Weight of tumor after 14 days of therapy. (b) Changes of body weight with time for different treatment groups.