Supplementary Information

Engineering of Small Molecular Organic Nanoparticles for Mitochondria-Targeted Mild Photothermal Therapy of Malignant Breast Cancers

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Materials and Methods

Materials

1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[square(polyethylene glycol)-2000] (DSPE-PEG), dipalmitoylphosphatidylethanol-polyethylene glycol-triphenyl phosphate (DSPE-PEG-TPP), and indocyanine green (ICG) were purchased from Ruixi Biological Technology (Xi'an, China). Roswell park memorial institute-1640 medium (RPMI 1640), phosphate buffered saline (PBS), trypsin-EDTA solution and 3-(4,5-dimethylthiazol-2-*yl*)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sangon Biotech (Shanghai, China). Fetal bovine serum (FBS) was purchased from Biological Industries.

Characterization

NMR spectra were obtained using a Bruker 500 NMR spectrometer. UV-vis (Shimadzu Model UV-1700 spectrometer) was used to test the absorption properties of NP suspension. The size and zeta-potential of synthesized NPs samples was measured on NanoBrook Omni Particle Size Analyzer (Brookhaven Instruments, USA). The morphology of the NPs samples was characterized by transmission electron microscope (TEM) on a Tecnai G2 F30 S-Twin system

(Philips-FEI, Netherlands), operating at an acceleration of 100 kV. The photoluminescence spectra and fluorescent decay kinetics was measured with a FLS 980 fluorescence spectrometer (Edinburgh Instruments, Livingston, UK) equipped with an Xe lamp and μs flash Xe lamp as the excitation source. DFT and time-dependent DFT (TDDFT) calculations were done using the Gaussian 09 program on a trimer model *via* the B3LYP functional with the 6-31G(d,p) basis set. To reduce the computational time, the alkyl side-chains were replaced with the methyl groups.

Synthesis of TD0

A solution of 4-(octyloxy)-*N*-(4-(octyloxy) phenyl)-*N*-(4-(thiophen-2-yl) phenyl) aniline (compound 1, 234 mg, 0.4 mmol) and dried tetrahydrofuran (40 mL) was stirred at -78 ℃ of dry ice/acetone bath under argon atmosphere. Then butyl lithium (0.48 mmol) was added drop by drop. The mixture was further stirred at -78 ℃ for 3 h. In the following, tributyltin chloride (187 mg, 0.58 mmol) was added in one portion. After that, the reaction was slowly warmed to room temperature and further stirred overnight. When the reaction was finished, the mixture was poured into 500 mL of water and subsequently extracted with dichloromethane for two times. The combined organic mixture was dried over $MgSO_4$ and concentrated *via* a rotary evaporator to yield 4-(octyloxy)-*N*-(4-(octyloxy) phenyl)-*N*-(4-(5-(tributylstannyl) thiophen-2-yl) phenyl) aniline (compound 2) as viscous oil. Compound 2 was used directly without further purification for next step. A mixture of TBZ (44.7 mg, 0.1 mmol), Pd $(PPh_3)_4$ (11.6 mg, 0.01 mmol) and anhydrous toluene (20 mL) was stirred at 95℃ under argon atmosphere overnight. After the reaction was completed, the solvent was removed by rotary evaporator. The crude product was purified by silica column with hexane/dichloromethane (v: v = 6/4) as the eluent to afford TD0 as green solids (41 mg, yield 28%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.77 (s, 2H), 7.60 (br, 4H), 7.38 (br, 2H), 7.13 (d, J = 8.52, 10H), 6.99 (d, J = 8.4, 4H), 6.89 (d, J = 8.68, 10H), 4.86 (s, 2H), 3.97 (t, 8H), 2.38 (m, 1H), 1.82 (m, 8H), 1.50-1.33 (m, 48H), 1.07 (t, 3H), 0.93-0.92 (m, 15H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 155.69, 149.90, 148.55, 142.50, 140.37, 137.35, 135.91, 132.20, 126.79, 126.44, 122.55, 120.29, 115.32, 111.28, 68.30, 60.73, 40.63, 31.87, 30.73,

29.43, 29.41, 29.30, 28.56, 26.14, 24.15, 23.00, 22.71, 14.15, 10.68. MALDI: calcd for $C_{90}H_{114}N_7O_4S_3$: 1452.81, found 1452.81.

Synthesis of TD1

A solution of 4-([2,2'-bithiophen]-5-yl)-*N,N*-bis(4-(octyloxy)phenyl)aniline (compound 3, 266.4 mg, 0.4 mmol) and dried tetrahydrofuran (40 mL) was stirred at -78 ℃ of dry ice/acetone bath under argon atmosphere. Then butyl lithium (0.48 mmol) was added drop by drop. The mixture was further stirred at -78℃ for 3h. In the following, tributyltin chloride (187 mg, 0.58 mmol) was added in one portion. After that, the reaction was slowly warmed to room temperature and further stirred overnight. When the reaction was finished, the mixture was poured into 500 mL of water and subsequently extracted with dichloromethane for two times. The combined organic mixture was dried over $MgSO_4$ and concentrated *via* a rotary evaporator to yield 4-(octyloxy)-*N*-(4-(octyloxy) phenyl)-*N*-(4-(5'-(tributylstannyl)-[2,2'-bithiophen]-5-yl) phenyl) aniline (compound 4) as viscous oil. Compound 4 was used directly without further purification for next step. A mixture of TBZ (44.7 mg, 0.1 mmol), Pd $(PPh_3)_4$ (11.6 mg, 0.01 mmol) and anhydrous toluene (20 mL) was stirred at 95℃ under argon atmosphere overnight. After the reaction was completed, the solvent was removed by rotary evaporator. The crude product was purified by silica column with hexane/dichloromethane (v: v = 5/5) as the eluent to afford TD1 as green solids (37 mg, yield 23%). ^{1}H NMR (400 MHz, CDCl3) δ 8.48 (br, 2H), 7.30 (br, 4H), 6.99 (d, J = 8.76, H), 6.84 (d, J = 8.60, H), 6.76 (d, J = 8.88, H), 4.74 (br, 2H), 3.86 (t, 8H), 2.26 (m, 1H), 1.70 (m, 8H), 1.38-1.18 (m, 48H), 0.98 (m, 3H), 0.87-0.80 (m, 15H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 155.67, 148.31, 140.39, 126.78, 120.21, 115.31, 68.29, 60.70, 40.71, 31.87, 30.75, 29.73, 29.43, 29.41, 29.30, 28.59, 26.13, 24.18, 23.03, 22.70, 14.15, 10.71. MALDI: calculated for $C_{98}H_{118}N_5O_4S_5$: 1616.78, found 1616.78.

Construction of nanoparticles

The synthetic route for TD1 NPs and M-TD1 NPs is shown as follows. For TD1 NPs, TD1 (1 mg) and DSPE-PEG (3 mg) were dissolved in 1 mL of THF to form a homogeneous solution. The mixture was

added drop by drop to ultrapure water (10 mL) under ultrasound sonification at the power of 15 W within one minute. For M-TD1 NPs, The Mixture of TD1 (1 mg), DSPE-PEG (2 mg) and DSPE-PEG-TPP (1mg) in THF were added into 10 mL water under ultrasound sonification. The obtained emulsion was further stirred in the dark by rotary evaporation to remove THF at 200 rpm overnight. Then, the solution was purified by dialysis against water with a membrane with molecular cutoff of 8 kDa for 2 days. The obtained TD1 NPs and M-TD1 NPs were concentrated to 1 mg/mL (based on TD1 mass concentration) through an ultrafiltration tube, respectively. To fabricate the Alex-TD1 NPs and M-Alex TD1 NPs, the Alexa Fluor 647 were co-encapsulated with TD1, according to weight ratio of 1:1.

Photothermal Properties of the NPs

The temperature rise of NPs samples was measured by the FLIR ONE PRO (FLIR Systems Inc, USA). Different concentrations of NPs were dispersed in 100 μL of ultrapure water, and concentrations range from 0, 10, 20, 50 $\mu g \cdot mL^{-1}$. The different samples were irradiated 10 min in 96-well plates by 808 nm near infrared light (NIR) laser at 2.0 $W \cdot cm^{-2}$. We recorded three continuous changes of ten-minute heating and ten-minute cooling with different NPs at 50 $ug \cdot mL^{-1}$, 2.0 $W \cdot cm^{-2}$. The photothermal conversion efficiency was then calculated according to the published literature [1].

Cell Culture

The mouse breast 4T1 cancer cells line was received from Zhejiang University. 4T1 cells line was cultured in RPMI 1640 medium, supplemented with 10% fetal bovine serum, at 37 ℃ in a humidified atmosphere containing 5% CO_2.

Cellular Internalization and Mitochondrial Colocalization

We fabricated the Alex-TD1 NPs and M-Alex TD1 NPs to trace NPs as mentioned above. 4T1 cells were treated with Alex-TD1 NPs or M- Alex-TD1 NPs and incubated for 6 h, to trace the cellular uptake by flow cytometer (BD Biosciences, USA). For colocalization measurement, 4T1 cells were treated with Alex NPs or M- Alex NPs (20 μg·ml^{-1}, 6 h) and then stained with Hoechst and Mito-Tracker Green at 37 ºC for another 30 min. Cells were washed three times by PBS and then photographed with a confocal laser scanning microscopy (CLSM) (Zeiss LSM 880, Germany). For Alex-TD1 NPs, M-Alex-TD1 NPs, ex = 645 nm, 670-780 nm band pass filter was used; for MitoTracker, ex = 543 nm, 575-625 nm band pass filter was used.

MTT Assays

The 4T1 cells were seeded into 96-well plates with 1×10^4 cells per cell for 24 h. Next, different concentrations of TD1 NPs and M-TD1 NPs (0, 10, 20, 30, 40, and 50 μg·mL^{-1}) were added to the culture media without FBS, respectively. After 24 h incubation, 100 μL of 0.5 mg·mL^{-1} MTT solution were then added into per well after washed three times with phosphate-buffered saline (PBS). For PTT treatment, the cells were irradiated with 808 nm laser (2.0 W·cm^{-2}) for 5 min. After 24 h culture, the cells were incubated with the MTT solution. After 4 h incubation, we removed MTT solution and added dimethyl sulfoxide (100 μL) into each well. The absorbance at 490 nm was detected (Themo Multiscan MK3, USA).

Living and Dead Cell Staining

The 4T1 cells culture and drug treatment were the same as the MTT assay. Finally, each hole is processed according to Calcein-AM/PI Double Stain Kit (Yeasen, shanghai). We observed the results through microscope (Oplenic Digital Camera, Nikon, Japan) after washed three times with PBS.

Wound-healing Assay

The 4T1 cells were seeded into 6-well plates with 2×10^5 cells per cell for 24 h. Then, different concentrations of TD1 NPs and M-TD1 NPs (0 and 20 $\mu g \cdot mL^{-1}$) were respectively added into the culture media and divided into dark and laser groups. After 24 h, using pipette tip made a scratch in a monolayer of 4T1 cells. The laser group was irradiated with 808 nm laser (2.0 $W \cdot cm^{-2}$) for 5 min. Then, images were collected with a microscope after 0 and 24 h. The experiments were repeated 3 times.

Trans-well Assays

Trans-well chambers (Corning, USA) were employed to evaluate the migration capabilities of 4T1 cells. The upper compartment was seeded with 4T1 cells. After 24 h culture, divided chambers into dark and laser groups and replaced by different concentrations of TD1 NPs and M-TD1 NPs (0 and 20 $\mu g \cdot mL^{-1}$). After 24 h incubation, the laser group was irradiated with 808 nm laser (2.0 $W \cdot cm^{-2}$) for 5 min. Then, upper compartment culture medium was replaced by FBS-free medium, whiling lower compartment of the chamber contained medium with 10% FBS. After incubation, removing the cells that failed to pass through the polycarbonate membrane, whereas the migratory cells were fixed and stained with purple crystal (0.1%) and then photographed by microscope. The experiments were repeated 3 times.

Analysis of Apoptosis

Annexin V-FITC/PI Apoptosis Detection kit was purchased from BD Biosciences (USA). The 4T1 cells were harvested and stained with Annexin V-FITC and PI for 15min in dark. After stained, flow cytometer (BD Biosciences, USA) was employed to detect and analyze the apoptosis.

Western Blot Analysis

Primary antibodies: β-actin, Bax, Bcl-2, Caspase 3, GAPDH, β-tublin, LC3B and P62 antibodies were obtained from Cell Signaling Technology (USA). The 4T1 cells were harvested in RIPA Lysis Buffer (EMD Millipore Corp, USA) containing 1% Phosphatase Inhibitor Cocktail (CW Biotech, Beijing, China) and 1% Protease Inhibitor Cocktail (CW Biotech, Beijing, China). Equal amounts of protein of each sample were used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Then, protein was transferred to PVDF membranes. After being blocked with KPL Detector Block (SeraCare Life Scienes, USA), the membranes were incubated with appropriate primary antibody for 2 h. Tris-buffered saline solution mixed with Tween-20 (TBST) was used to wash the samples for three timesbefore and after second antibody was incubated for 1 h, respectively. The protein bands were detected with the Enhanced Chemiluminescence (ECL) Substrate Kit (Millipore) by chemiluminescent substrate (Thermo Fisher Scientific, USA).

Animal Model Construct and Treatment

Female BaLb/c mice of 4 weeks of age were received from the China National Laboratory Animal Resource Center. When the tumor size reached about 100 mm^3 ($V = (length \times width^2)/2$), 4T1 tumor-bearing mice were randomly divided into five groups (n = 5 per group) and recorded day 0. Recorded the five groups as saline, TD1 NPs, TD1 NPs + Laser, M-TD1 NPs, M-TD1 NPs + Laser and intratumorally injected with 100 μL of saline, TD1 NPs (2 $mg \cdot Kg^{-1}$) and M-TD1 NPs (2 $mg \cdot Kg^{-1}$) in day 0, 3, 6. Subsequently, the groups with laser underwent a PTT treatment (808 nm, 2 $W \cdot cm^{-2}$) for 10 min at 12 h post-injection. The tumor volume and body weight were recorded every three day. 12 days after the last treatment, the mice were euthanized and got the serum after collected blood centrifuge for 10 minutes at 3000 rpm, after which the livers, spleens, lungs and tumors were dissected, weighed, imaged and fixation of freshly excised organs in 4% paraformaldehyde for histological assay.

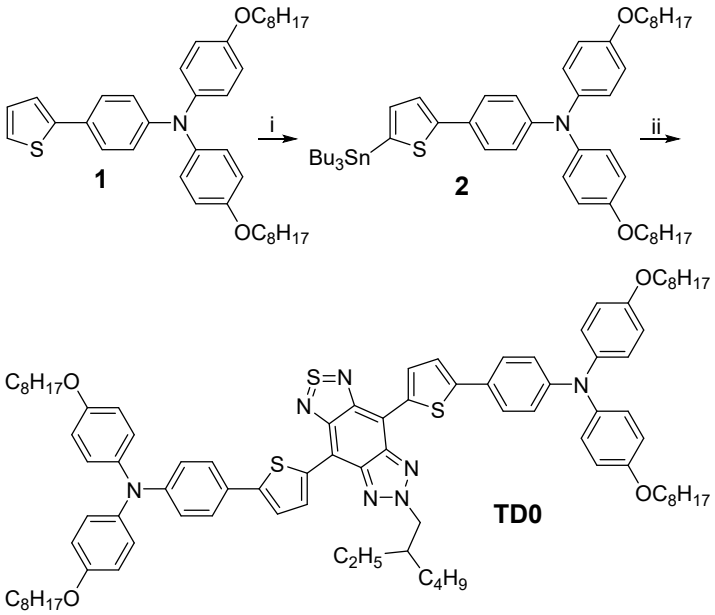
Histopathological and Safety Evaluation

Collected tissues were processed into paraffin for 24h, and sectioned hematoxylin and eosin (H&E) were employed to stain tumors, livers, spleens, lungs, respectively. The one-step terminal deoxyribonucleotidyl transferase (TDT)-mediated dUTP nick-end labeling (TUNEL) apoptosis assay kit was used to evaluate the apoptosis of tumor tissues. The fluorescence microscopy was used to capture photographs.

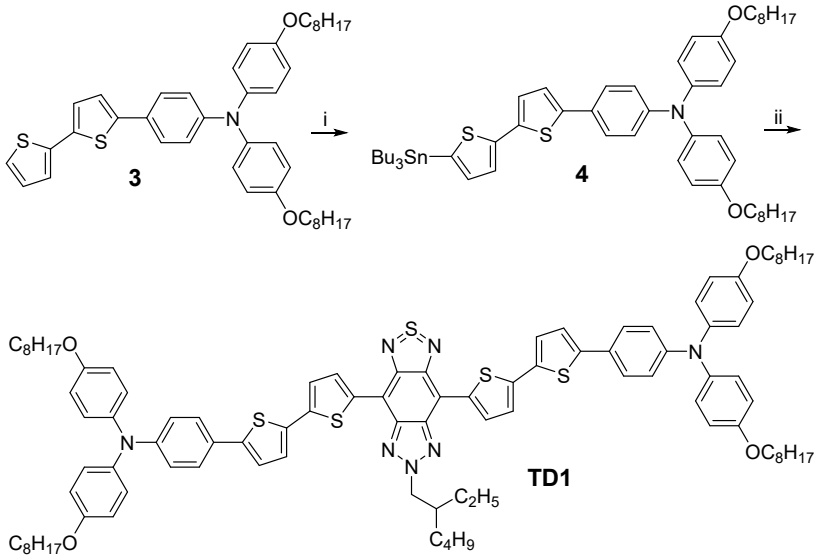
Mouse IL-12 ELISA Kit was purchased from Meilian biological (Shanghai, China). Mouse TNF-α ELISA Kit was purchased from Multisciences (China). The IL-12 and TNF-α level of mouse blood supernatant was detected according to the instructions.

Statistical Analysis

All data were characterized as mean ± standard deviation and analyzed by two-tailed Student's t-test. The statistically significant was strictly accepted at $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$, and $^{****}p<0.0001$.



Scheme S1. The synthetic route for TD0.



Scheme S2. The synthetic route for TD1.

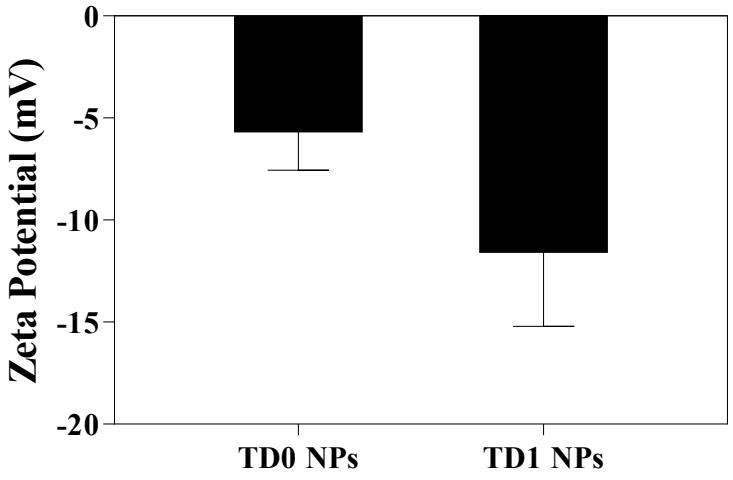


Figure S1. Zeta potential of TD0 NPs and TD1 NPs, n = 3.

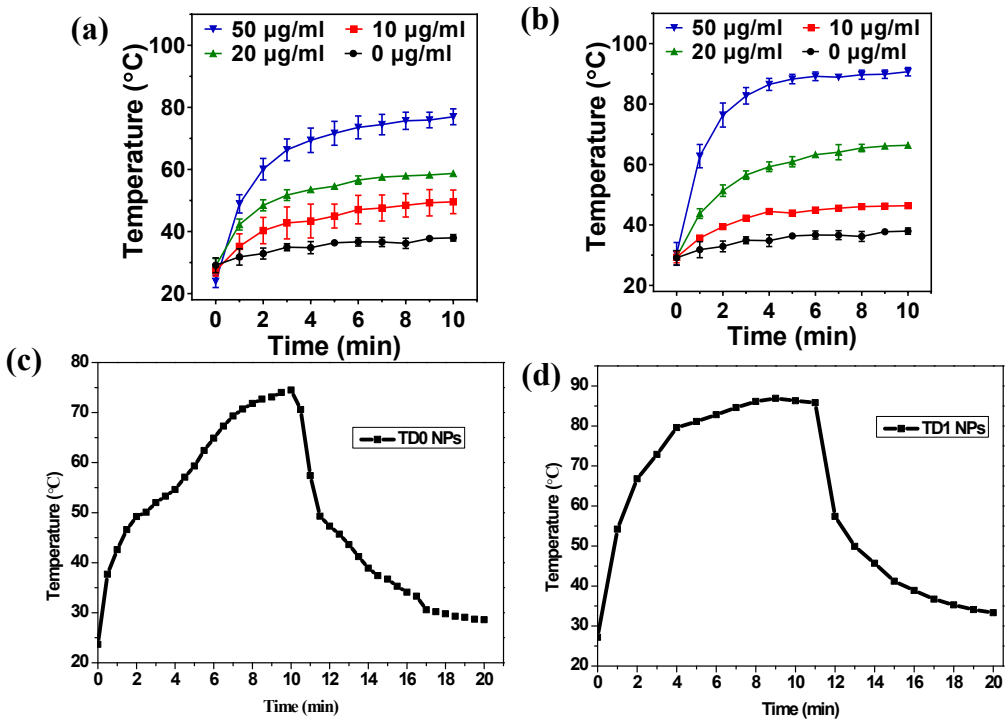


Figure S2. Photothermal heating processes of TD0 (a) and TD1 NPs (b) at different concentrations (0-50 μg/mL) under 808 nm light irradiation (2 W/cm2) for 10 min. Determination of photothermal conversion efficiency of TD0 NPs (c) and TD1 NPs (d) under the excitation of an 808 nm laser at 2 W cm^{-2}. All NPs were tested in water solution.

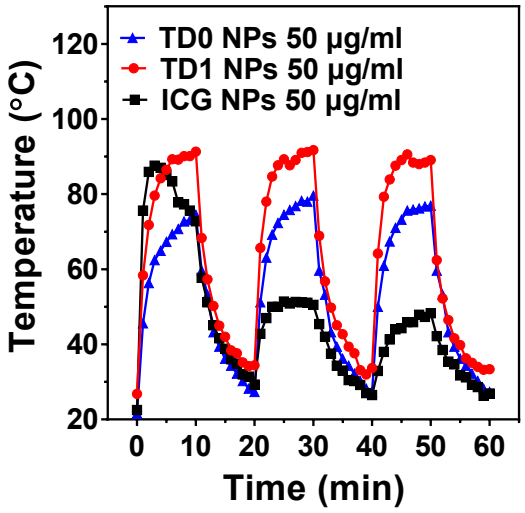


Figure S3. Heating and cooling cycle 3 times of TD0 NPs, TD1 NPs, and ICG NPs, phototherapy laser every 10 mins, then cool down for 10 mins at 50 μg·mL^{-1}.

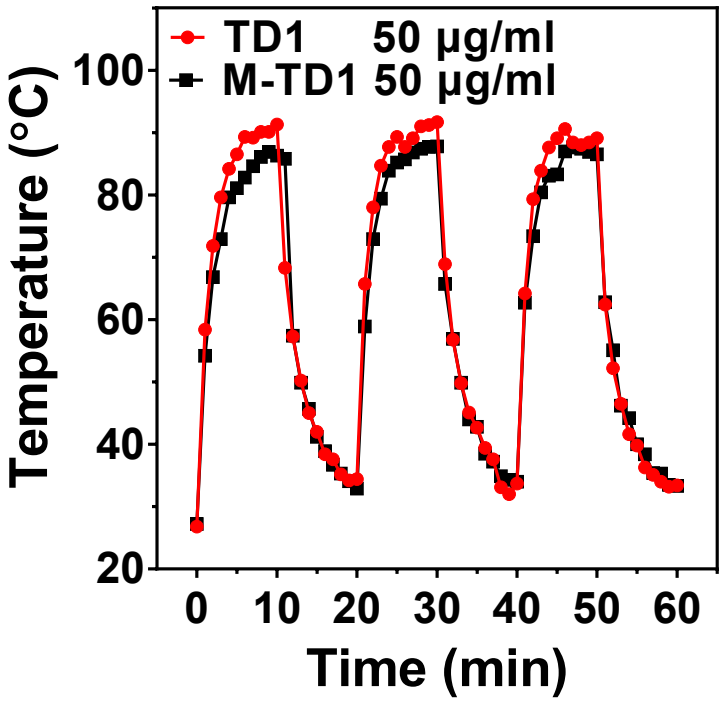


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Figure S4. Heating and cooling cycle 3 times of TD0 NPs, TD1 NPs, and ICG NPs, phototherapy laser every 10 mins, then cool down for 10 mins at 50 μg·mL^{-1}.

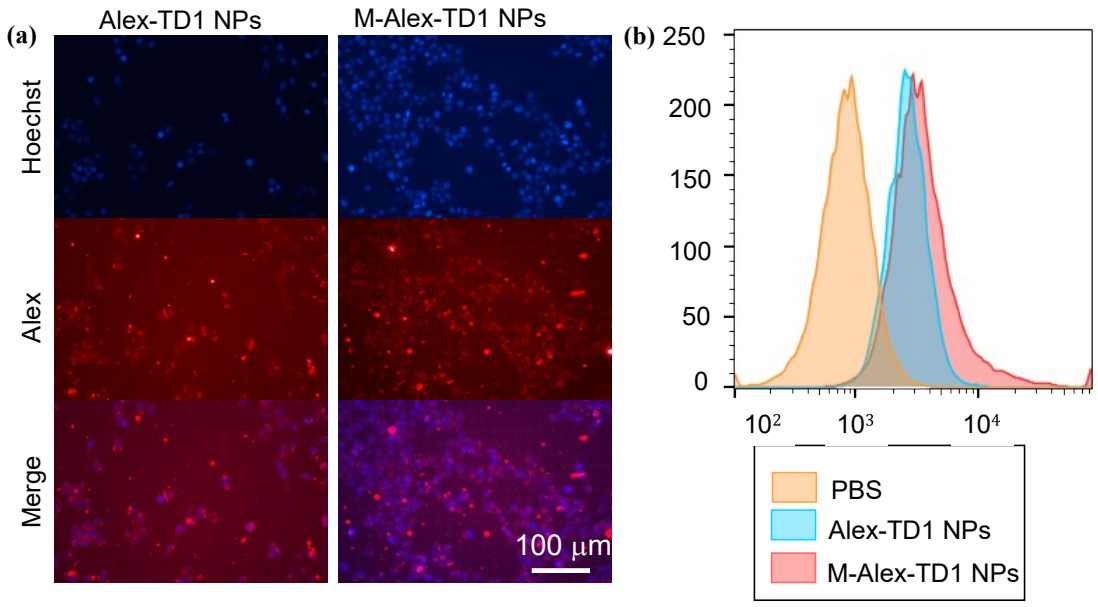

Figure S5. (a)Fluorescence of cellular uptake, red: Alex NPs or M-Alex NPs, blue: cell nucleus (b) Fluorescence intensity was detected by flow cytometry. Scale bar : 100 μm.

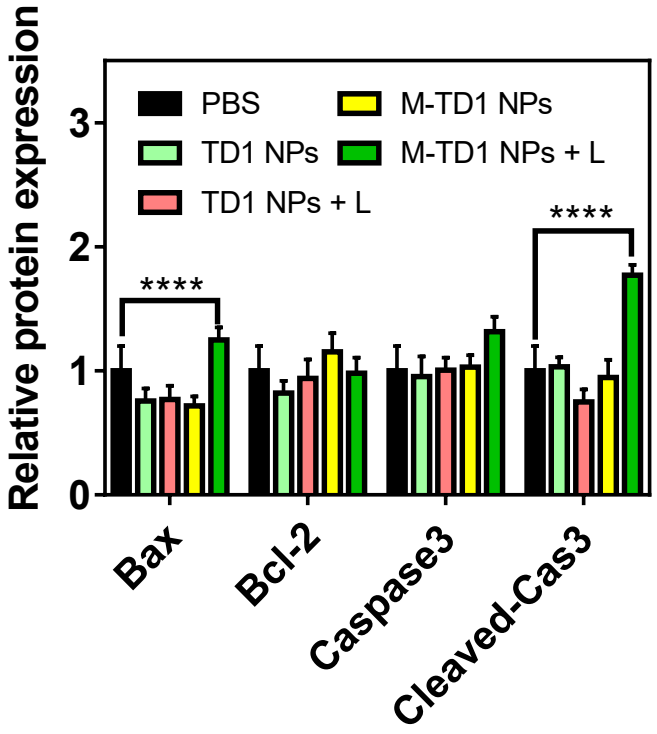

Figure S6. Relative expression quantification of Bax, Bcl-2, Caspase3 and cleaved Caspase 3 in 4T1 cells after incubation with TD1 NPs and M-TD1 NPs for 12 h with or without lase irradiation.

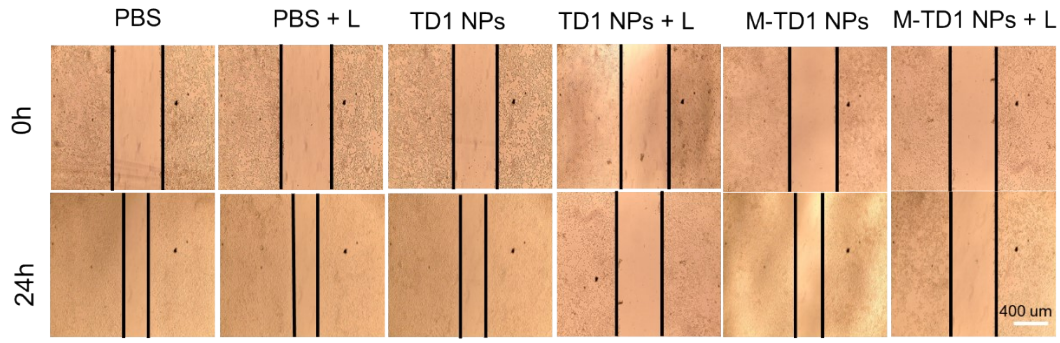

Figure S7. Wound-healing Assay. Wound closure was delayed in TD1 NPs + L and M-TD1 NPs + L groups compared with in the control group at 24 h (magnification, ×40).

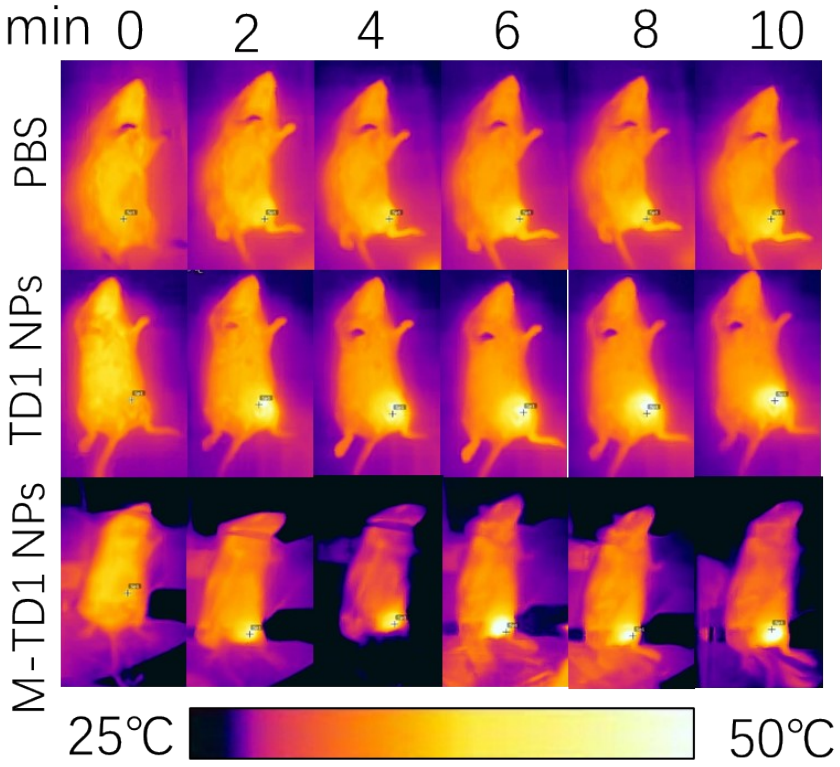

Figure S8. Photothermal picture of mice bearing 4T1 tumors under 808 nm light (2.0 W·cm^{-2}).

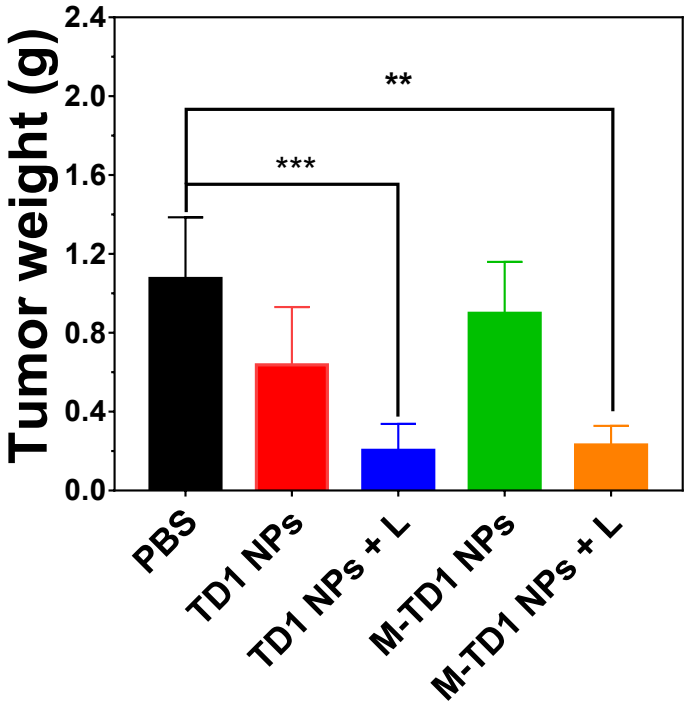

Figure S9. The tumor weights of mice after different treatment.

References

[1] B. Guo, Z. M. Huang, Q. Shi, E. Middha, S. D. Xu, L. Li, M. Wu, J. W. Jiang, Q. L. Hu, Z. W. Fu, B. Liu. *Adv Funct Mater.* **2020**, *30*, 1907093.