The mitochondria-targeted and IR780-regulated theranosomes for imaging and enhanced photodynamic/photothermal therapy

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Supporting information

1. Materials and methods

1.1 Materials

IR780 and Ce6 were purchased from J&K Scientific Ltd. (China). 4',6-diamidino-2-phenylindole (DAPI) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich (USA). 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000](DSPE-PEG_{2000}) was purchased from Shanghai Advanced Vehicle Technology Co., Ltd (Shanghai, China). Dipalmitoyl phosphatidylcholine (DPPC) 1,2-Distearoyl-sn-glycero-3-phophoethanolamine-N-[amino(polyethyleneglycol)2000] (ammonium salt) (DSPE-PEG_{2000}-NH_2) were purchased from Laysan Bio Inc. (Arab, AL, USA). (3-carboxypropyl)triphenyl-phosponium bromide (CTPP), N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC), triethylamine (TEA), and 1-Hydroxybenzotriazole (HOBT) were purchased from Adamas Reagent Co., Ltd. (Swiss). Mitotracker Green FM was supplied by Invitrogen-life technologies (USA).

1.2 Synthesis of DSPE-PEG_{2000}-TPP

Into the solution of CTPP (8.8 mg, 20 μM) in chloroform, 20 μL TEA, EDC (11.5 mg, 60 μM) and HOBT (8.1mg, 60 μM) were added. The mixed solution was stirred at room temperature for 3 h followed by addition of 50 mg of DSPE-PEG_{2000}-NH_2 in chloroform (25 mg/mL). The reaction mixture was stirred at room temperature overnight under nitrogen. Then the chloroform was evaporated to obtain the crude reaction mixture. The mixture was diluted with water and dialyzed against water using cellulose ester membrane (MWCO, 2 KDa) for 24 h. The dialysate was frozen, lyophilized to obtain a solid powder (DSPE-PEG_{2000}-TPP), which was dissolved in chloroform-d ($CDCl_3$) and analyzed by ^{1}H-NMR using a AVANCE III 600MHz spectroscope. The obtained DSPE-PEG_{2000}-TPP polymer was dissolved in chloroform at a concentration of 5-10 mg/mL and stored at -80 ℃ for future study.

1.3 Preparation and characterization of theranosomes

The theranosomes were prepared using film hydration method via sonication followed by membrane extrusion. IR780, Ce6 and lipid mixture (See **Table S1**) were dissolved in mixed solvent of chloroform and methanol. Then the organic solvent was removed with rotavapor to generate a thin lipid film on the glass vial. The lipid film was then hydrated with PBS (pH 7.4) via sonication and followed by sequential extrusions through 800, 400, 200, and 100 nm polycarbonate filters (five times each). Then free IR780 and Ce6 were removed by dialysis method. The content of IR780 and Ce6 in TNS was determined using IR780 and Ce6 UV-vis calibration curve at 780 nm and 405 nm, respectively (**Fig. S1-S2**).

The morphology of theranosomes was characterized by JEM-100CXII transmission electron microscopy (TEM, JEOL, Japan) with an accelerating voltage of 100 kV, followed by negative staining procedure using phosphotungstic acid. A droplet containing samples was dried on a carbon coated copper grid for staining and after drying, the specimen was observed under the microscope as a tiny hollow vesicle with surrounding darkness. The mean diameter and particle size distribution were measured by dynamic light scattering (DLS) using Malvern Zetasizer Nano (Malvern Instruments Ltd., U.K.). UV-Vis spectrum of theranosomes was detected by a Cary 60 UV-vis spectrometer (Agilent, USA).

1.4 Fluorescence and Singlet oxygen generation

Fluorescent spectrums of free IR780/Ce6, Ce6-TNS and IR780/Ce6-TNS were measured by FLS980 Spectrofluorophotometer (Edinburgh Instruments) with the excitation wavelength at 405 nm. Then the three solutions irradiated by 808 nm laser (1 W/cm^2) for 1, 2, 5, 8 and 10 minutes were also measured, respectively.

Singlet oxygen ($^1O_2$) generation was determined by using 1, 3-diaphenylisobenzofuran (DPBF) as a chemical $^1O_2$ probe, which reacted irreversibly with $^1O_2$ to cause a decrease in the DPBF absorption at about 400 nm.[1] Briefly, DPBF (30 μL, 1.5 mg/mL in acetonitrile) was added to TNS solution (containing 2 μg/ml Ce6, 2 mL), and then subject to 660 nm laser irradiation (0.5 W/cm^2), 808 nm laser irradiation (1.0 W/cm^2), or 808 nm plus 660 nm laser for different periods of time. Thereafter, the absorbance of DPBF at 410 nm was measured.

1.5 Photothermal effect measurement

The solutions of IR780/Ce6-TNS at the concentrations of 1, 2, 5, 10, 20, and 40 μg/mL IR780 were stored in the glass vials (each 0.5 mL), respectively. Then the solutions were irradiated at 808 nm laser (1 W/cm^2). Simultaneously, the temperature of the solutions was measured during 300 s. Thereafter, centrifuge tubes were added 0.5 mL of PBS, free IR780/Ce6, IR780/Ce6-TNS or TPP-IR780/Ce6-TNS (all containing 10 μg/mL IR780), respectively. The 808 nm laser irradiated the tubes. The temperature of the solution was monitored using a thermocouple microprobe. Flukr Ti27 infrared (IR) thermal imaging camera obtained IR thermographic maps and maximum temperature.

1.6 Cell culture and cell viability

HeLa cell line was culture in DMEM culture medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin and 1 % streptomycin. Cells were maintained at 37 ℃ in a 5% CO_2 atmosphere. MTT assay was performed to evaluate the potential cytotoxicity of free IR780/Ce6, IR780/Ce6-TNS and TPP-IR780/Ce6-TNS (all C_{Ce6}=4 μg/mL) irradiated by 660 nm laser, 808 nm laser or 808nm plus 660 nm laser, respectively. Briefly, HeLa cells were seeded to 96-cell plates (1×10^4 cells/well) for 24 h cultivation prior to incubation with theranosomes. After 4 h incubation, the supernatants were discarded, every well was washed with PBS and the medium containing theranosomes was changed with fresh medium. Then 20 μL of MTT solution solution (5 mg/mL) was added to each well and the mixture was incubated for another 4 h. Finally, the incubation solution was removed, and 100 μL of dimethyl sulfoxide (DMSO) was added. The absorption of MTT was recorded at 570 nm using

a microplate readed (Thermo Scientific Varioskan Flash, USA).

1.7 Subcellular localization of theranosomes

HeLa cells (1×10^{4} cells) were seeded to a glass-bottomed dish (NEST, Φ15 mm, China) for 24 h cultivation under 5% CO_2 at 37 ℃. After 24 h incubation, cells were treated with IR780/Ce6-TNS or TPP-IR780/Ce6-TNS for another 6 h incubation. After being washed by PBS, the cells were stained with Mitotracker Green FM (100 nM) for 30 min and DAPI (5 μg/mL) for 15 min. Stained cells were washed with PBS three times to remove free dye and observed by a Leica TCS SP5 (Leica, Germany) CLSM. The fluorescence signal was imaged at excited wavelength of 405 and at emission wavelength of 660 nm for Ce6 (red). The Mitotracker Green was excited at 490 nm, and its emission was collected from 516 to 530 nm (green).

1.8 CD-31 Antibody Staining

The nude mice model (20 ± 2 g, 4-6 weeks old) bearing HeLa cells were randomly divided into three groups (n=6) and received intravenous administration of PBS (control), IR780/Ce6-TNS and TPP-IR780/Ce6-TNS at a dose of 0.5 mg/kg Ce6. The mice were sacrificed 12 h after the administration. Thereafter, the tumor was embedded in OCT (Sakura, Torrance, CA, USA) and frozen at -20 ℃, sectioned at 14 μm. In order to observe the vascular damage, the sections were incubated with a CD31 primary antibody (Abcam, Cambridge, MA) at 4℃ overnight followed by incubation with Dylight 549-labeled secondary antibody for 1 h at room temperature. The sections were also stained by DAPI and covered with a coverslip. Finally, the sections were observed by CLSM.

Table S1 Composition of the theranosomes formulations.

Formulations	DPPC (mol)	Chol (mol)	$DSPE\text{-}PEG_{2000}$ (mol)	$DSPE\text{-}PEG_{2000}$-TPP (mol)	Ce6 (mol)	IR780 (mol)
IR780/Ce6-TNS	65	30	5	--	2	2
TPP-IR780/Ce6-TNS	65	30	4	1	2	2

2. Supporting figures:

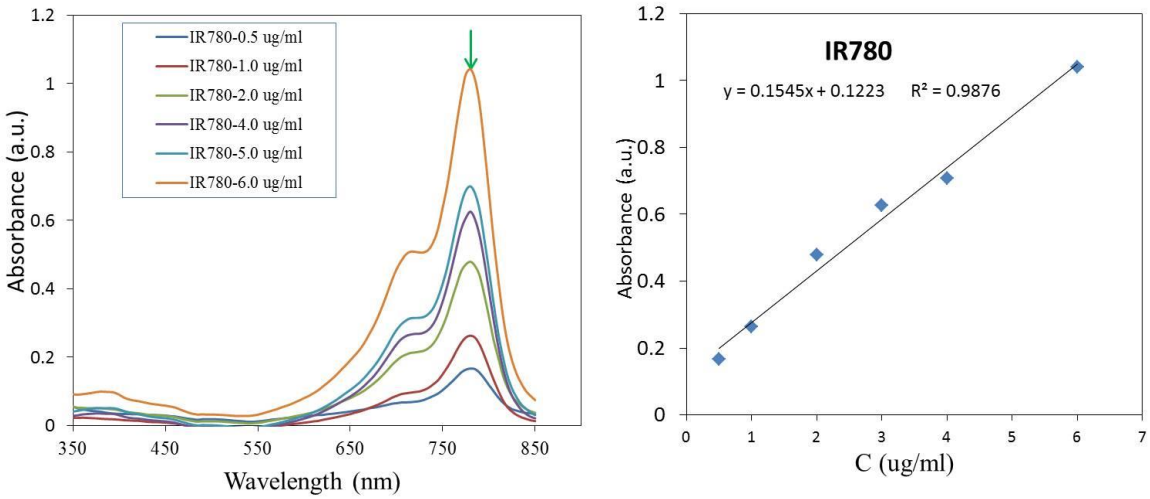

Fig. S1 (a) UV-VIS absorption spectra of IR780. (b) The linear relationship between concentration and absorbance of IR780 at 780 nm.

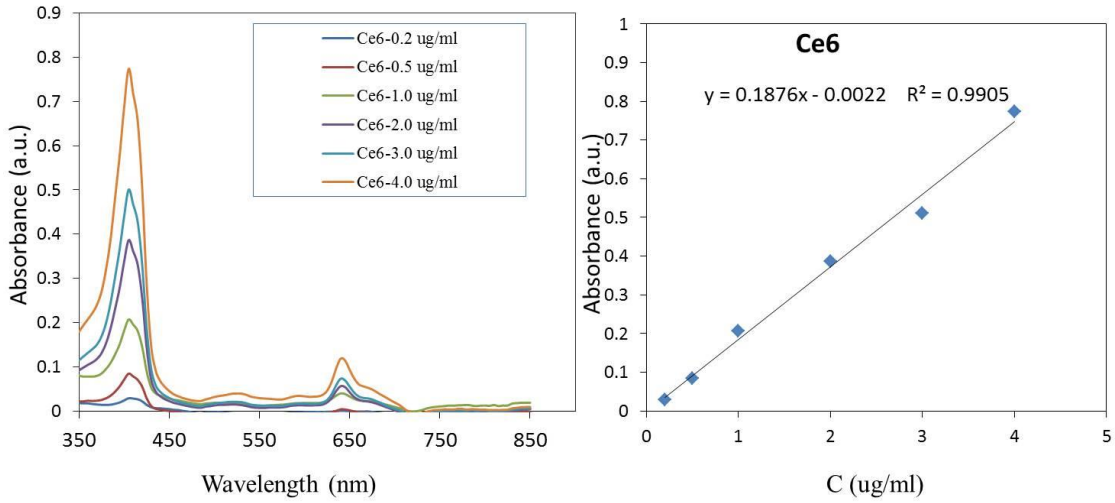

Fig. S2 (a) UV-VIS absorption spectra of Ce6. (b) The linear relationship between concentration and absorbance of Ce6 at 405 nm.

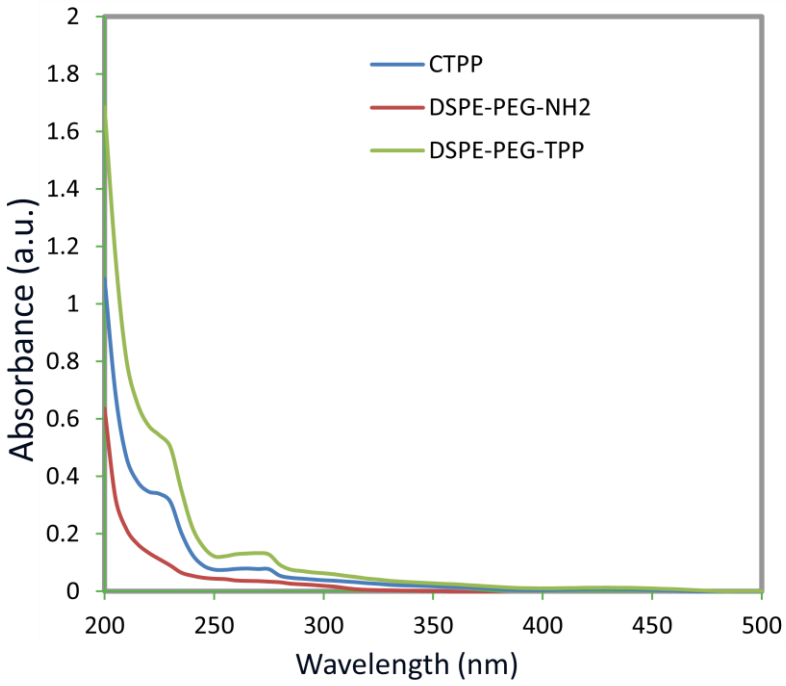

Fig. S3 The UV-Vis spectrum of TPP, DSPE-PEG-NH_2 and DSPE-PEG-TPP.

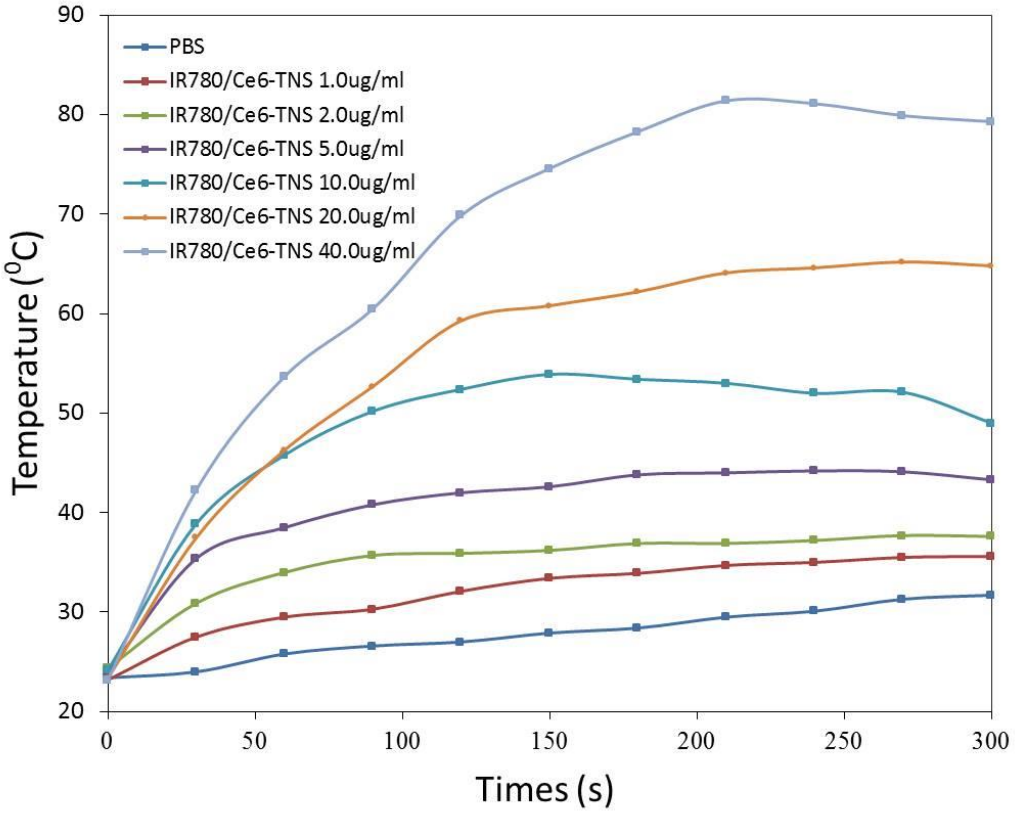

Fig. S4 Change of temperature of 0.5 mL IR780/Ce6-TNS at various concentrations of IR780 as the function of photoirradiation time (1.0 W/cm^2)

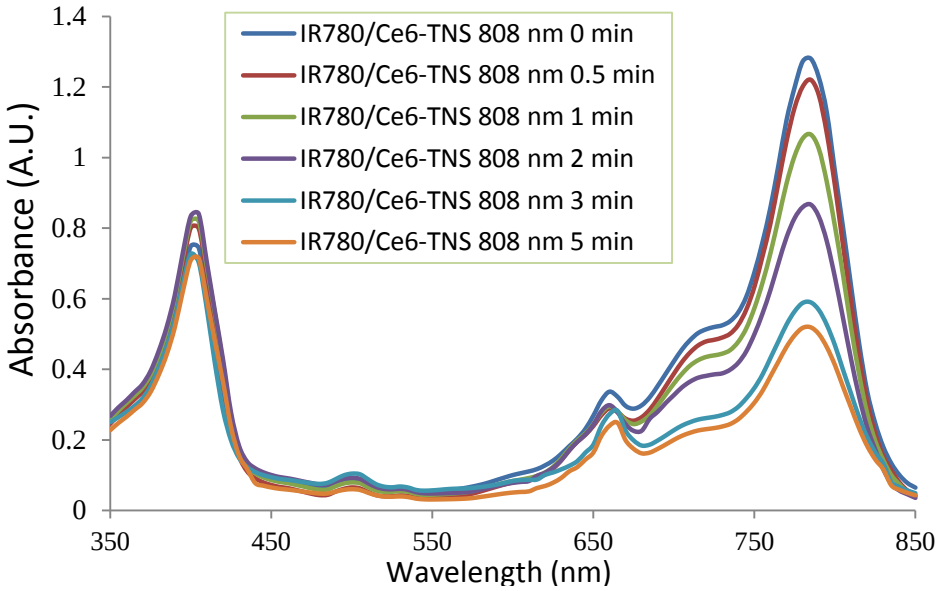

Fig. S5 The UV-Vis absorption of IR780/Ce6-TNS at different time under 808 nm photoirradiation.

Reference:

1. Zhao, Z.; Shi, S.; Huang, Y.; Tang, S.; Chen, X. Simultaneous Photodynamic and Photothermal Therapy Using Photosensitizer-Functionalized Pd Nanosheets by Single Continuous Wave Laser. *ACS Appl. Mater. Interfaces* 2014, 6, 8878−8885

2. Nicole, Y. M.; Wei, C.; Victor, C.; Angelo, R.; Paul, D. S.; Amir, G. Real time in vivo non-invasive optical imaging using near-infrared fluorescent quantum dots. *Acad. Radiol.* 2005, 12, 313-323.