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Supporting Information

Ultra-high Photoactive Thiadiazolo[3,4-g]quinoxaline Nanoparticles with Active-targeting Capability for Deep Photodynamic Therapy

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Table of Contents

1. Experimental Section

All of solvents were purified according to standard methods. All reagents and chemicals were purchased from Alfa Aesar Chemical Co. and J&K Chemical Co. and used without further purification unless otherwise stated. Deionized water (Millipore Milli-Q grade) with a resistivity of 18.2 MΩ was employed in all experiments. All manipulations involving air-sensitive reagents were performed in an atmosphere of dry argon. 3-(4,5-dimethylthiahiazozy1)-3,5-di-phenytetrazoliumromide (MTT) reagent, Dulbecco's modified eagle's medium (DMEM), Roswell park memorial institute-1640, were purchased from Hyclone Laboratories Inc. (USA). Calcein-AM and propidium iodide (PI) dyestuff, Mito-tracker and Hoechst 33342 were purchased from Beyotime Biotechnology Inc.

NMR spectra were recorded using a Bruker AV400 (400 MHz) instrument. The residual solvent protons (^{1}H) or the solvent carbons (^{13}C) were used as internal standards. ^{1}H NMR data are presented as follows: the chemical shift in ppm (δ) downfield from tetramethylsilane (multiplicity, coupling constant (Hz), and integration). The following abbreviations are used in reporting NMR data: s, singlet; d, doublet; t, triplet; q, quartet; and m, multiplet. Mass spectra were acquired using a Bruker Daltonics Inc spectrometer. UV-vis absorption spectra were recorded using a Shimadzu U-3900 spectrophotometer. Fluorescence tests were carried out on a HITACHI F4500 spectrophotometer and Edinburgh LP-920 photoluminescence spectrometer. TEM test were performed using HITACHI HT7700 electronic microscope. Dynamic laser scatter and zeta potential tests of nanoparticles were recorded on a Malvern Zetasizer Nanozse instrument.

Measurement of $^{1}O_2$ quantum yield of TQs-PEG4

To evaluate the $^{1}O_2$ quantum yield of TQs-PEG4, TPP and DPBF were used as a standard PS and $^{1}O_2$ trapping reagent, respectively. DPBF can react with $^{1}O_2$ and cause a decreased absorption. To avoid the inner-filter effect, the absorption of TPP, TQs-PEG4 in toluene at 635 nm was regulated to approximately 0.10 OD, the absorption of DPBF at 415 nm was regulated to approximately 1.0 OD. Then the mixture was exposed to 635 nm laser illumination (1.5 mW cm^{-2}) for different time

and the absorption spectra of DPBF were recorded immediately after each irradiation. The absorption intensity of DPBF at 440 nm was plotted against irradiation time, and the slop of the decay curve was fitted by applying a first-order exponent using Origin 9.0 software, which is proportional to the reaction rate of DPBF with $^1O_2$. The $^1O_2$ quantum yield of TQs-PEG4 can be calculated by Equation (1) as followed.

$$\Phi_{\Delta=}^{S} \frac{K_S}{K_R} \times \Phi_{\Delta}^{R} \quad (1)$$

where K is slop for the decay of the DPBF absorption, S represents the sample to be tested, R stands for the reference and Φ_{Δ}^{R} represents the $^1O_2$ quantum yield of the reference that is reported as 0.68 for TPP in toluene.

The $^1O_2$ generating abilities of TQs-PEG4 NPs in aqueous solution were evaluated by a similar method except using methylene blue (MB) as a standard PS and Anthracenediyl-bis(methylene)dimalonic acid (ABDA) as $^1O_2$ trapping agent, respectively.

Electron spin resonance (ESR) spectra experiments

The ESR spectra were recorded on a Bruker E500 spectrometer. A mixture of the TQs-PEG4 NPs and $^1O_2$ trapper (TEMPO) in aqueous solution was transferred to a standard quartz capillary and irradiated by a 635 nm laser (50 mW cm^{-2}) for different time (0, 2, 4, 6 min) to monitor $^1O_2$ signals.

Photobleaching experiment

The solution of TQs-PEG4 NPs in water (20 μg mL^{-1} based on TQs-PEG4) was exposed to a 635 nm laser illumination (60 mW cm^{-2}) for different time (5, 10, 15 and 30 min) and the absorption spectra were recorded immediately after each irradiation.

Aqueous dispersibility test of TQs-PEG4 NPs

The solution of TQs-PEG4 NPs (20 μg mL^{-1} based on TQs-PEG4) were prepared in water, PBS, FBS and DMEM, respectively, before the further characterization.

DLS test: Hydrodynamic diameter of TQs-PEG4 NPs in different media were characterized on a

Zetasizer Nano instrument by dynamic laser scatter method after the solutions were stored for different time (0, 1, 3, 5 and 7 day).

Zeta potential test: Zeta potential of TQs-PEG4 NPs in different media were characterized on a Zetasizer Nano instrument after the solutions were stored for different time (0, 1, 3, 5 and 7 day).

Cell culturing and Confocal Imaging of HeLa cells

HeLa cells were incubated on the cell culture plate in DMEM medium supplemented with 10% FBS and 1% penicillin-streptomycin at 37℃ in a humidified, 5% CO_2 atmosphere.

HeLa cells were firstly seeded in 35 mm fluoro-dishes and incubated for 24 h. Then the cells were continually incubated with TQs-PEG4 NPs (0.6 μg mL^{-1}) for 4 hours. After washing twice with PBS to remove residual TQs-PEG4 NPs, the cells were imaged by a Nikon A1si laser scanning confocal microscope at different excitation wavelengths of 488, 560 and 640 nm, respectively.

Live/Dead cell co-staining

HeLa cells were seeded in 35 mm fluoro-dishes and incubated for 24 h. Then the cells were continually incubated with TQs-PEG4 NPs (0.4 μg mL^{-1}) for 2 hours. After washing with PBS twice to remove residual TQs-PEG4 NPs, the cells were irradiated upon 635 nm light (20 mW cm^{-2}) for 5 min. After light treatment, the confocal dishes were rinsed in 2 mL PBS solution supplemented with 4 μL Calcein-AM (1.0 mg mL^{-1}) and 6 μL propidium iodide (PI, 1.0 mg mL^{-1}). The cells were stained with Calcein-AM and PI for 15 min, then washed twice with PBS and evaluated by confocal fluorescence microscopy (Nicon A1 R-si).

Intracellular ROS detection

HeLa cells were seeded in 35 mm confocal dishes and incubated for 24 h. After that, the cells were continually incubated with TQs-PEG4 NPs (0.4 μg mL^{-1}) for 4 hours, then the old medium was discarded, washed twice with PBS and stained with DCFH-DA (10 μM) in fresh medium for another 30 min. After all treatment, the cells were washed twice with PBS. The control group was stored in the dark, and the test group was irradiated with 635 nm laser (20 mW cm^{-2}) for 5 minutes.

The green fluorescence of DCFH-DA was measured by confocal fluorescence microscopy (Nicon A1 R-si). The excitation wavelength was 488 nm, and the capture emission region was from 500 nm to 530 nm.

Annexin V-FITC and PI co-staining

HeLa cells were seeded in 35 mm fluoro-dishes and incubated for 24 h. Then the cells were continually incubated with TQs-PEG4 NPs (0.4 μg mL^{-1}) for 2 hours. After washing with PBS twice to remove residual TQs-PEG4 NPs and adding into fresh medium, the cells were irradiated upon 635 nm light (20 mW cm^{-2}) for 5 min. After light treatment, the cells were incubated for different times (1 h, 4 h, 24 h,) and stained by Annexin V-FITC and PI for 20 min. Finally, the cells were washed twice with PBS and evaluated by confocal fluorescence microscopy (Nicon A1 R-si).

PDT experiments of TQs-PEG4 NPs *in vitro*

Dark toxicity: The cells (L929, HeLa, 4T1 or MDA-MB-231) were seeded in 96-well plate at a density of 5×10^4 per well and cultured in 5% CO_2 at 37 °C for 24 h. Then, the TQs-PEG4 NPs at different concentrations (0, 5, 10, 15, 20, 25 and 30 μg mL^{-1}) were added into each well and incubated for 24 h. The standard MTT assay was carried out to determine the cell viabilities relative to control group (untreated cells).

Light toxicity: The cells (L929, HeLa, 4T1 or MDA-MB-231) were seeded in 96-well plate at a density of 5×10^4 per well and cultured in 5% CO_2 at 37 °C for 24 h. Then, TQs-PEG4 NPs at different concentrations (0, 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 μg mL^{-1}) were added into each well and incubated for 4 h. The cells were irradiated upon 635 nm light (10, 20, 40, 60 mW cm^{-2}, 80 mW cm^{-2} + pork tissue) for 10 or 20 min. After light treatment, the cell medium was replaced with 200 μL fresh medium and cells were incubated for another 16 h. The standard MTT assay was performed to determine the cell viabilities relative to control group (untreated cells).

Flow cytometry experiments

L929 or HeLa cells were firstly seeded in 12-well plate at a density of 5×10^4 per well and cultured in 5% CO_2 at 37 °C for 24 h. Then, TQs-PEG4 NPs (5.0 μg mL^{-1}) were added into each well and

incubated for different times (1, 2, 4, 12, 24 h), and the cells without TQs-PEG4 NPs were used as control group. Then the cells were centrifuged for 5 minutes followed by rinsed with 2 mL PBS solution. This process repeated for three times and a final cell suspension in 1 mL PBS was obtained and used for cell flow cytometry analysis.

Table S1 Photophysical parameters of TQs-PEG4 and TQs-PEG4 NPs in different solvents

Solvent	λ_{max}^{abs}[a] (nm)	ε_{max} (10^4 $M^{-1}cm^{-1}$)	λ_{max}^{fl}[b] (nm)	Δvss (cm^{-1})	Φ_f[c]	Φ_Δ[d]
Toluene	561	2.01	663	2740	0.018	1.04
Chloroform	573	4.21	710	3360	0.016	0.42
Methanol	571	3.56	727	3757	---	0.17
DMF	579	3.27	652	1933	---	0.34
Water[e]	584	---	749	3772	---	---

[a] Absorption spectra and [b] fluorescence emission spectra in toluene solution (1.0×10^{-5} M) at room temperature. [c] fluorescence quantum yield. [d] singlet oxygen quantum yield. [e] TQs-PEG4 NPs

2. Supplementary data

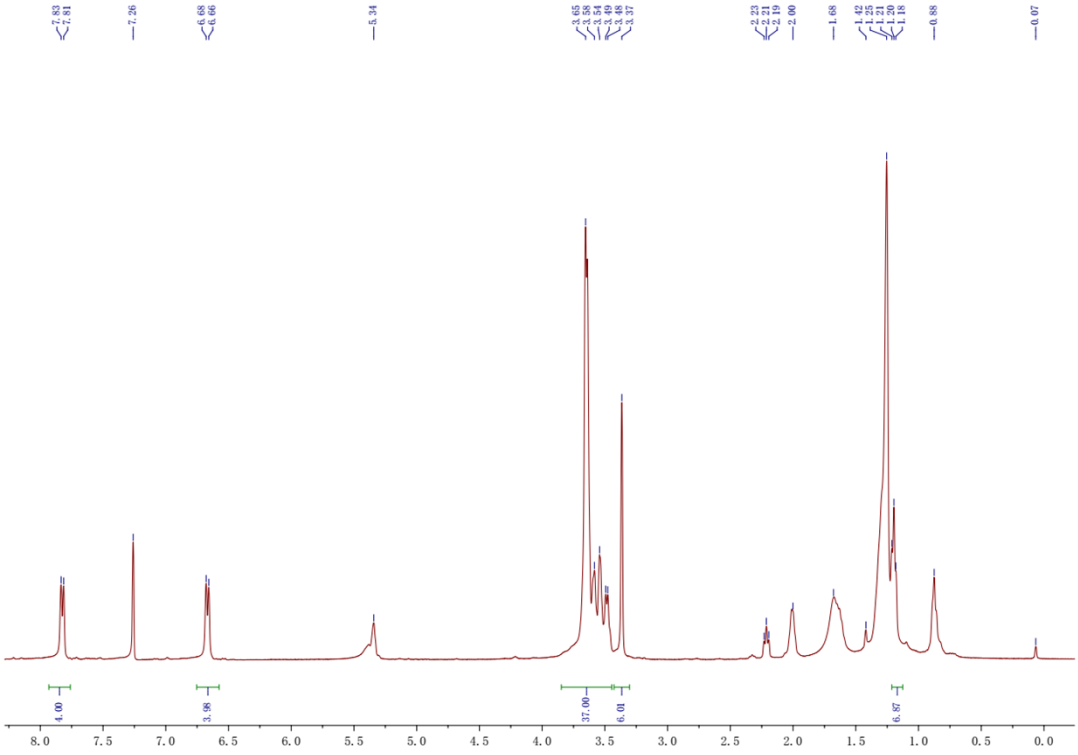

Figure S1 ^{1}H NMR spectrum of TQs-PEG4

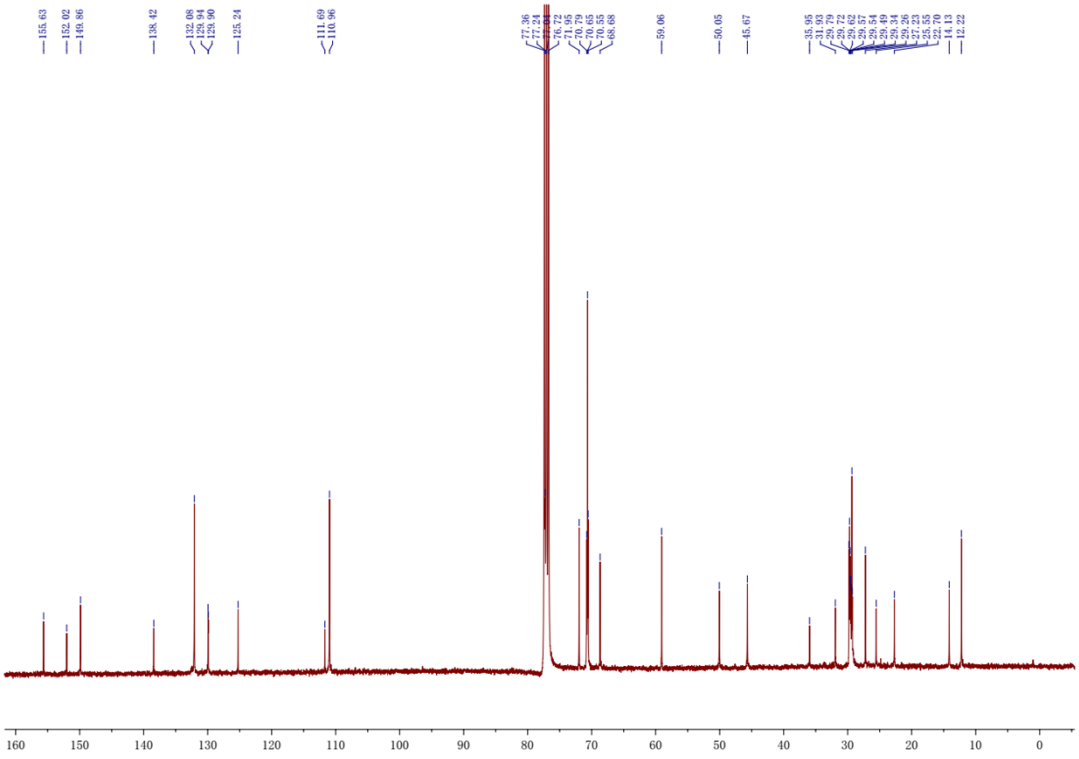

Figure S2 ^{13}C NMR spectrum of TQs-PEG4

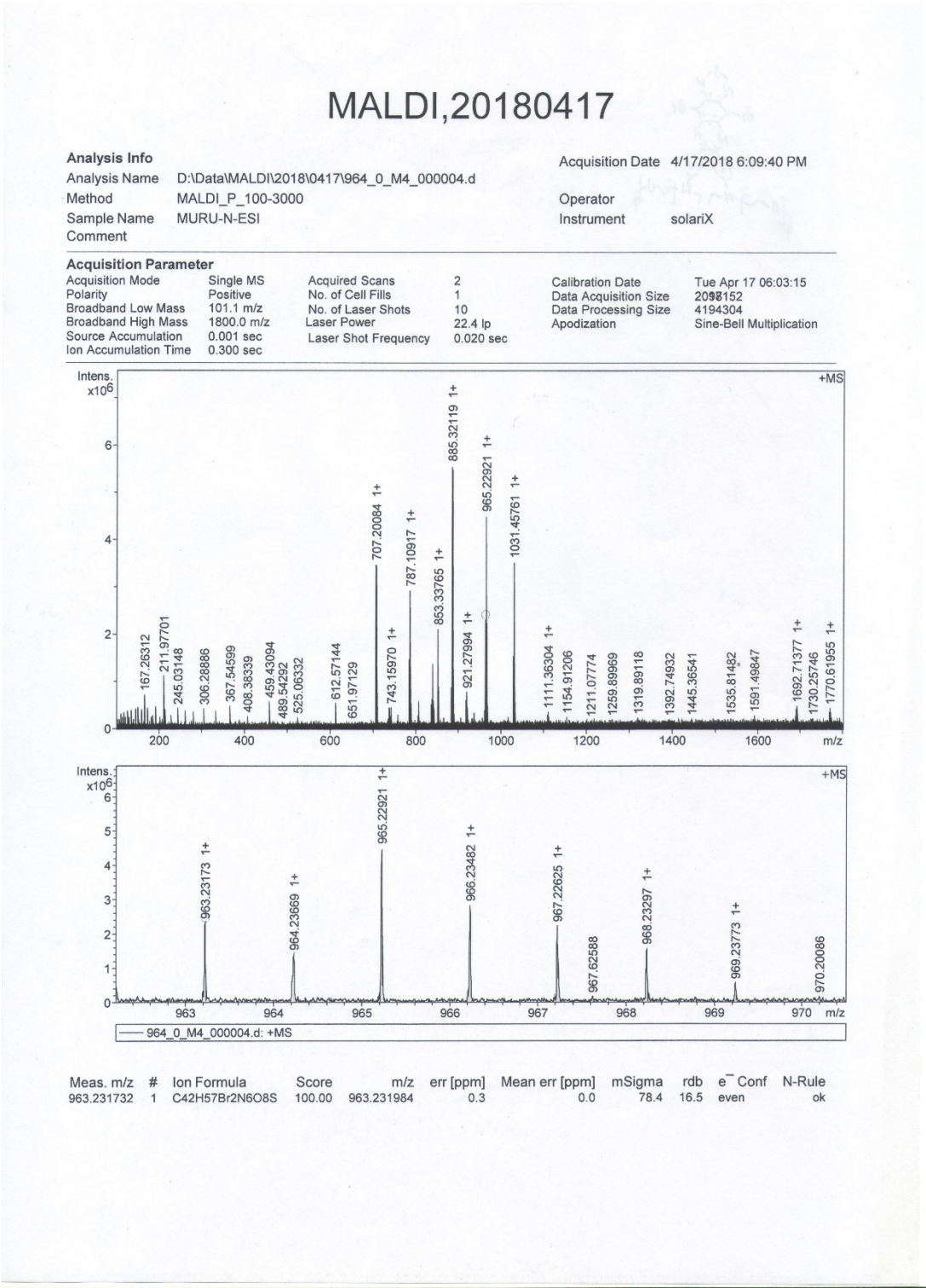

Figure S3 HRMS (MALDI-TOF) spectrum of TQs-PEG4

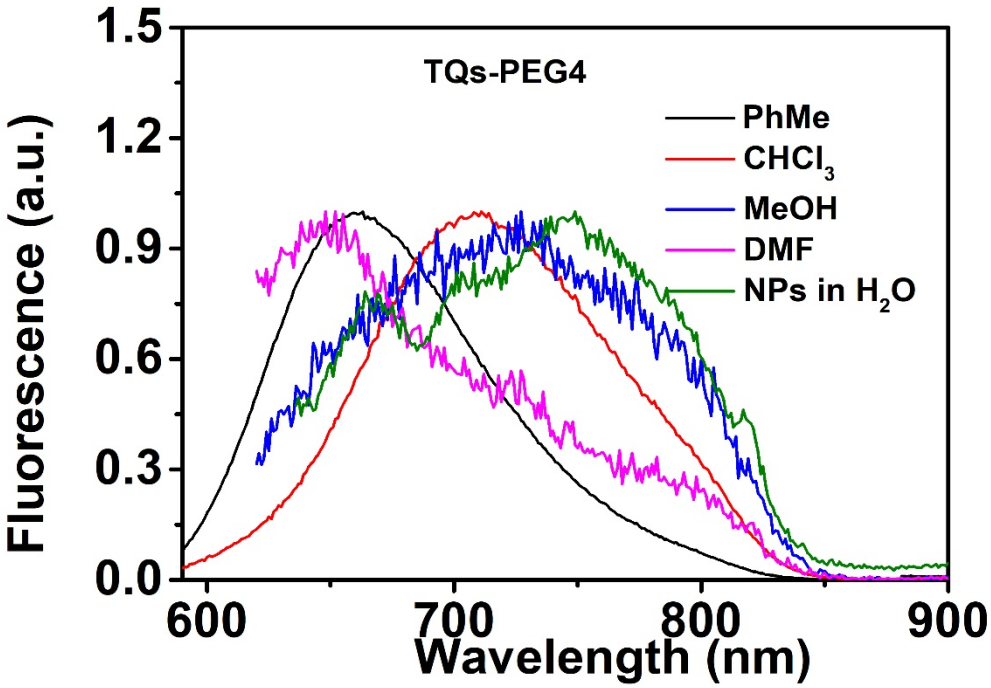

Figure S4 Fluorescence spectra of TQs-PEG4 in different solvents.

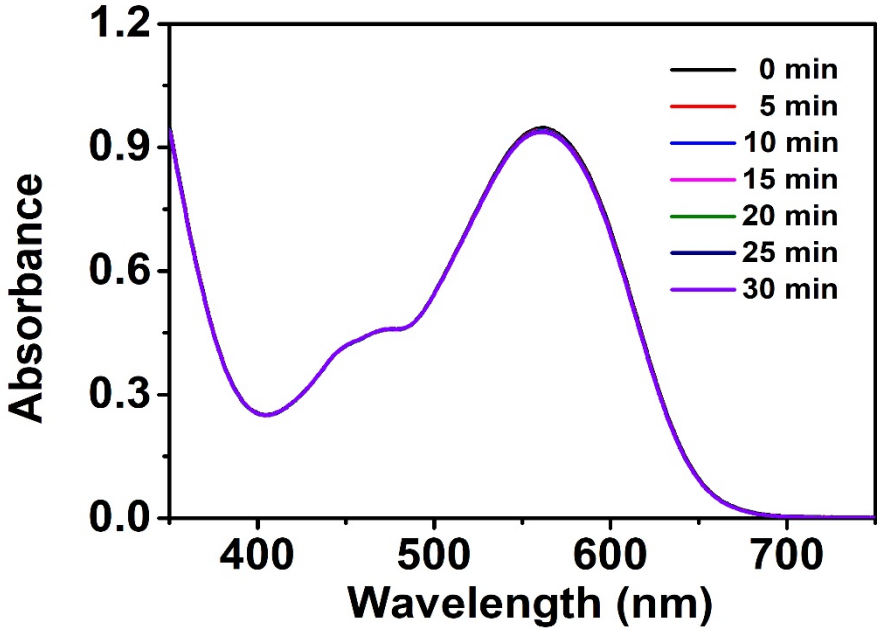

Figure S5 UV-vis absorption spectra of TQs-PEG4 in toluene irradiated by laser for different time. Irradiation condition: 635 nm, 60 mW cm^{-2}.

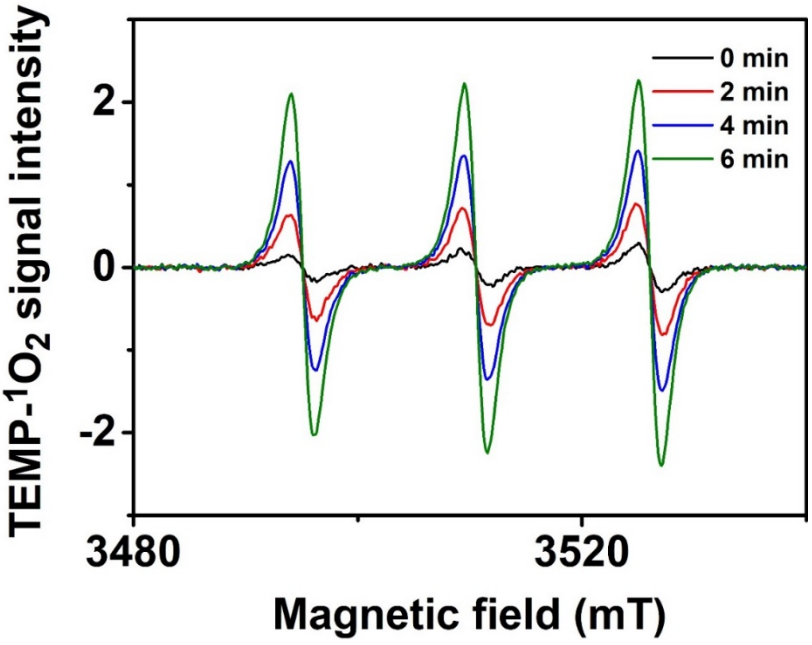

Figure S6 TEMP-$^1O_2$ signal intensities of TQs-PEG4 NPs + TEMP in water after irradiated by laser for different time. Irradiation condition: 635 nm, 40 mW cm^{-2}.

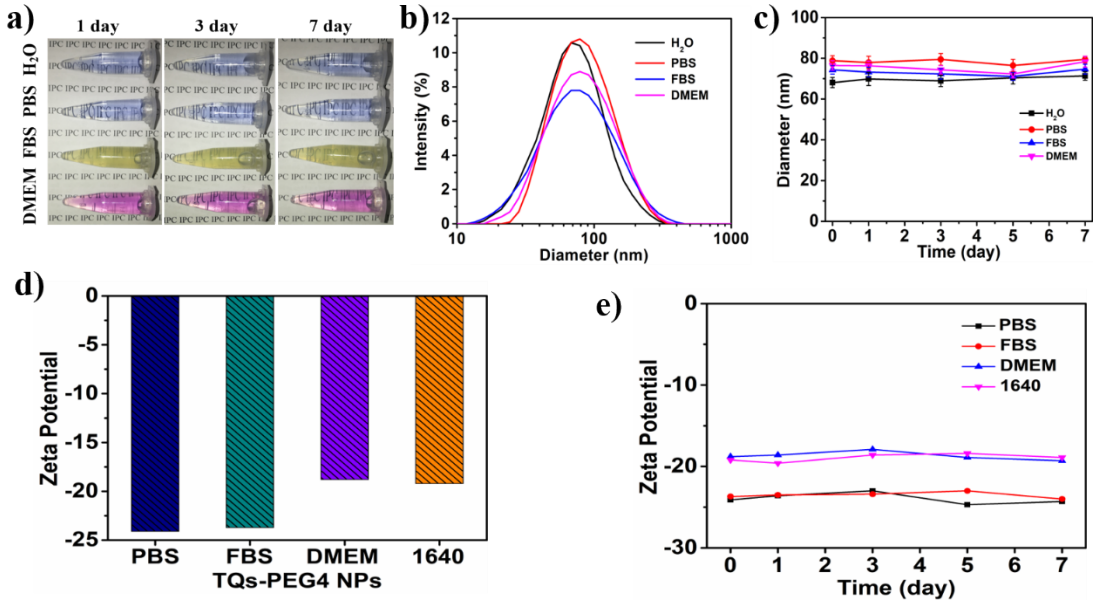

Figure S7 a) Photographs of TQs-PEG4 NPs in different mediums at different time; b) DLS profile of TQs-PEG4 NPs in different culture media; c) The diameter results of TQs-PEG4 NPs characterized by DLS in different culture media at different time; d) Zeta potentials of TQs-PEG4 NPs in different culture media; e) Zeta potential results of TQs-PEG4 NPs in different culture media at different time.

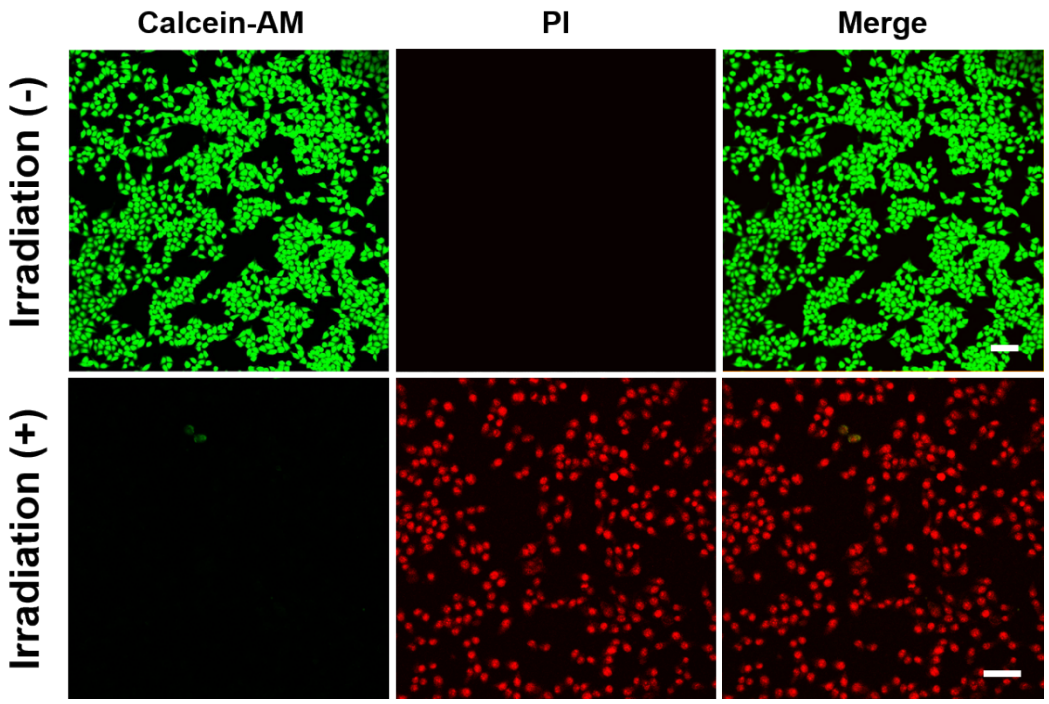

Figure S8 CLSM images of HeLa cells after co-stained with Calcein-AM and PI with or without laser irradiation. Scale bar: 100 μm. Irradiation condition: 635 nm, 20 mW cm^{-2}, 5 min.

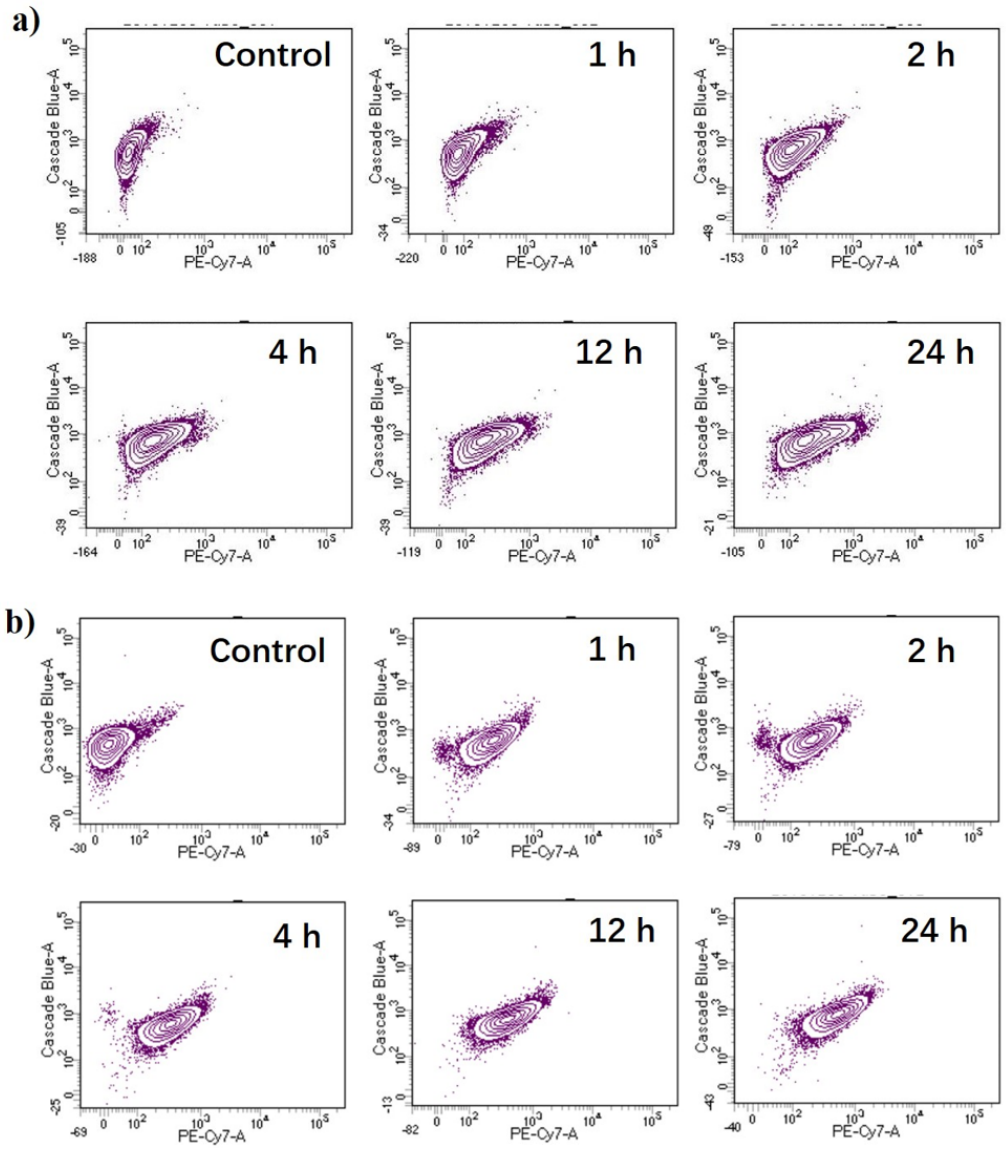

Figure S9 Cell flow cytometry analysis towards of L929 cells a) and HeLa cells b) after incubation with TQs-PEG4 NPs (5.0 μg mL^{-1}) for different time.

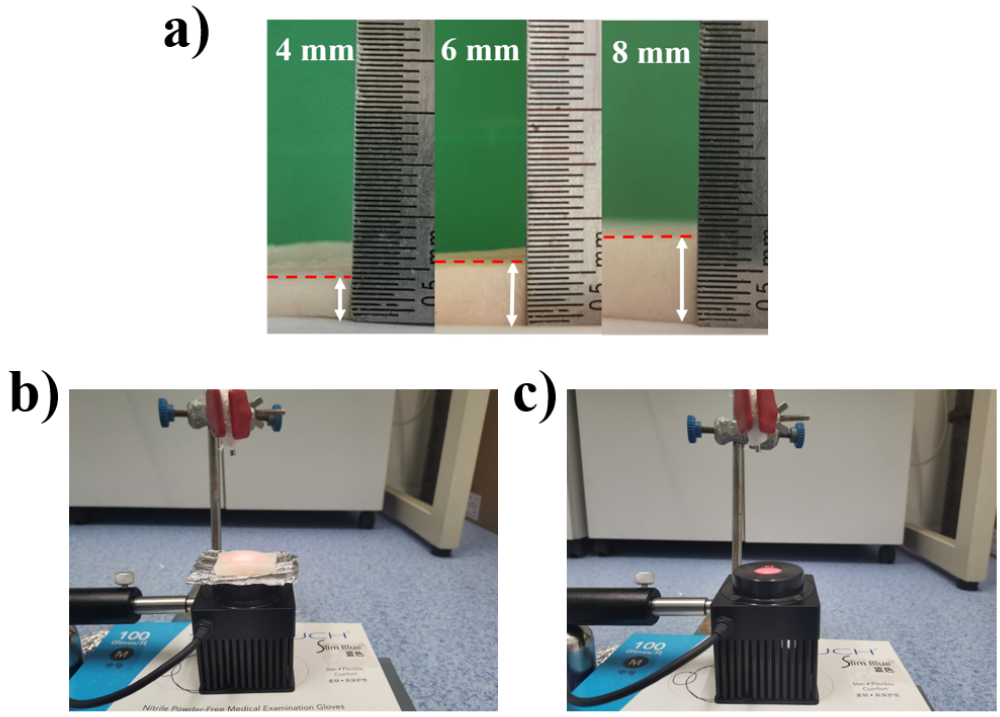

Figure S10 a) Pictures of pork tissues with different thickness; The output power of laser (635 nm) was detected with b) and without c) pork.

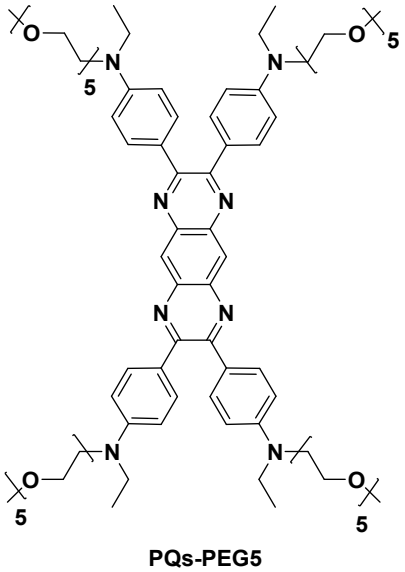

Figure S11 The chemical structure of PQs-PEG5.

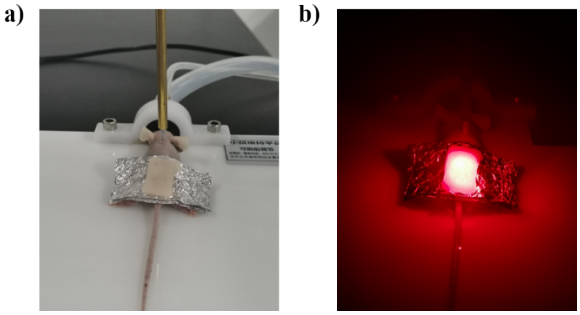

Figure S12 Photograph of mouse covered with pork tissue at tumor site a) and under irradiation by a 660 nm dioxide laser b). Irradiation condition: 150 mW cm^{-2}, 30 min.

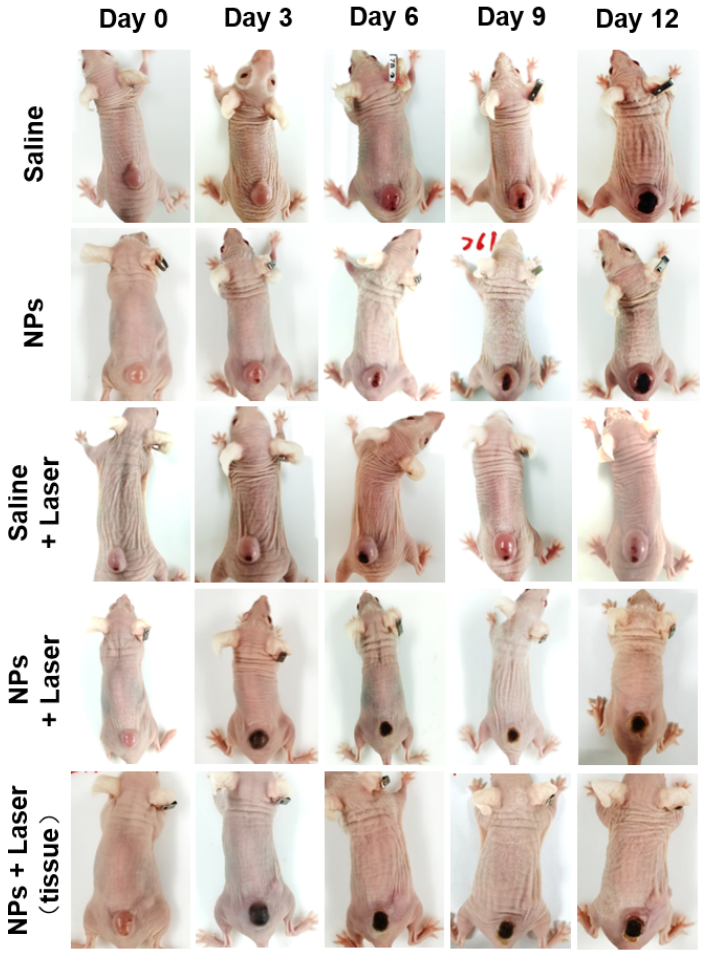

Figure S13 Photos of tumor-bearing mice after different treatments at different time

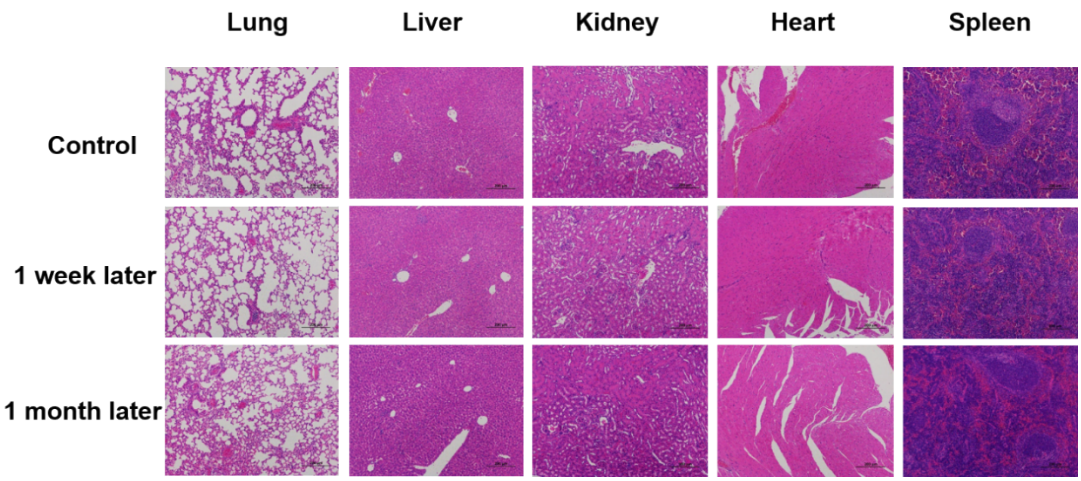

Figure S14 Histological hematoxylin and eosin (H&E) analysis of the major organs (lung, liver, kidney, heart, and spleen) tissues collected from mice of untreated group (control) and TQs-PEG4 NPs group at 1 week and 1 month. Scale bar: 200 μm