Electronic Supplementary Information

Novel mitochondrial-targeted thiadiazolo[3,4-g]quinoxaline dyes as efficient photosensitizers for ultra-low dose operable photodynamic therapy

Xianqiang Li, [a,b] Li-Peng Zhang, *[a] Lin Kang, [a,b] and Yuxia Zhao*[a]

[a] Key Laboratory of Photochemical Conversion and Optoelectronic Materials, Technical Institute of Physics and Chemistry, Chinese Academy of Sciences, 29 Zhongguancun East Road, Haidian District, Beijing, 100190, China.

E-mail: lipzhang@mail.ipc.ac.cn; yuxia.zhao@mail.ipc.ac.cn;

[b] University of Chinese Academy of Sciences, No.19A Yuquan Road, Beijing, 100049, China.

Materials and measurement

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.

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 were presented as follows: the chemical shift in ppm (δ) downfield from tetramethylsilane (multiplicity, coupling constant (Hz), and integration). The following abbreviations were used in reporting NMR data: s, singlet; d, doublet; t, triplet; q, quartet; and m, multiplet. UV-vis absorption spectra were recorded using a Shimadzu U-3900 spectrophotometer and fluorescence tests were carried out on a HITACHI F4500 spectrophotometer. Mass spectra were acquired using

a Bruker Daltonics Inc. spectrometer.

Synthesis of TQs-3 and TQs-4

The synthetic routes of TQs-3 and TQs-4 are shown in Scheme S1.

Synthesis of 4,4'-(4,9-dibromo-[1,2,5]thiadiazolo[3,4-g]quinoxaline-6,7-diyl)bis(N,N-di-p-tolylaniline) (TQs-3). Under argon atmosphere, compound **1** (0.768 g, 2.0 mmol) and iron powder (2.0 g, 35.7 mmol) were added in glacial acetic acid (25 mL) and the mixture was stirred at 70 °C for 4 h to form intermediate **2**. The excess iron powder was removed using magnetic bar followed by adding 1,2-bis(4-1,2-bis(4-(di-p-tolylamino)phenyl)ethane-1,2-dione **3** (1.2 g, 2.0 mmol). After stirring at 90 °C for 36 h, the solvent was removed by evaporated under a reduced pressure and the residue was dissolved with dichloromethane (100 mL), washed with saturated $NaHCO_3$ solution (100 mL) and water (100 mL), respectively. The organic phase was dried over anhydrous Na_2SO_4 and concentrated. The crude was purified by silica gel column chromatography (eluting with dichloromethane/petroleum ether, 50/50, v/v) to afford the product **TQs-3**, (0.783 g, 72.4%) as a dark blue solid. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 2.35 (s, 12H), 6.96 (d, 4H), 7.06 ~ 7.13 (m, 16H), 7.68 (d, J = 8.5 Hz, 4H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 161.10, 155.48, 152.21, 138.10, 131.99, 130.07, 114.41, 113.33, 63.86, 14.76. HR-MS (MALDI-TOF): m/z $[M+H]^+$ cacld for $C_{24}H_{19}Br_2N_4O_2S$, 889.1136; found, 889.1147.

Synthesis of 4,4'-(4,9-dibromo-[1,2,5]thiadiazolo[3,4-g]quinoxaline-6,7-diyl)bis(N,N-diethylaniline) (TQs-4). TQs-4 was prepared according to the similar procedure of **TQs-3**, except using 1,2-bis(4-(diethylamino)phenyl)ethane-1,2-dione as starting material. **TQs-4** was obtained as a blue solid, yield 40%. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.21 (t, J = 6.9, 12H), 3.43 (q, J =6.9, 8H), 6.66 (d, J = 7.4, 4H), 7.85 (d, J = 8.5, 4H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 155.67, 152.00, 149.73, 138.47, 132.12, 130.89, 128.84, 110.81, 44.55, 12.65. HR-MS (MALDI-TOF): m/z $[M+H]^+$ cacld for $C_{28}H_{29}Br_2N_6S$, 639.0536; found, 639.0531.

Preparation of TQs-3 NPs and TQs-4 NPs

A solution of DSPE-mPEG2000 (10 mg) and PS (5 mg) in THF (3 mL) was added slowly dropwise into 10 mL deionized water. Then the mixture was stirred for 12 hours at room temperature and filtered by a 0.22 μm membranes to remove the insoluble large particles. Finally, the mixture was concentrated to 1.0 mg mL^{-1} by evaporated under a reduced pressure to generate the PS NPs aqueous dispersion, which was stored in fridge at 4 °C before further usage.

Measurement of $^1O_2$ quantum yield of TQs-3 and TQs-4

To evaluate the $^1O_2$ quantum yield of **TQs-3** and **TQs-4**, TPP and DPBF were respectively used as a standard PS and $^1O_2$ trapping agent. DPBF can react with $^1O_2$ and cause a decreased absorption. To avoid the inner-filter effect, the absorption of TPP, **TQs-3** and **TQs-4** in chloroform 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 7.0 software, which is proportional to the reaction rate of DPBF with $^1O_2$. The $^1O_2$ quantum yield of **TQs-3** and **TQs-4** 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-3 NPs and TQs-4 NPs in aqueous solution were evaluated by a similar method 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 NPs and $^1O_2$ trapper (TEMP) 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, 8, 10 min) to monitor $^1O_2$ signals.

Photobleaching experiment

The solution of **TQs-3 or TQs-4** in toluene was exposed to 635 nm laser illumination (60 mW cm^{-2}) for different time (5,10,15,20, 25 and 30 min) and the absorption spectra were recorded immediately after each irradiation.

Cell Culturing and Confocal Imaging of HeLa cells

Firstly, 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.

Secondly, HeLa cells were seeded in 35 mm confocal dishes and incubated for 24 h. Then the cells were continually incubated with **TQs-3 NPs** or **TQs-4 NPs** (0.6 μg mL^{-1}) for 2 hours, after washing twice with PBS to remove residual **TQs-3 NPs** or **TQs-4 NPs**, the cells were imaged by a Nikon A1si laser scanning confocal microscope at an excitation wavelength of 488, 560 and 640 nm.

Live/Dead cell co-staining

HeLa cells were seeded in 35 mm confocal dishes and incubated for 24 h. Then the cells were continually incubated with **TQs-4 NPs** (0.4 μg mL^{-1}) for 2 hours. After washing with PBS twice to remove residual **TQs-4 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 for 15 min, then washed twice with PBS and evaluated by confocal fluorescence microscopy (Nicon A1 R-si).

Intracellular $^1O_2$ detection

HeLa cells were seeded in 35 mm confocal dishes and incubated for 24 h. After that, the cells were continually incubated with **TQs-4 NPs** (0.4 μg mL^{-1}) for 2 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 and then divided into two groups. The control group was stored in the dark, and the illumination group was irradiated with 635 nm laser (20 mW cm^{-2}) for 5 minutes. The green fluorescence of DCFH was measured by confocal fluorescence microscopy (Nicon C2 R-si). The excitation wavelength was 488 nm, and the capture emission region was from 500 nm to 530 nm.

PDT experiments of the TQs-3 NPs and TQs-4 NPs in vitro.

Dark toxicity: HeLa cells 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-3 NPs** and **TQs-4 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 untreated cells.

Light toxicity: HeLa cells 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-3 NPs** and **TQs-4 NPs** at different concentrations (0, 0.12, 0.24, 0.36, 0.48, 0.60 and 0.72 μg mL^{-1}) were added into each well and incubated for 4 h. The cells were irradiated upon 635 nm light (20,40 and 60 mW cm^{-2}) for 10 min. After light treatment, the cell medium was replaced with 200 μL fresh medium and cells were allowed to continue growing for 16 h. The standard MTT assay was carried out to determine the cell viabilities relative to control untreated

cells.

PDT experiments towards other kinds of cells, such as 4T1, MCF-7 and L929 cells, were taken on according to the similar procedure above.

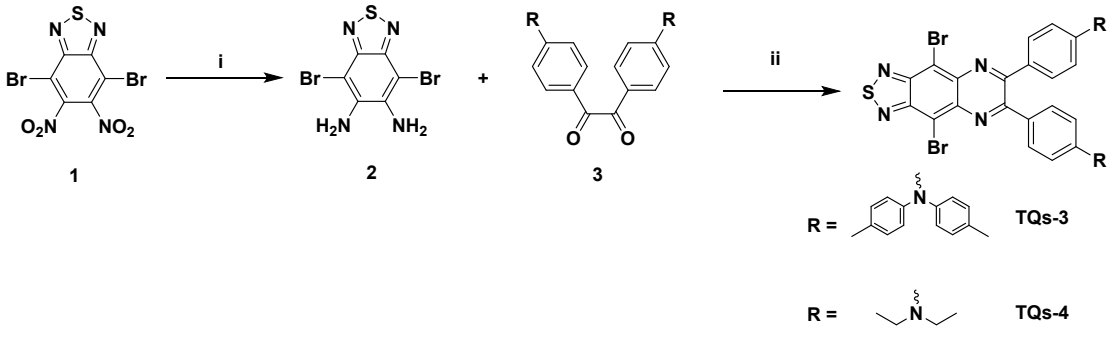
Annexin V-FITC and PI co-staining

HeLa cells were seeded in 35 mm confocal dishes and incubated for 24 h. Then the cells were continually incubated with **TQs-4 NPs** (0.4 μg mL^{-1}) for 2 hours. After washing with PBS twice to remove residual **TQs-4 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 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).

Table S1 Photophysical parameters of **TQs-3** and **TQs-4** in toluene

Compound	λ_{max}^{abs}[a] (nm)	ε_{max} (10^4 $M^{-1}cm^{-1}$)	λ_{max}^{fl}[b] (nm)	$\Delta\nu_{ss}$ (10^{-4} nm^{-1})	Φ_f[c]	Φ_Δ[d]
TQs-3	578	2.25	707	2.53	0.030	0.97
TQs-4	574	2.30	670	2.64	0.022	1.00

[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.



Scheme S1 The synthetic routes of **TQs-3** and **TQs-4**. Reagents and conditions: i) Fe, HOAc, Ar, 70 °C; ii) HOAc, Ar, reflux.

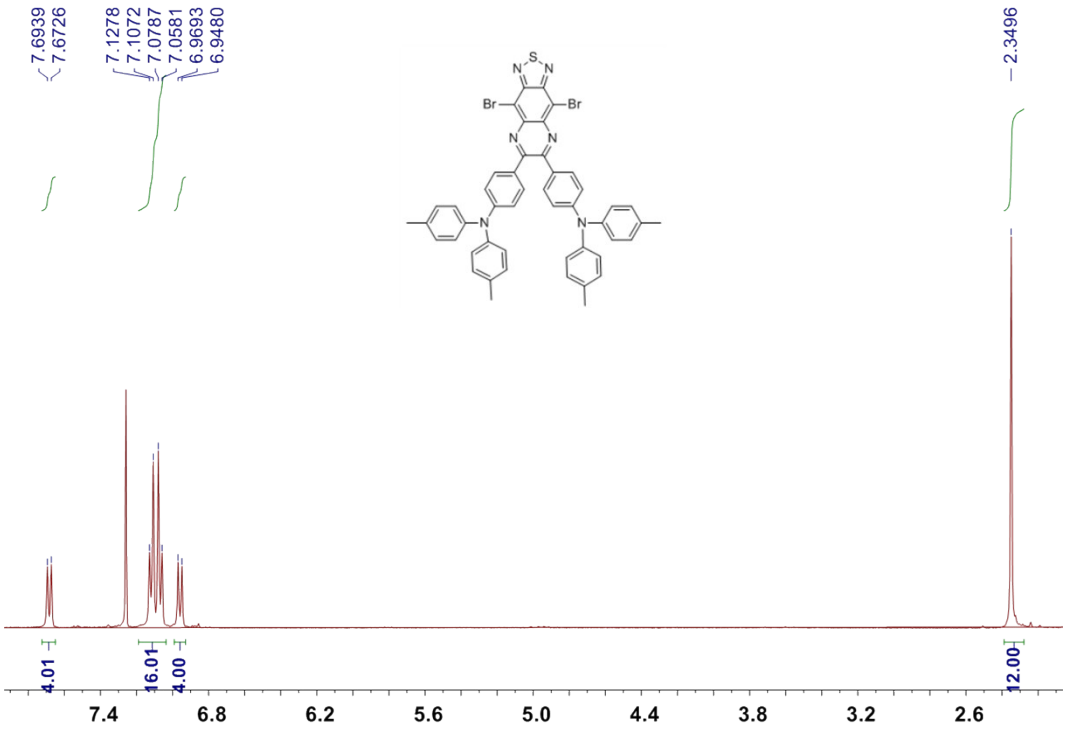


Fig. S1 ^{1}H NMR spectrum of **TQs-3** in chloroform-d.

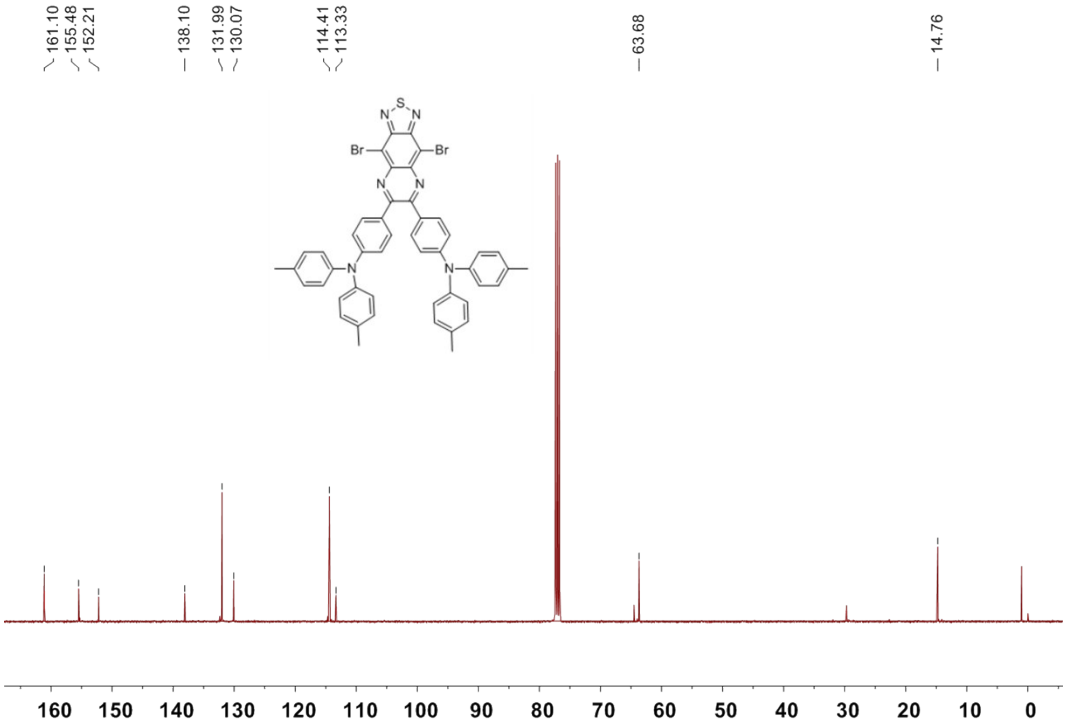


Fig. S2 ^{13}C NMR spectrum of **TQs-3** in chloroform-d.

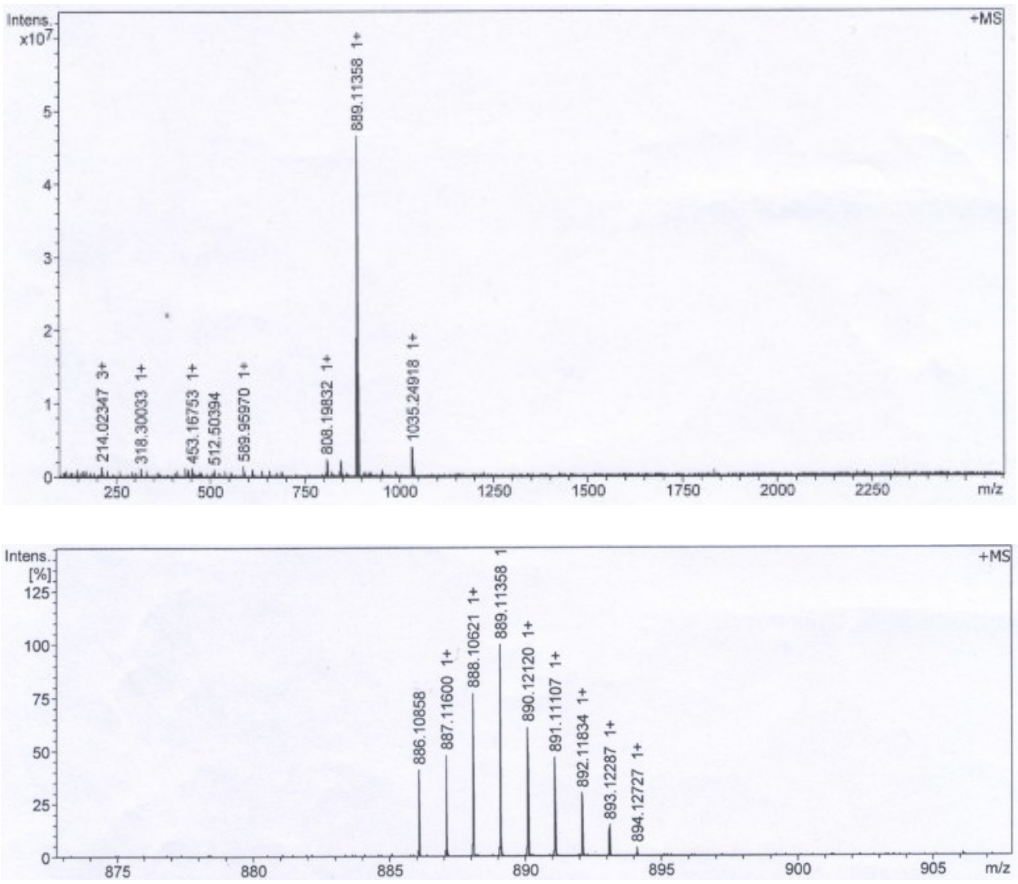


Fig. S3 High resolution mass spectrum of **TQs-3**.

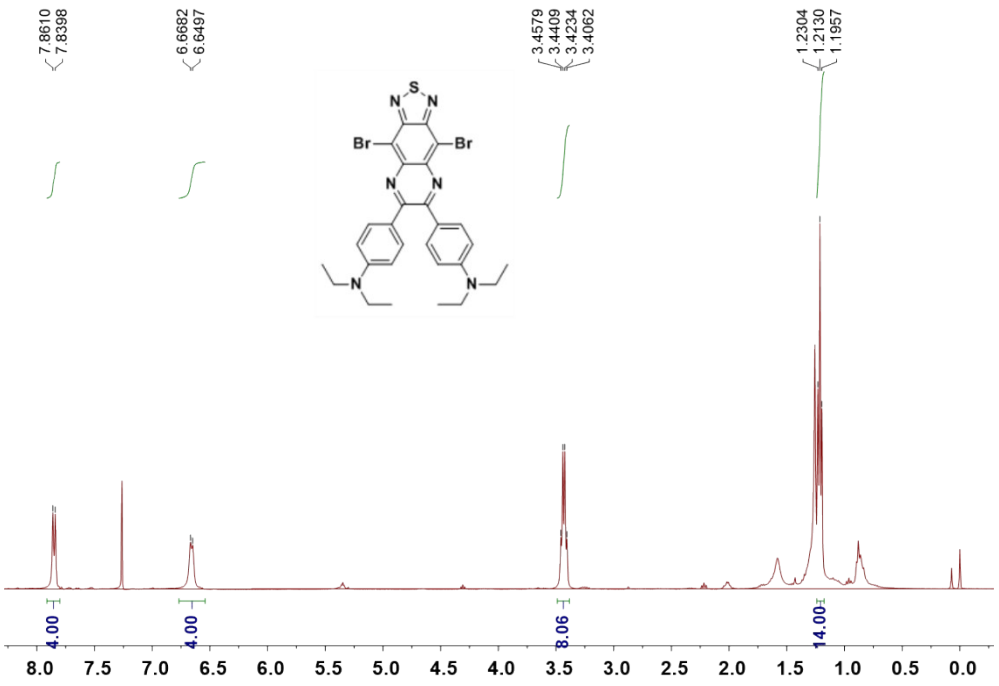


Fig. S4 ^{1}H NMR spectrum of **TQs-4** in chloroform-d.

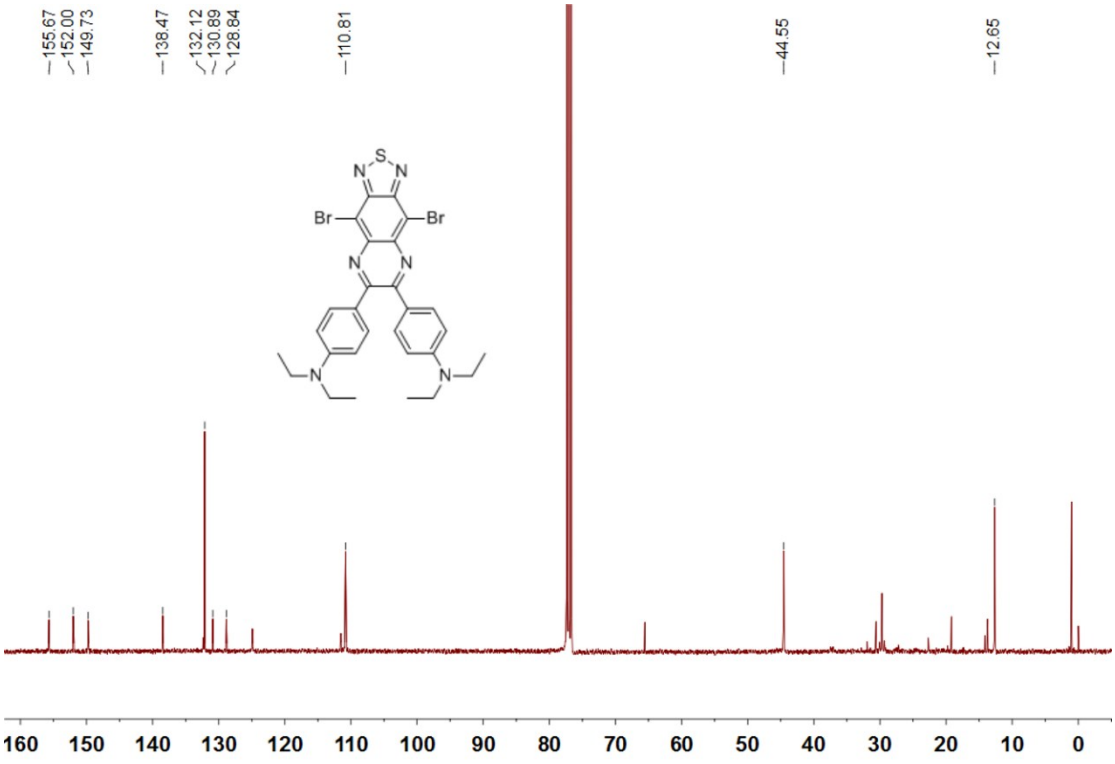


Fig. S5 ^{13}C NMR spectrum of **TQs-4** in chloroform-d.

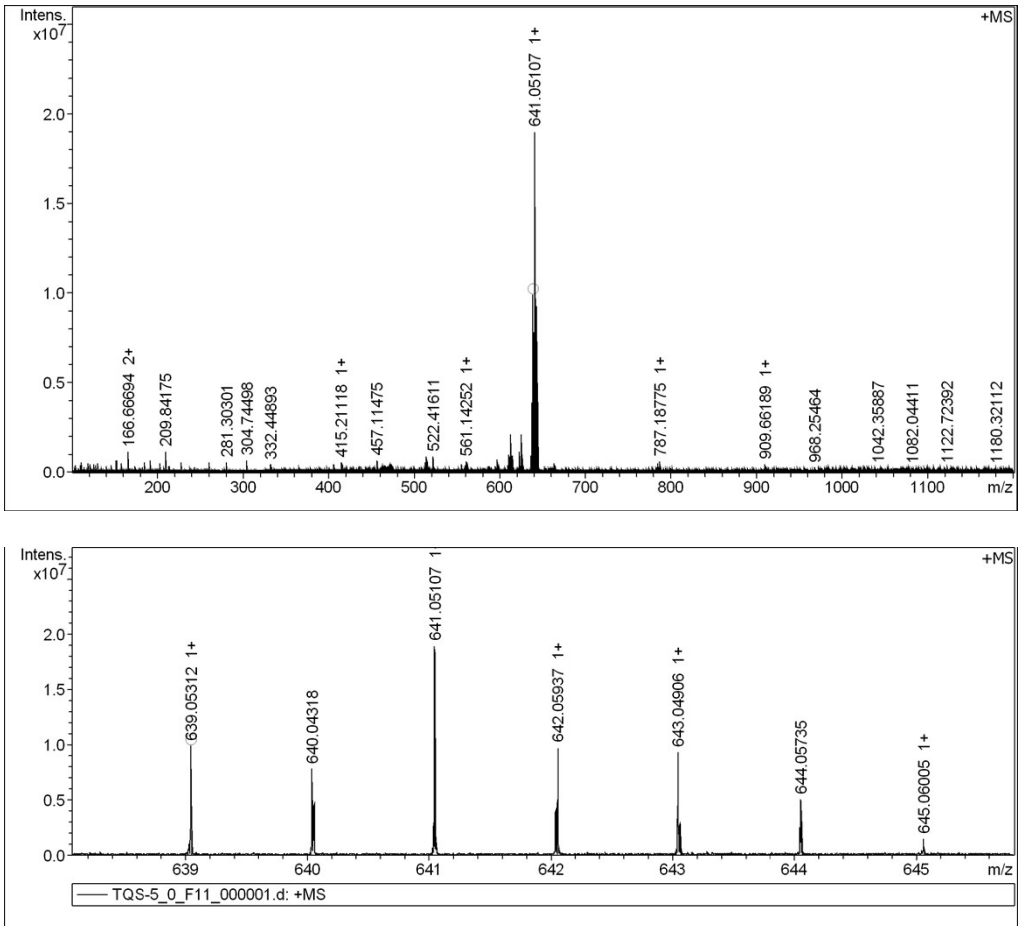


Fig. S6 High resolution mass spectrum of **TQs-4**

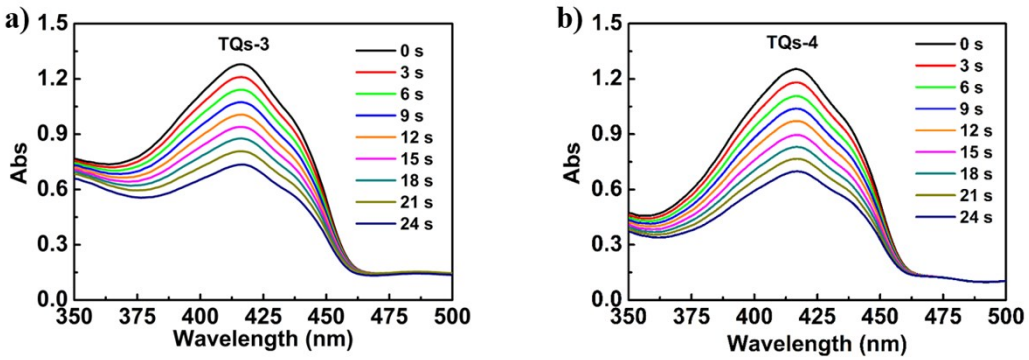


Fig. S7 Absorption spectra of DPBF mixed with a) **TQs-3** and b) **TQs-4** in toluene with 635 nm laser irradiation.

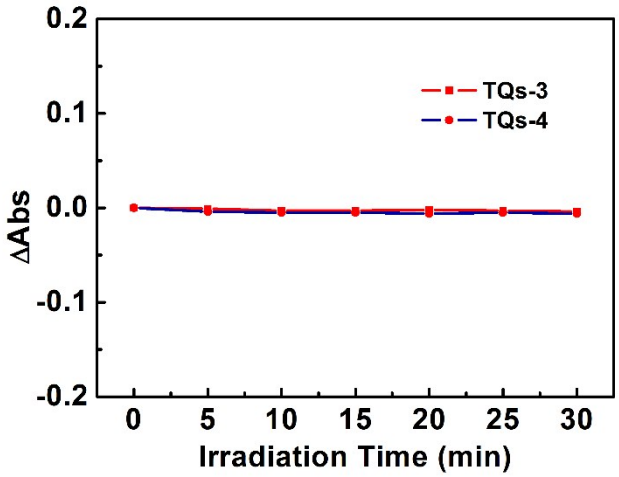


Fig. S8 the photostability of **TQs-3** and **TQs-4** irradiated by a 635 nm laser (60 mW cm^{-2})

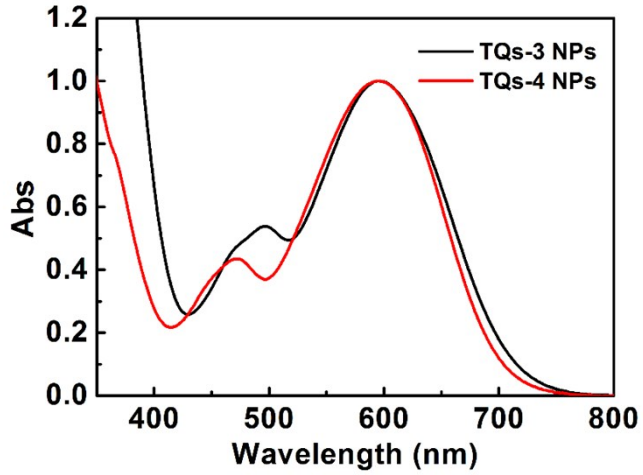


Fig. S9 Normalized UV-Vis spectra of **TQs-3 NPs** and **TQs-4 NPs** in aqueous solution.

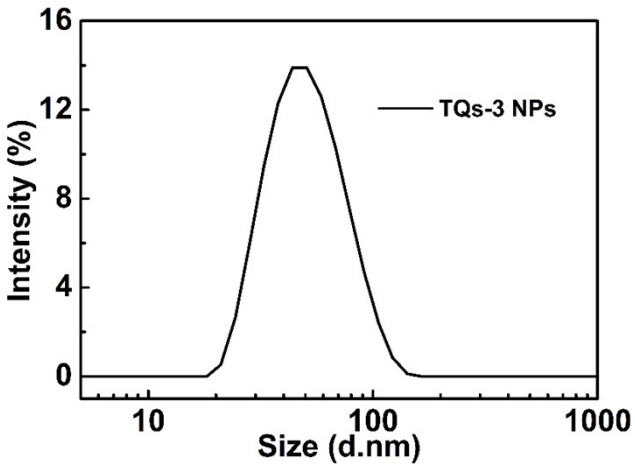


Fig. S10 The DLS analysis of **TQs-3 NPs** in aqueous solution.

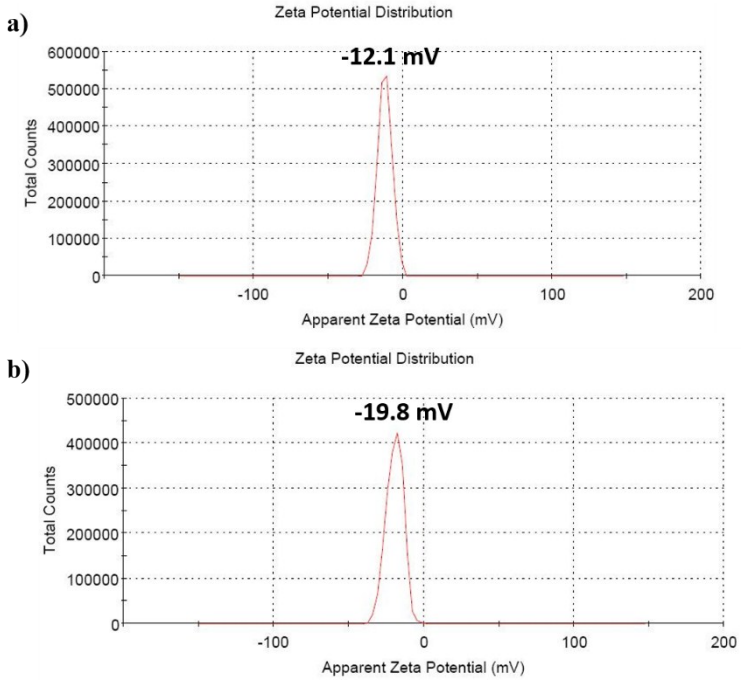


Fig. S11 Zeta potentials (a) of **TQs-3 NPs** and (b) **TQs-4 NPs** in aqueous solution.

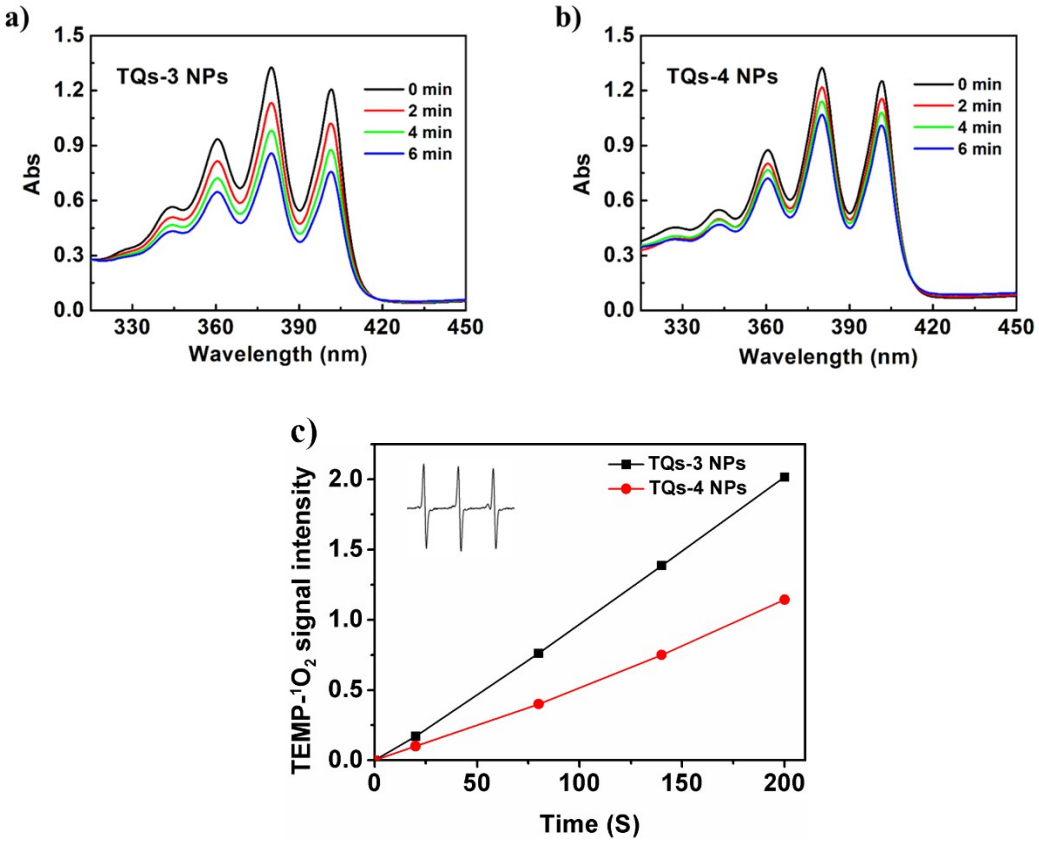


Fig. S12 Absorption spectra of ABDA mixed with a) **TQs-3 NPs** and b) **TQs-4 NPs** in aqueous solution with 635 nm laser irradiation; c) TEMP-$^1O_2$ signal intensities plots versus time of TQs NPs in aqueous solution irradiated by 635 nm laser.

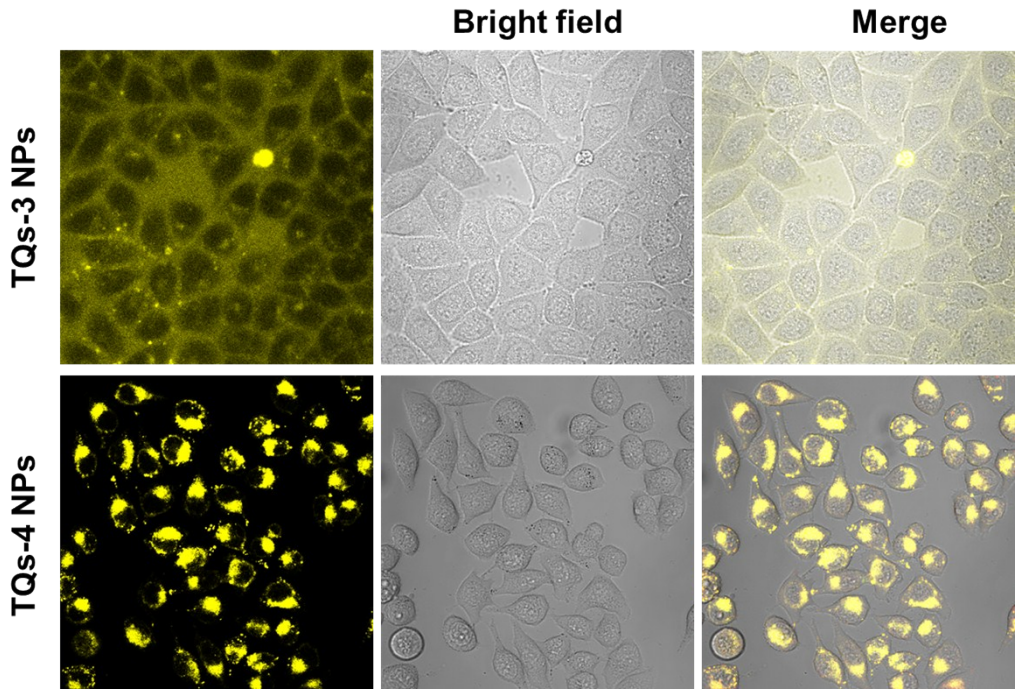


Fig. S13 Confocal FL images of **TQs-3 NPs** and **TQs-4 NPs** incubated with HeLa cells

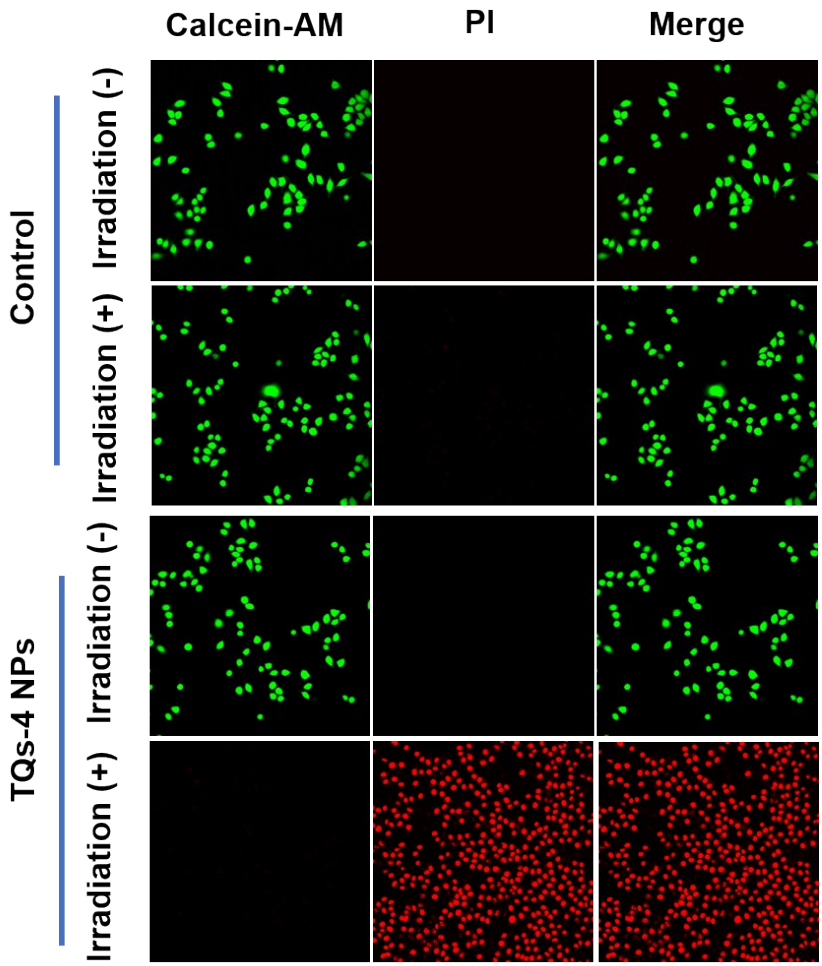


Fig. S14 Confocal FL images of Calcein-AM and PI co-staining HeLa cells after **TQs-4 NPs** incubation

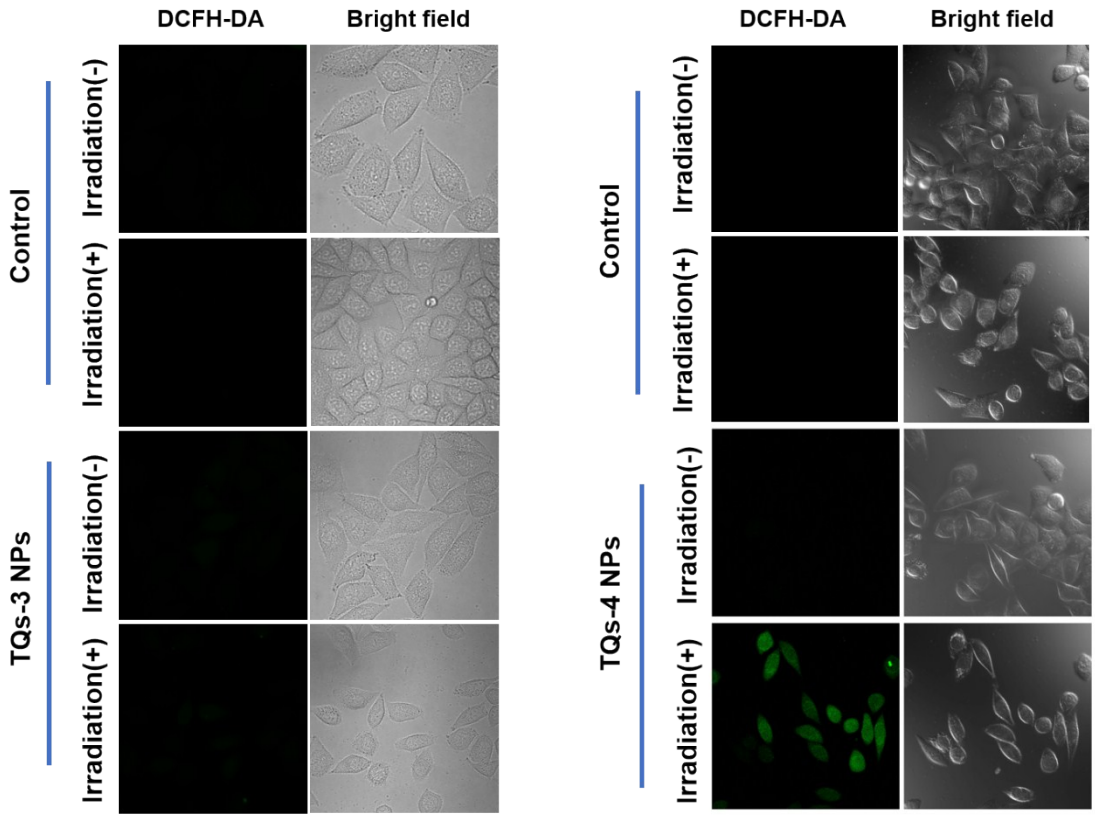


Fig. S15 Confocal FL images of DCFH-DA staining HeLa cells incubated with a) **TQs-3 NPs** and b) **TQs-4 NPs** before and after irradiation by a 635 nm laser (20 mW cm^{-2}, 5 min).

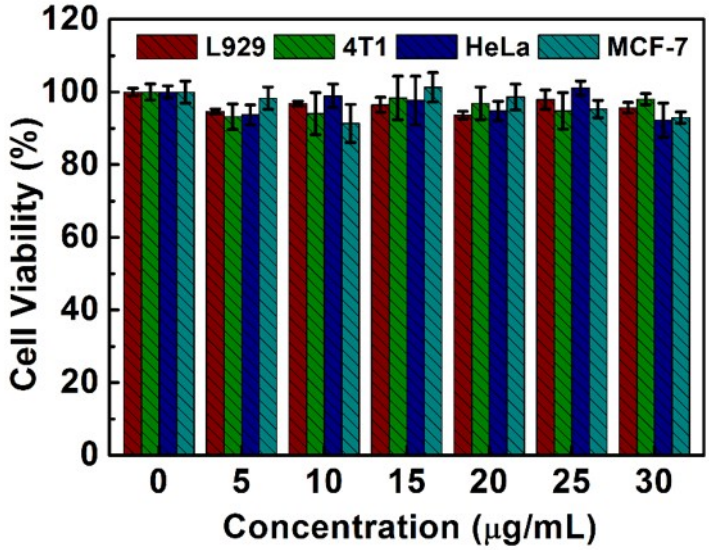


Fig. S16 Relative cell viability of L929, HeLa, MCF-7 and 4T1 cells incubated with **TQs-4 NPs** under different concentrations in dark.

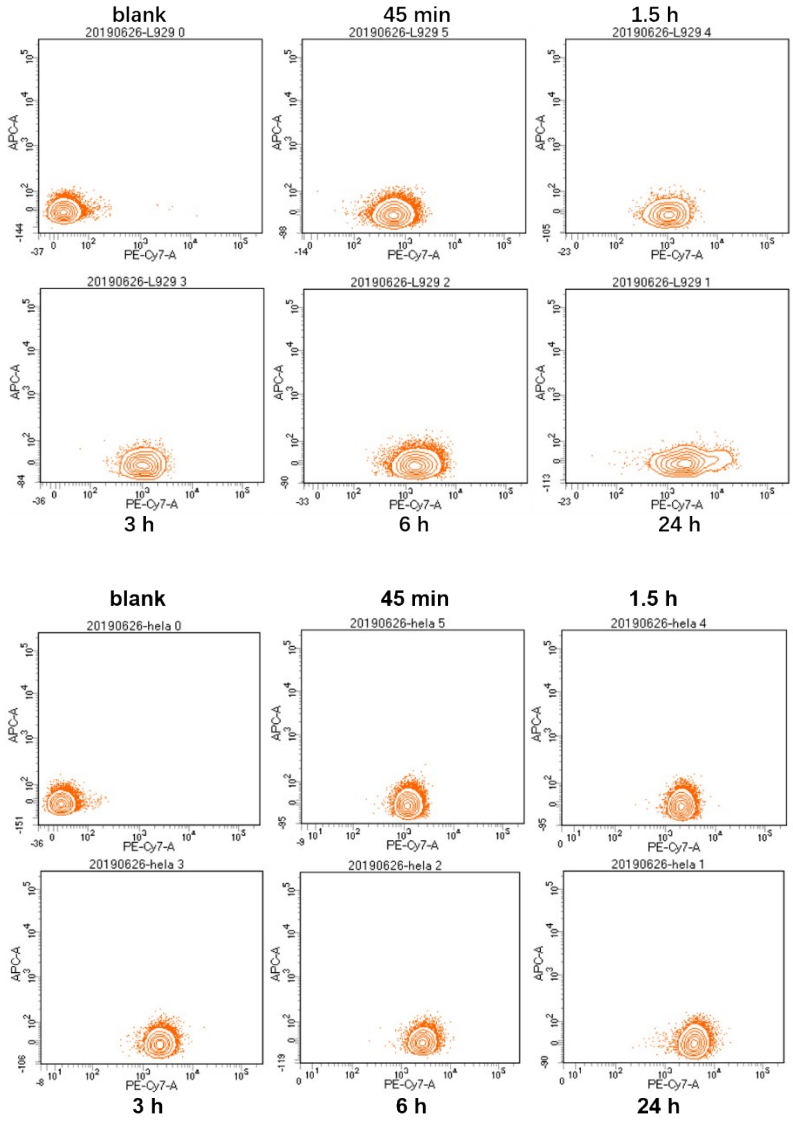


Fig. S17 the uptake of TQs-4 NPs by L929 and HeLa cells at different incubated time

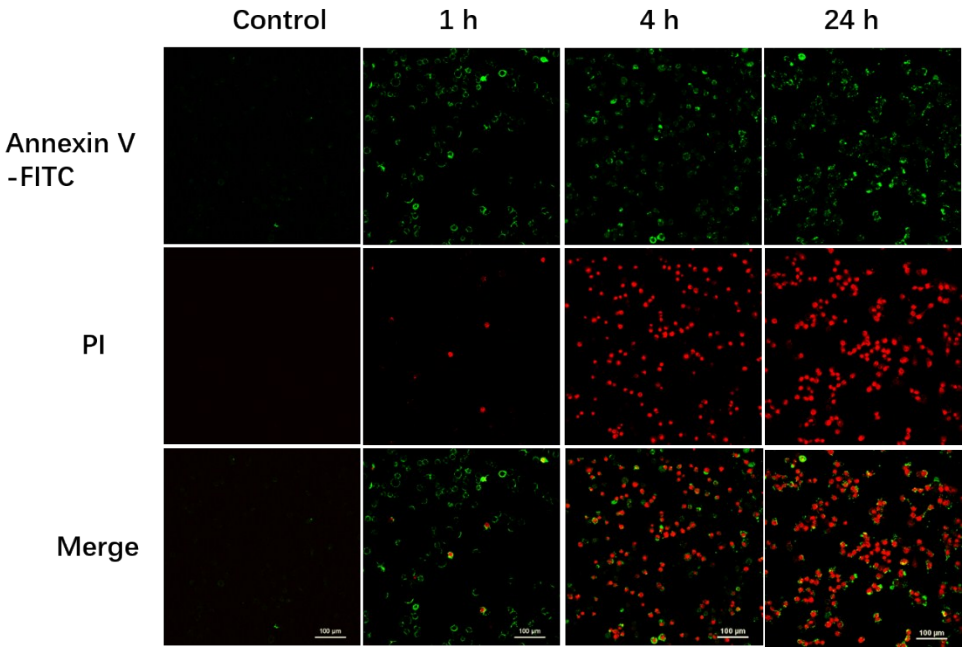


Fig. S18 Confocal laser scanning microscope images of HeLa cells co-staining by Annexin V-FITC and PI at different incubated time after irradiation by 635 nm laser.

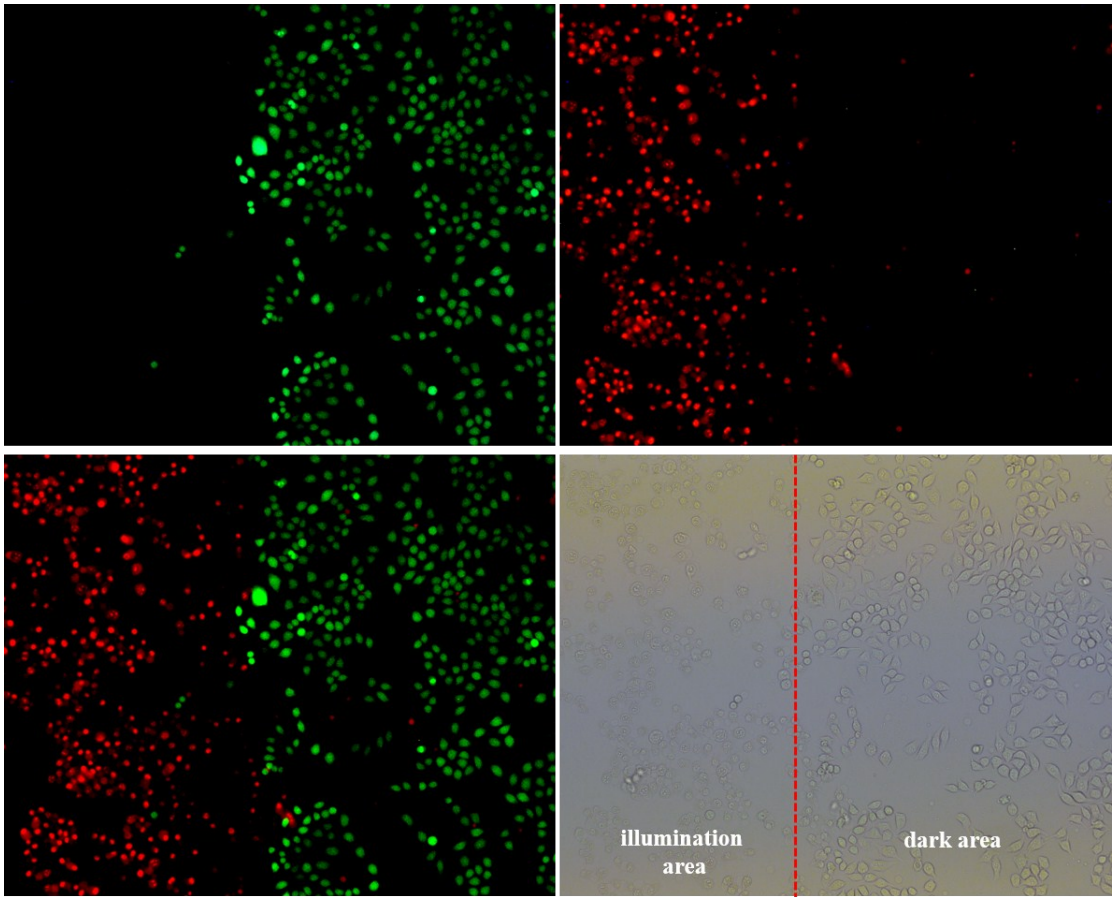


Fig. S19 FL microscope images of HeLa cells incubated with **TQs-4 NPs** with or without irradiation by 635 nm laser, red dash line in right down represented the margin of light beam.