Electronic Supplementary Information

A water-soluble pyrazino[2,3-g]quinoxaline photosensitizer for high-efficient one- and two-photon excited bioimaging and photodynamic therapy

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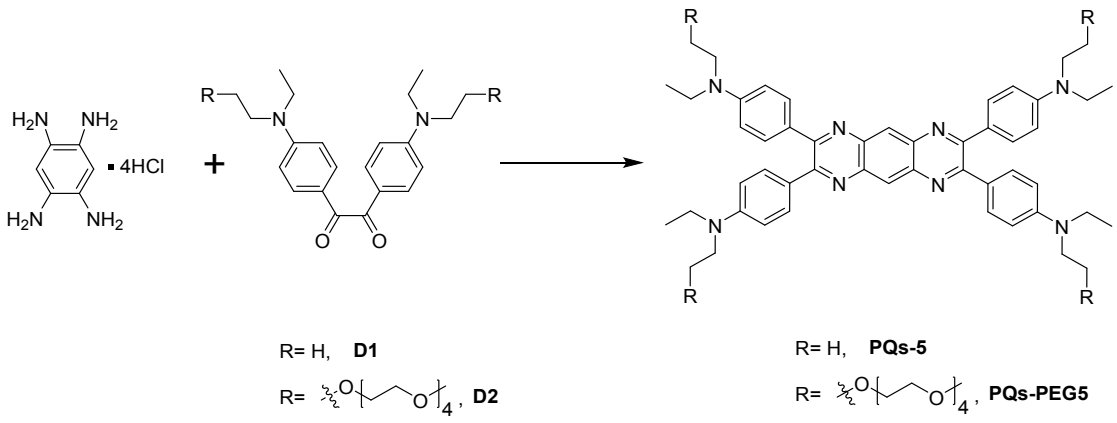
Materials and measurement

All of solvents were purified according to standard methods. All reagents and chemicals for photosensitizers synthesis were purchased from Alfa Aesar Chemical Co.， Ark 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 (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 AVANCE 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 and fluorescence emission spectra were carried out on a HITACHI F4500 spectrophotometer. Fluorescence lifetime and anisotropy were tested on an Edinburgh FLS 1000 instruments. Cell imaging were performed using a Nikcon A1 R-si confocal laser scan microscopy using three diode lasers (405 nm, 488 nm and 561 nm) as the light sources, respectively. Two-photon PDT experiment was also performed on the Nikcon A1 R-si confocal laser scan microscopy using an 820 nm fs laser (100 mW, 80 MHz, 140 fs) as the light source. PDT experiment was used a 635 nm diode laser (Changchun New Industries Optoelectronic Tech.) as the light source.



Scheme S1 The synthetic route to PQs-5 and PQs-PEG5. Reagents and conditions: HOAc, N_2, reflux, 24 h

Synthesis of PQs-5 and PQs-PEG5

Under nitrogen atmosphere, benzene-1,2,4,5-tetraamine tetra hydrogen chloride (1.0 mmol) was dissolved in glacial acetic acid (30 mL), followed by adding the corresponding dione compound D1 or D2 [1](2.0 mmol). After reflux for 24 h, the solvent was removed by evaporated under the reduced pressure and the residue was diluted with dichloromethane (100 mL), washed with saturated $NaHCO_3$ solution (100 mL), water (100 mL), and brine (100 mL), successively. The organic phase was dried over anhydrous $MgSO_4$, concentrated in vacuum and purified by silica gel column chromatography (eluting with Methyl alcohol/dichloromethane) to afford photosensitizers (PSs) as a deep red solid (**PQs-5**) or a dull red oily solid (**PQs-PEG5**).

4,4',4",4"'-(pyrazino[2,3-g]quinoxaline-2,3,7,8-tetrayl)tetrakis(N,N-diethylaniline) (**PQs-5**). Yield: 60%. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.19 (t, *J* = 12.0 Hz, 24H), 3.40 (q, *J* = 8.0 Hz, 16H), 6.65 (d, *J* = 8.0 Hz, 8H), 7.60 (d, *J* = 8.0 Hz, 8H), 8.70 (s, 2H). ^{13}C NMR ($CDCl_3$, 150 MHz): δ 153.51, 147.39, 139.15, 130.34, 125.53, 125.42, 110.03, 43.42, 11.62. HR-MS (MALDI): m/z $[M]^+$ cacld for $C_{50}H_{58}N_8$, 770.4784; found, 771.4857.

4,4',4",4"'-(pyrazino[2,3-g]quinoxaline-2,3,7,8-tetrayl)tetrakis(N-ethyl-N-(2-(2-methoxytetraethoxy)ethyl)aniline) (**PQs-PEG5**). Yield: 23%. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.18 (t, *J* = 12.0 Hz, 12H), 3.37 (s, 12 H), 3.46 (q, *J* = 8.0 Hz, 8H), 3.55 (m, 16 H), 3.66 (s, 64 H), 6.66 (d, *J* = 8.0 Hz, 8 H), 7.59 (d, *J* = 8.0 Hz, 8 H), 8.70 (s, 2 H). ^{13}C NMR ($CDCl_3$, 150 MHz): δ 153.43, 147.45, 139.14, 130.33, 125.63, 110.04, 70.92, 69.72, 69.60, 67.71, 58.03, 48.99, 44.77, 44.53, 30.89, 28.68, 28.31, 28.23, 26.20, 21.67, 13.10, 11.20, 7.60. HR-MS (MALDI): m/z $[M]^+$ cacld for $C_{86}H_{130}N_8O_{20}$, 1594.9471; found, 1596.9474.

Measurement of fluorescence quantum yield

The fluorescence quantum yields (Φ_f) of PQs-5 and PQs-PEG5 in toluene were measured by HITACHI F4500 spectrophotometer at room temperature. The absorbance of all samples at the absorption maximum (λ_{max}[a]) were adjusted about 0.05. Rhodamine B (RB) in methanol was used as reference (Φ_f = 0.70). Fluorescence spectra of these samples were recorded and their integrates were also calculated. The excitation wavelength was 520 nm. The Φ_f of tested PSs can be calculated by Equation as followed,

$$\Phi_{s=} \frac{\Phi_R F_s A_R n_s^2}{F_R A_s n_R^2}$$

where F is the integral area of fluorescence spectra for PSs, and A is the absorbance value of the tested PSs at the excited wavelength. n is the refractive index of solvent in test. S represents tested sample; R stands for the reference RB and Φ_R is the Φ_f of RB.

Measurement of $^1O_2$ quantum yield

To evaluate the $^1O_2$ quantum yield (Φ_Δ) of **PQs-5** and **PQs-PEG5** in toluene, tetraphenylporphyrin (TPP) and 1,4-diphenyl-2,3-benzofuran (DPBF) was used as a standard PS and $^1O_2$ trapping agent, respectively. DPBF can react with $^1O_2$ and cause a gradual decrease of its absorbance within UV-vis region. To avoid the inner-filter effect, the absorbance of PS in toluene at 532 nm was regulated to approximately 0.20 OD, the absorbance of DPBF at 440 nm was regulated to approximately 1.0 OD. Then the mixture was illuminated by a 532 nm diode laser (10 mW cm^{-2}) for different time and the absorption spectra of PS+DPBF in toluene were recorded immediately after each irradiation. The absorbance decay at 440 nm of these solution was plotted against irradiation time. After linear fitted using Origin 9.0 software, the slop of each set of

datapoints is proportional to the reaction rate of corresponding PS with $^1O_2$. The Φ_Δ of tested PSs can be calculated by Equation as followed.

$$\Phi_{\Delta=}^{S} \frac{K_S}{K_R} \times \Phi_\Delta^R$$

where K is the slop obtained for PSs, S represents tested sample, R stands for the reference TPP and Φ_Δ^R is the Φ_Δ of TPP.

Photobleaching experiments

The solutions of **PQs-5** and **PQs-PEG5** in toluene were exposed to 532 nm laser illumination (100 mW cm^{-2}) and the solution of **PQs-PEG5** in DI water were exposed to 635 nm laser illumination (100 mW cm^{-2}) for different time (5, 10, 15, 20 and 30 min) and the absorption spectra of them were recorded immediately after each exposure.

2PA cross section characterization

2PA cross-section (σ_2) values of PSs were determined by two-photon excited fluorescence (TPEF) method. The incident light from Mai Tai HP apparatus (740–1000 nm, 80 MHz, 100 fs) was focused into a cuvette containing the sample (**PQs-5** or **PQs-PEG5**) solution (1×10^{-5} M in toluene). 2PE fluorescence spectra were recorded by a fiber spectrometer (SD2000). The laser beam was aligned through two pinholes, placed 50 cm apart, before and behind the sample, which ensured that the excitation beams passed through the exact same location. RB solution (1×10^{-5} M in methanol) was used as the reference. In order to minimize the reabsorption effect, the focus of the laser was

located about 0.3 mm from the sample cuvette wall through which the 2PA induced fluorescence was collected. Each sample was repeatedly measured three times and the average value was taken for calculation. The σ_2 values of the PSs were calculated by the following equation:

$$\sigma_s = \frac{F_s \Phi_R \phi_R c_R}{F_R \Phi_s \phi_s c_s} \sigma_R$$

where R and S stand for the RB and sample, respectively; F is the integral area of the 2PE fluorescence; Φ is Φ_f of RB and samples; φ is the overall fluorescence collection efficiency of the experimental apparatus, and c is the molar concentration of the molecules in solution.

The logarithmic plots of the 2PE fluorescence emission integral area of PSs *versus* different excitation intensity of 800 nm laser were provided to calculate the linear dependence between them.

Cell imaging under 1PE and 2PE

All cells were incubated in DMEM (HeLa cell) medium or 1640 (4T1 cell) supplemented with 10% FBS and 1% penicillin-streptomycin at 37℃ in a humidified, 5% CO_2 atmosphere.

Cells were firstly seeded in 35 mm fluorescent dishes and incubated for 24 h. The cell medium was replaced with fresh medium containing **PQs-PEG5** (5.0 μM) and the cells were continually incubated for 4 h. The fluorescent dishes were rinsed in 2 mL PBS solution, and evaluated by laser confocal scanning microscopy (CLSM, Nikcon A1 R-si) under 1PE (561 nm) and 2PE (800–1000 nm). For subcellular localization experiments, Mito-tracker Green or Hoechst33342 was added after incubation of **PQs-PEG5** loaded cells and stained for 15 min. Then the cells were washed twice with PBS and evaluated by CLSM under 1PE.

Cell flow cytometry analysis

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, **PQs-PEG5** (5.0 μM) were added into each well and incubated for different times (1, 4, 8, 18, 24 h), and the cells without **PQs-PEG5** 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.

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 **PQs-PEG5** (4 μM) for 4 hours, then the culture medium was discarded, washed with PBS (3 mL×2) and stained with DCFH-DA (10 μM) in fresh medium for another 30 min. After all treatment, the cells were washed with PBS (3 mL×2) 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-DA was measured by CLSM. The excitation wavelength was 488 nm, and the capture emission region was from 500 nm to 530 nm.

In vitro PDT experiments

Dark toxicity: HeLa or 4T1 cells were firstly 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, **PQs-PEG5** at different concentrations (0, 2.5, 5, 10, 20, and 100 μM) 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: HeLa or 4T1 cells were firstly 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, **PQs-PEG5** at different concentrations (0, 0.05, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0 and 10.0 μM) were added into each well and incubated for 4 h. The cells were irradiated upon 635 nm light (60 mW cm^{-2}) for 5 min. After light treatment, the cell medium was replaced with 200 μL fresh medium and cells were allowed to continue growing for 24 h. The standard MTT assay was carried out to determine the cell viabilities relative to control group.

Live/Dead cell co-staining

4T1 cells were firstly seeded in 35 mm fluorescent dishes at a density of 5×10^4 per well and cultured in 5% CO_2 at 37 °C for 24 h, and the cells were continually incubated with **PQs-PEG5** (10 μM) for 24 h. After removal of culture, the cells was washed with PBS (3 mL×2) and soaked in PBS (1 mL). Then a square zone with 600×600 μm in central was set as the irradiation region, and the cells were exposed upon an 820 nm fs laser (100 mW, 80 MHz, 140 fs) for 5 min. The culture media was replaced with fresh DMEM (3 mL). Then the cells were incubated for another 8 h, and the culture was replaced with fresh DMEM (3 mL) containing 4 μL Calcein-AM (1.0 mg mL^{-1}) and 6 μL propidium iodide (PI, 1.0 mg mL^{-1}). After co-stained for 15 min, the cells were washed with PBS (3 mL×2) and evaluated by CLSM.

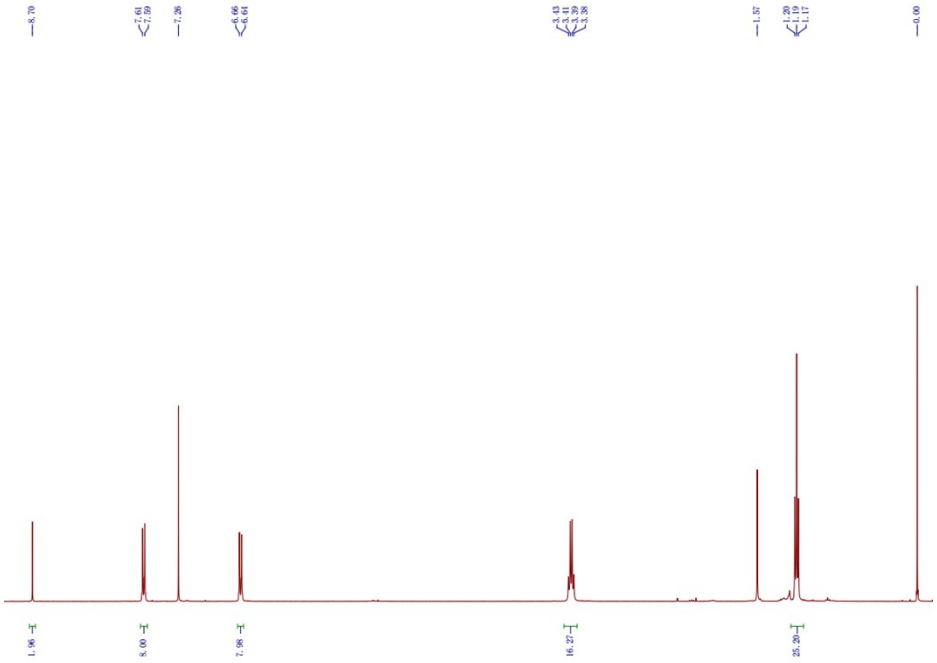


Fig. S1 [1]HNMR spectrum of **PQs-5**

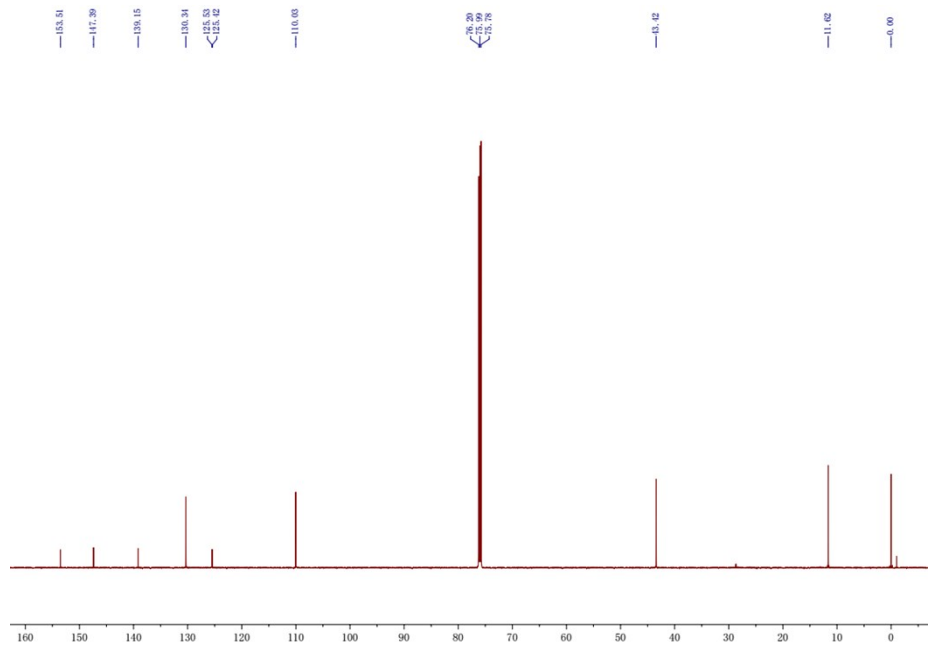


Fig. S2 [13]CNMR spectrum of **PQs-5**

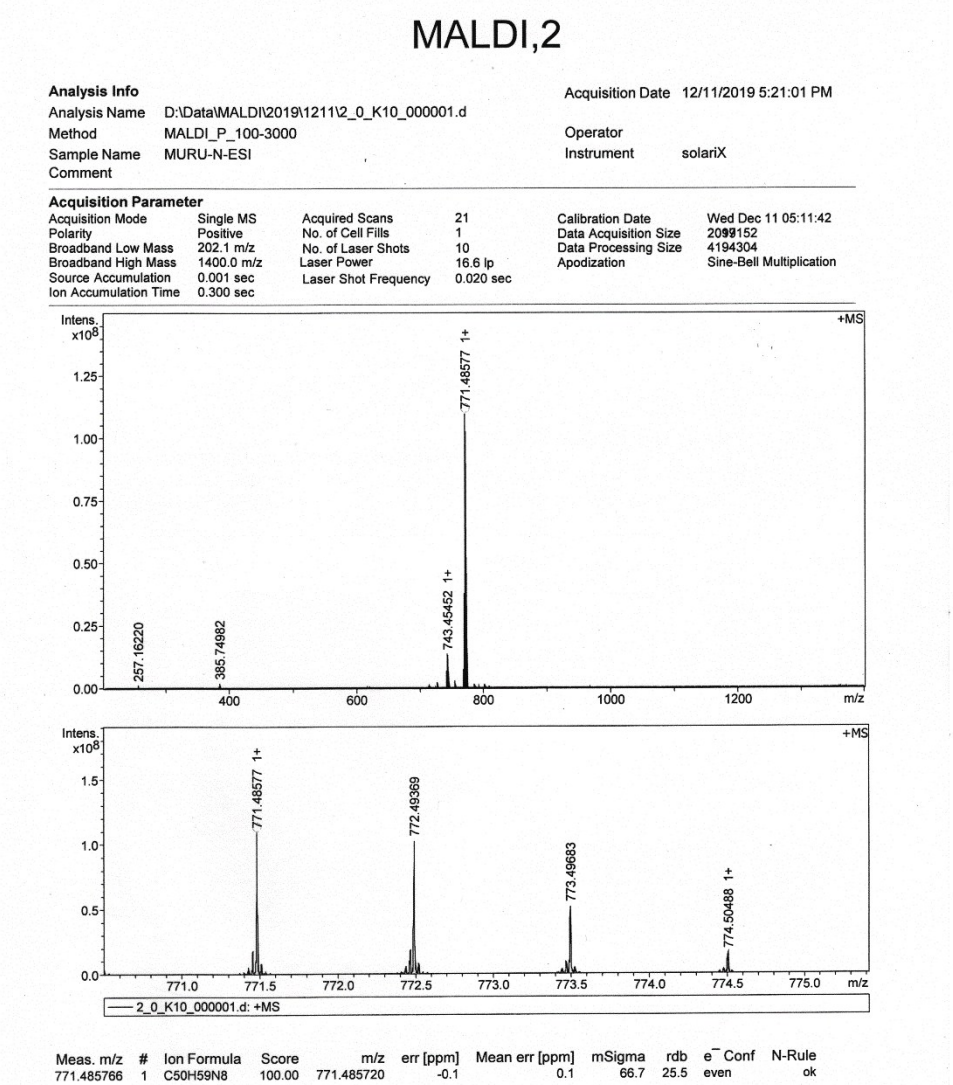


Fig. S3 HRMS (MALDI TOF) spectrum of **PQs-5**

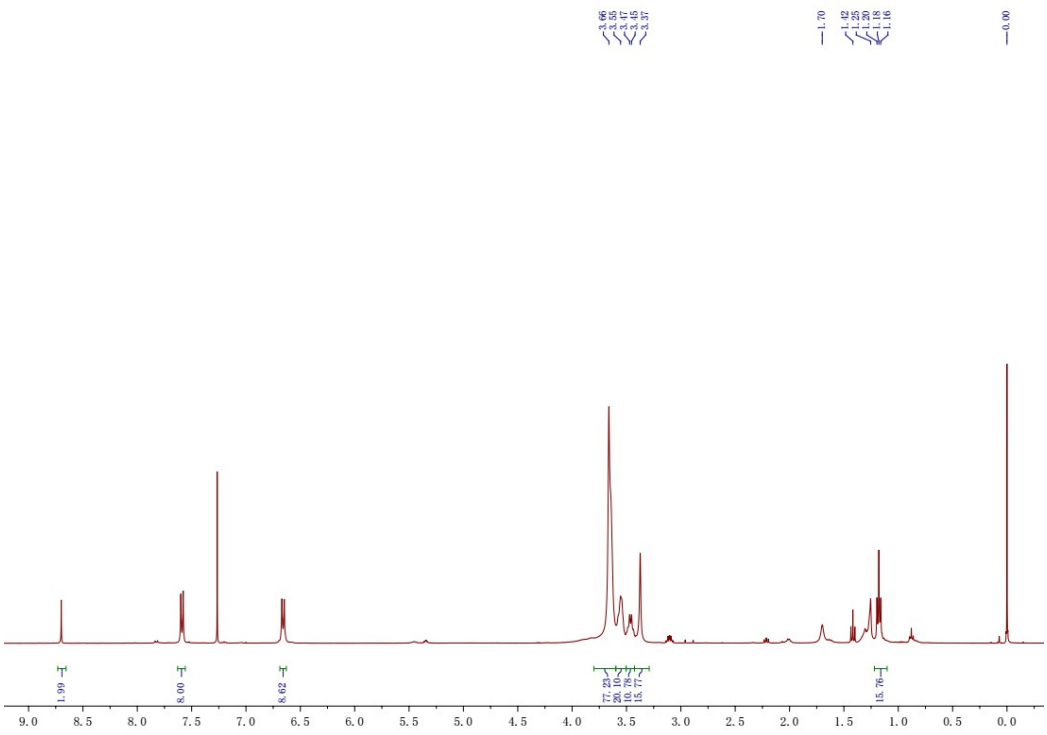


Fig. S4 ^{1}HNMR spectrum of **PQs-PEG5**

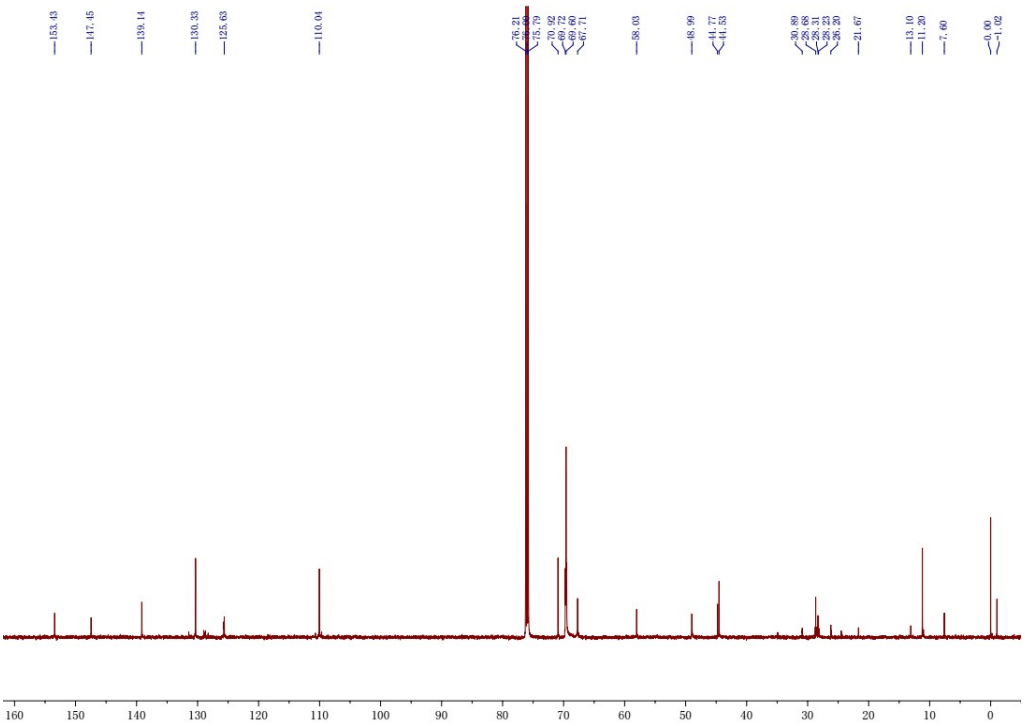


Fig. S5 ^{13}CNMR spectrum of **PQs-PEG5**

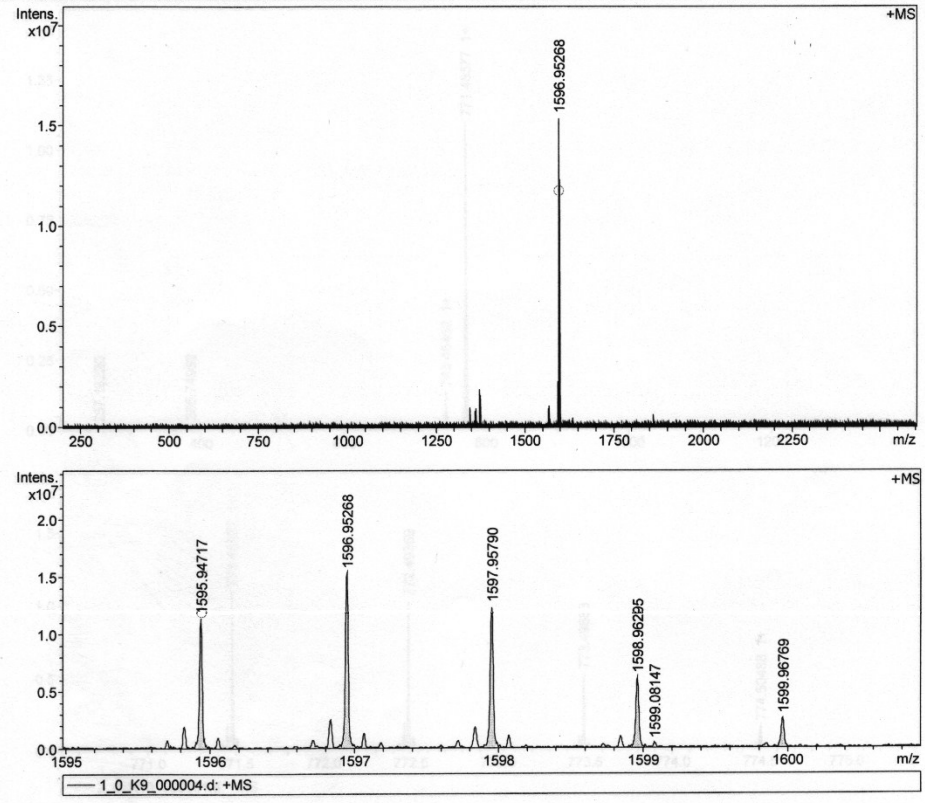
MALDI,1

Analysis Info

Analysis Name	D:\Data\MALDI\2019\1211\1_0_K9_000004.d	Acquisition Date	12/11/2019 5:19:37 PM
Method	MALDI_P_100-3000	Operator	
Sample Name	MURU-N-ESI	Instrument	solariX
Comment			

Acquisition Parameter

Acquisition Mode	Single MS	Acquired Scans	3	Calibration Date	Wed Dec 11 05:11:42
Polarity	Positive	No. of Cell Fills	1	Data Acquisition Size	2097152
Broadband Low Mass	202.1 m/z	No. of Laser Shots	10	Data Processing Size	4194304
Broadband High Mass	2600.0 m/z	Laser Power	16.6 lp	Apodization	Sine-Bell Multiplication
Source Accumulation	0.001 sec	Laser Shot Frequency	0.020 sec		
Ion Accumulation Time	0.300 sec				



Meas. m/z	#	Ion Formula	Score	m/z	err [ppm]	Mean err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
1595.947170	1	C86H131N8O20	100.00	1595.947415	0.2	-1.9	185.0	25.5	even	ok

Fig. S6 HRMS (MALDI TOF) spectrum of **PQs-PEG5**

Table S1 the photophysical and photochemical properties of **PQs-5** in toluene and **PQs-PEG5** in toluene and DI water

PSs	solvent	$\lambda_{max}{}^{a}$ (nm)	ε_{max} (10^5 M^{-1} cm^{-1})	$\lambda_{max}{}^{FLb}$ (nm)	$\Delta_{vss}{}^{c}$ (10^2 cm^{-1})	$\tau_f{}^{d}$ (ns)	$\Phi_f{}^{e}$	$\Phi_\Delta{}^{f}$
PQs-5	toluene	551	0.58	579	8.78	1.99	0.55	0.49
PQs-PEG5	toluene	549	0.53	578	9.14	2.0	0.54	0.47
	DI water	551	---	682	349	1.50	---	---

[a] Absorption maximum, [b] fluorescence emission maximum, [c] stokes shift, [d] fluorescence average lifetime, [e] fluorescence quantum yield and [f] singlet oxygen quantum yield of **PQs-5** and **PQs-PEG5** in toluene or DI water (1.0×10^{-5} M) at room temperature.

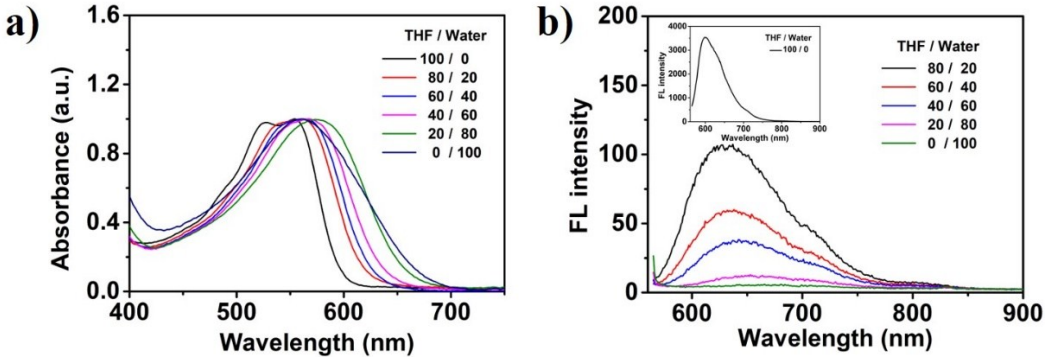


Fig. S7 UV-vis spectra a) and fluorescence spectra b) of **PQs-PEG5** in the mixed solvents of THF and water with different ratios.

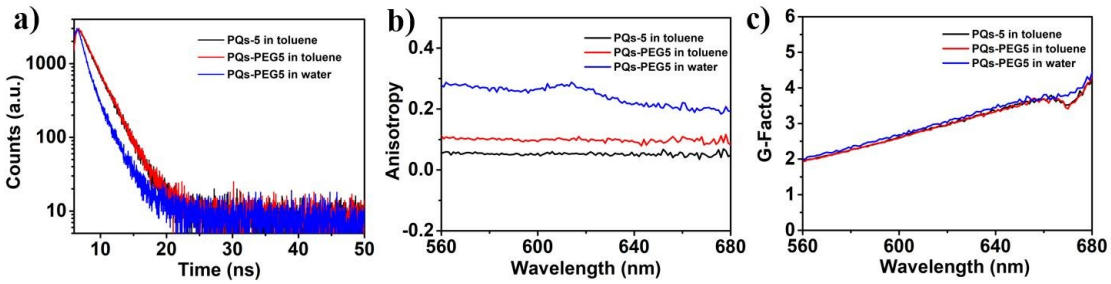


Fig. S8 a) Fluorescence decay plots; b) Anisotropy spectra; and c) G-Factor spectra of **PQs-5** and **PQs-PEG5** irradiated by a 510 nm laser.

Table S2 The linear fitting results of the data in Fig.1c

Equation	y = a + b*x		
Weight	No Weighting		
Residual Sum of Squares	0.00375	0.00119	0.00215
Pearson's r	-0.99556	-0.99714	-0.99469
Adj. R-Square	0.98894	0.99285	0.98675
		Value	Standard Error
TPP	Intercept	-0.03757	0.02217
	Slope	-0.03874	0.00183
PQs-5	Intercept	-0.02095	0.0125
	Slope	-0.02722	0.00103
PQs-PEG5	Intercept	-0.02757	0.01679
	Slope	-0.02679	0.00139

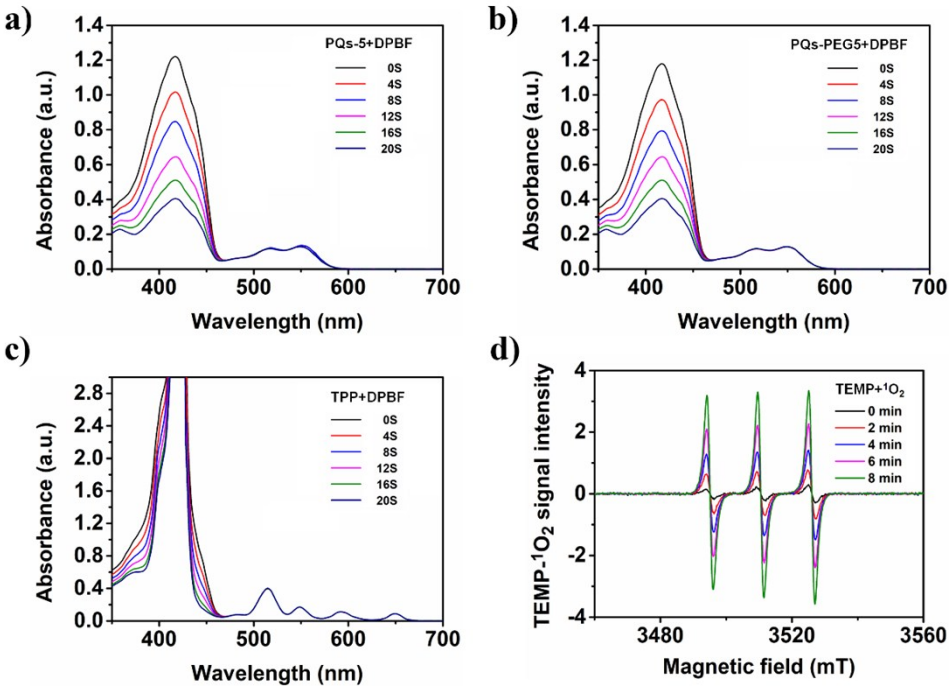


Fig. S9 UV-vis spectra of **PQs-5**+DPBF (a), **PQs-PEG5**+DPBF (b) and **TPP**+DPBF (c) in toluene after irradiated by a 532 nm diode laser (10 mW cm^{-2}) for different time; (d) TEMP-$^1O_2$ signal intensities of **PQs-PEG5**+TEMP in DI water after irradiated by a 635 nm diode laser for different time.

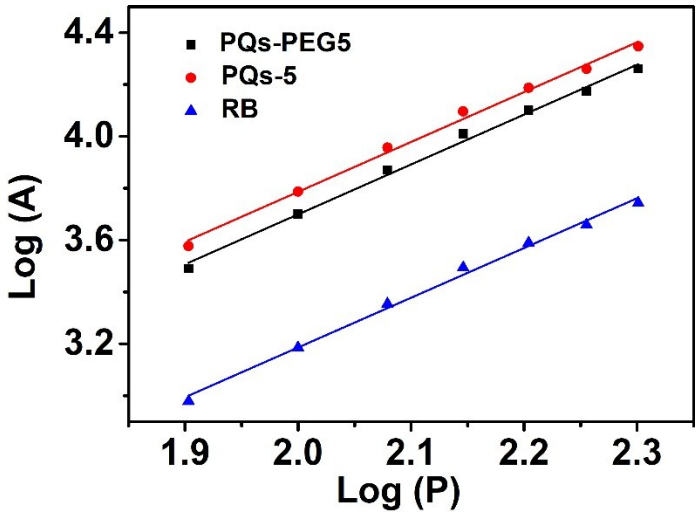


Fig. S10 Logarithmic plots of 2PE fluorescence emission integral area of **PQs-PEG5**, **PQs-5** and **RB** versus excitation power of an 800 nm fs pulse laser. A represented 2PE fluorescence emission integral area, and P was the excitation power intensity.

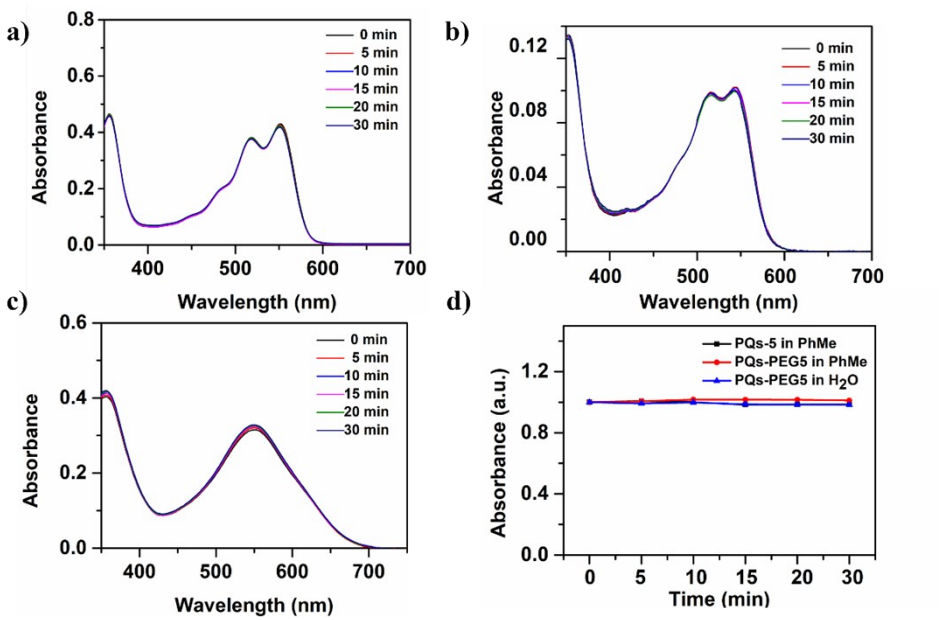


Fig. S11 UV-vis spectra of **PQs-5** in toluene (a), **PQs-PEG5** in toluene (b) and water (c) after irradiated by diode lasers (100 mW cm^{-2}, 532 nm for a and b, 635 nm for c) for different time; (d) λ_{max} values of the three solutions versus irradiation time.

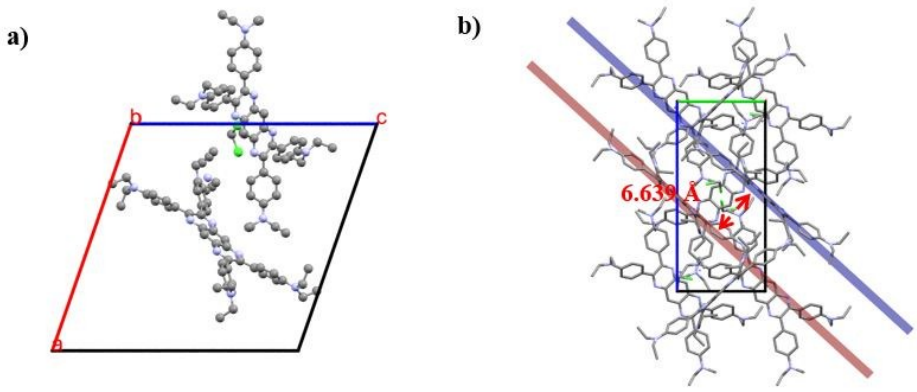


Fig.S12 a) View of single crystal of **PQs-5** along with b axis; b) The stacking patterns of **PQs-5** in its single state;

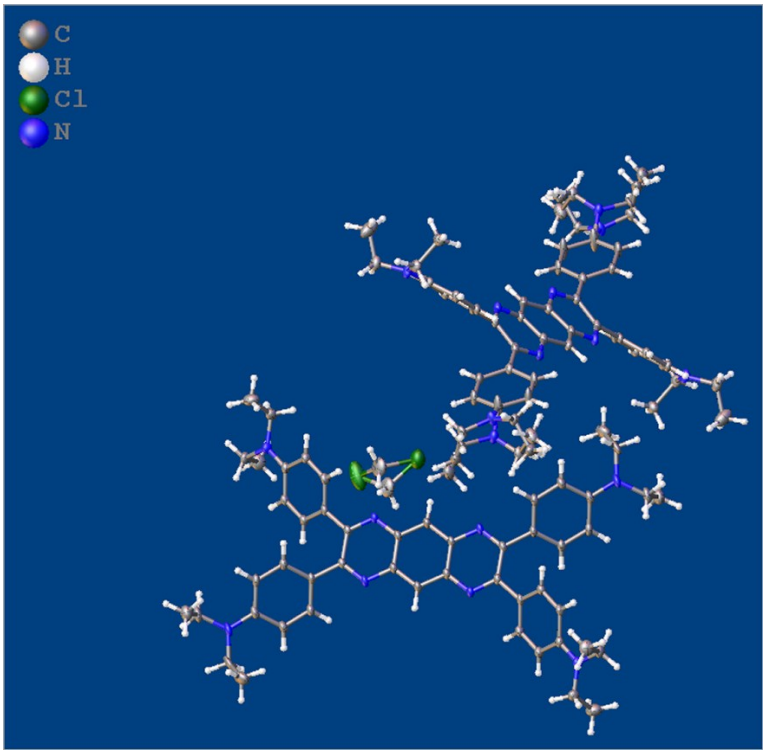


Fig. S13 The single crystal X ray structure of **PQs-5**

Table S3 Crystal data and structure refinement for PQs-5

Identification code	PQs-5 (20180523d)
Empirical formula	$C_{51}H_{60}Cl_2N_8$
Formula weight	855.97
Temperature/K	100.00(10)
Crystal system	monoclinic
Space group	$P2_1/c$
a/Å	21.9296(6)
b/Å	9.8470(3)
c/Å	22.4505(7)
α/°	90
β/°	109.159(3)
γ/°	90
Volume/Å^3	4579.5(2)
Z	4
ρ_{calc}g/cm^3	1.242
μ/mm^{-1}	1.615
F(000)	1824.0
Crystal size/mm^3	0.3 × 0.3 × 0.2
Radiation	CuKα (λ = 1.54184)
2Θ range for data collection/°	8.024 to 146.582
Index ranges	$-25 \leq h \leq 26$, $-8 \leq k \leq 12$, $-27 \leq l \leq 23$
Reflections collected	18686
Independent reflections	8976 [R_{int} = 0.0272, R_{sigma} = 0.0347]
Data/restraints/parameters	8976/0/610
Goodness-of-fit on F^2	1.105
Final R indexes [I>=2σ (I)]	R_1 = 0.0894, wR_2 = 0.2619
Final R indexes [all data]	R_1 = 0.0970, wR_2 = 0.2672
Largest diff. peak/hole / e Å^{-3}	0.95/-1.38

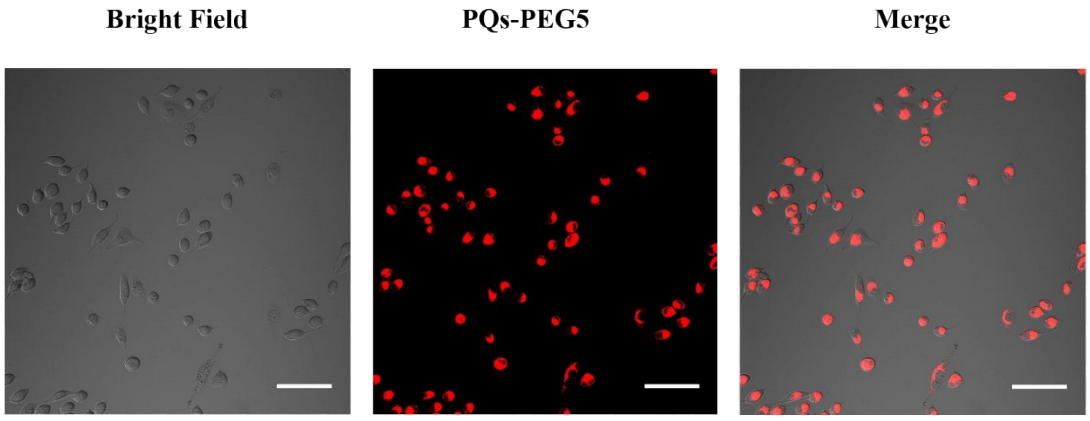


Fig. S14 CLSM images of HeLa cells incubated with **PQs-PEG5** (excited with 561 nm laser). Scale bar: 100 μm

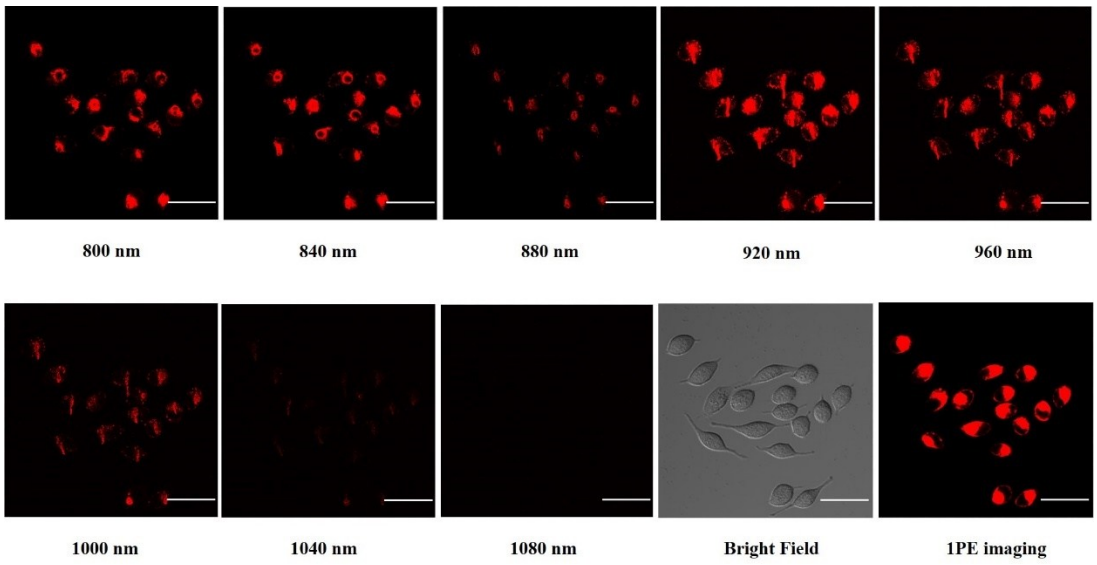


Fig. S15 CLSM images of **PQs-PEG5** incubated with HeLa cells under irradiation of a fs pulse laser with different wavelengths, and the images in bright field and 1PE (excited with 561 nm laser). Scale bar: 50 μm

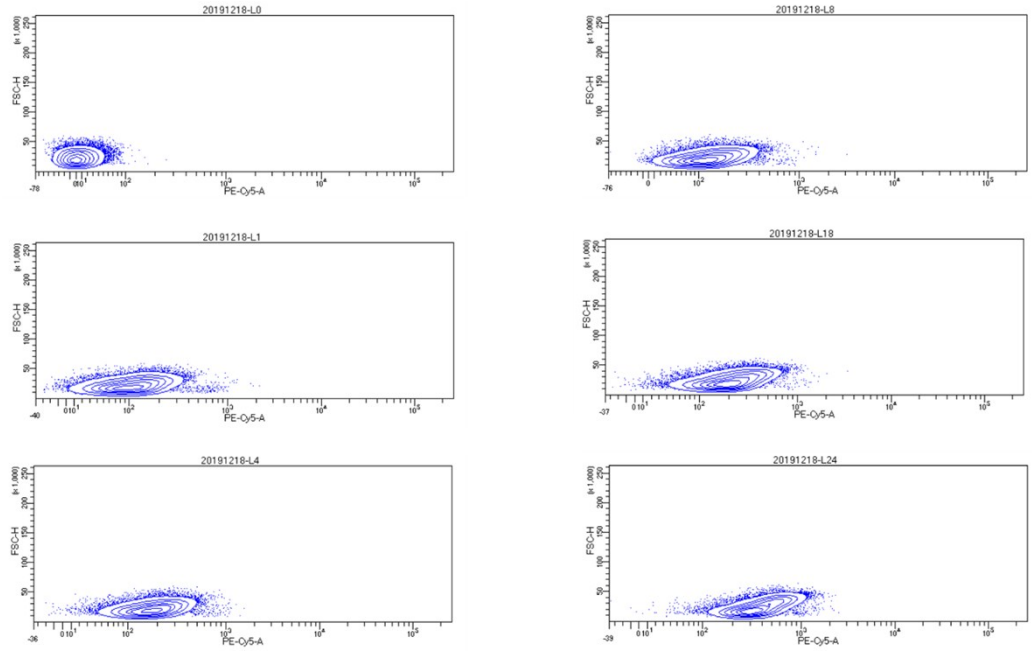


Fig. S16 Cell flow cytometry analysis towards of L929 cells after incubation with **PQs-PEG5** (6.0 μM) for different time.

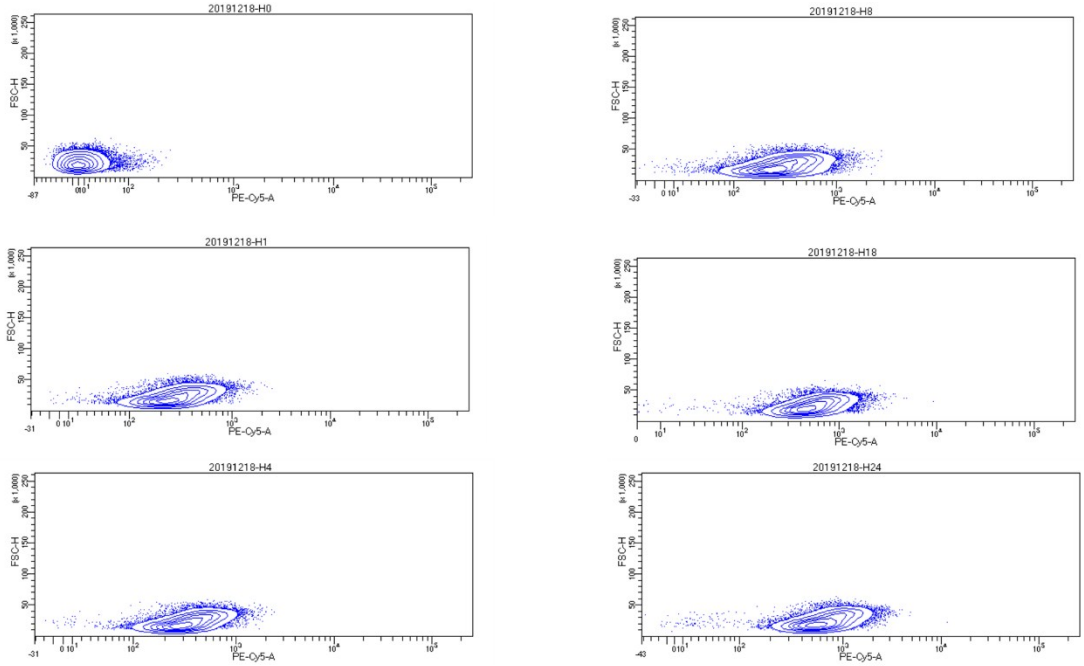


Fig. S17 Cell flow cytometry analysis towards of HeLa cells after incubation with **PQs-PEG5** (6.0 μM) for different time.

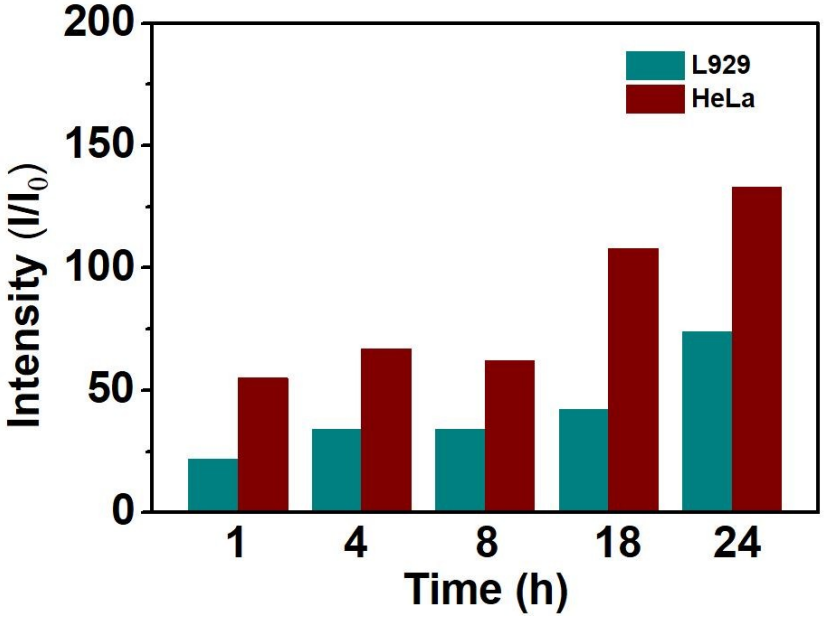


Fig. S18 The fluorescence intensities of L929 and HeLa cells after incubation with **PQs-PEG5** (6.0 μM) for different time detected by cell flow cytometry test.

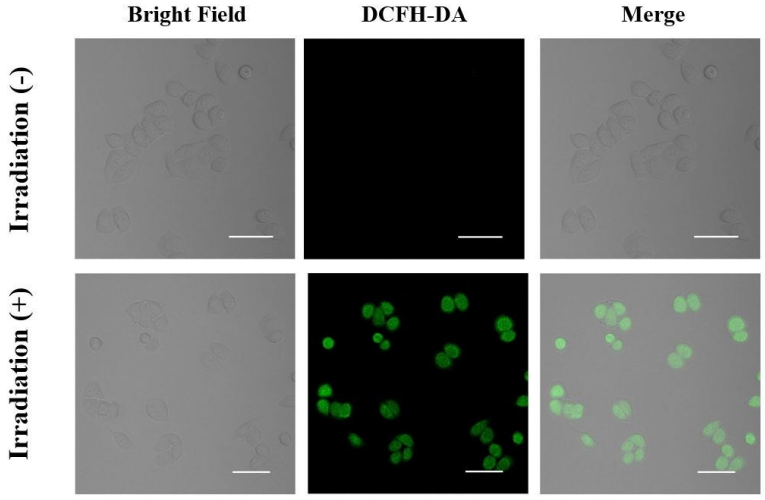


Fig. S19 CLSM images of DCFH-DA staining HeLa cells incubated with **PQs-PEG5** (4 μM) without and with irradiation by a 635 nm laser (20 mW cm^{-2}, 5 min), Scale bar: 50 μm.

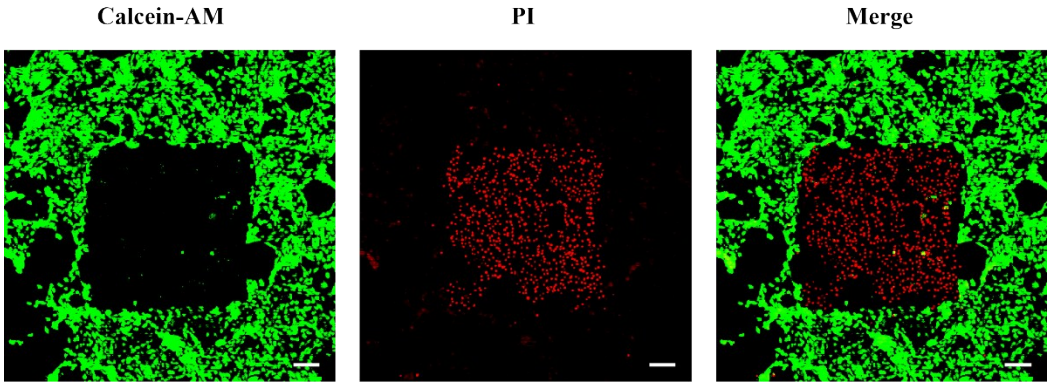


Fig. S20 CLSM images of 4T1 cells incubated with 10 μM **PQs-PEG5** after irradiation by an 820 nm fs pulse laser (100 mW, 80 MHz, 140 fs) for 5 min, then continued to be incubated for 8 h and followed by co-stained with Calcein-AM (left) and PI (middle), and the merge image of them (right), Scale bar: 100 μm.

References:

1、L-P. Zhang, K-J. Jiang, G. Li, Q-Q. Zhang and L-M. Yang, *J. Mater. Chem. A.*, 2014, **2**, 14852