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A Novel AIEgen Photosensitizer with Elevating Intersystem Crossing Rate for Tumor Precise Imaging and Therapy

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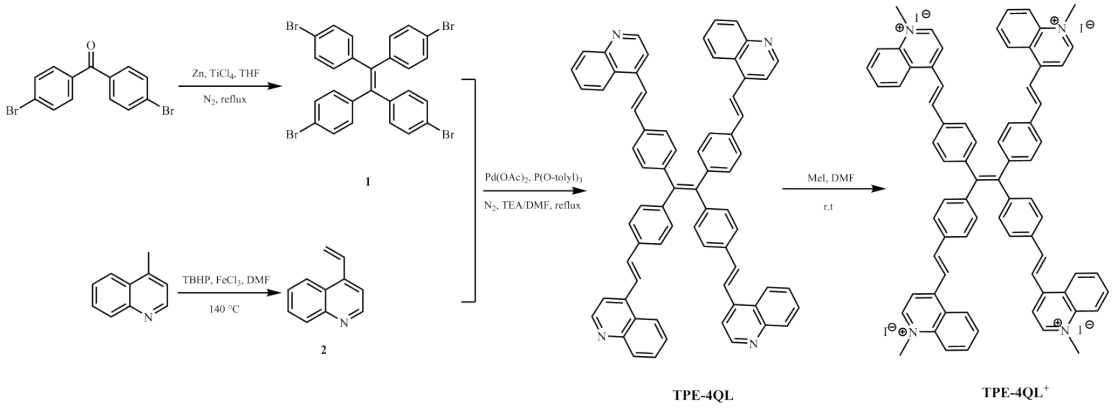
1. Materials

All chemical and biological reagents are purchased from local suppliers. Mass spectrometry uses matrix-assisted laser dissociation time of flight mass spectrometry imager (Bruker, Germany). NMR spectra were recorded on a Bruker 400MHz Advance spectrometer (Bruker, Germany) using TMS as an internal standard. The molar absorption coefficient was measured by Hitachi U-4100 UV absorption spectrometer (Hitachi, Kyoto, Japan). The fluorescent images of cells were obtained Nikon confocal two-photon microscope (Nikon, Japan).

2. Synthesis of TPE-4QL$^+$



Synthesis of TPE-4QL. Compound 1[1] (100 mg, 0.155 mmol), compound 2[2] (144.5mg, 0.932 mmol), $Pd(OAc)_2$ and tris(2-methylphenyl)phosphine were mixed with DMF/TEA (V/V = 1/2, 10 mL) at N_2 atmosphere in a round-bottom flask. Reflux overnight at 80℃. After the reaction, the organic phase was washed with saturated sodium chloride solution, and the solvent was reduced to vacuum evaporation. The crude product was purified by silica gel column chromatography (DCM/MeOH = 30/1) to obtain yellow solid product TPE-4QL (88 mg, 0.09 mmol) with a yield of 60%. ^{1}H NMR (400 MHz, Chloroform-d) δ 8.93 (d, *J* = 4.6 Hz, 1H), 8.24 (d, *J* = 8.4 Hz, 1H), 8.17 (d, *J* = 8.5 Hz, 1H), 7.85 (d, *J* = 16.0 Hz, 1H), 7.77 (t, *J* = 7.7 Hz, 1H), 7.62 (dd, *J* = 9.8, 6.2 Hz, 2H), 7.52 (d, *J* = 7.9 Hz, 2H), 7.35 (d, *J* = 16.1 Hz, 1H), 7.24 (d, *J* = 7.9 Hz, 2H). ^{13}C NMR (101 MHz, Chloroform-d) δ 144.04, 142.82,

140.95, 135.20, 134.60, 132.10, 130.15, 129.40, 126.87, 126.55, 126.34, 123.37, 122.90, 116.92. MALDI-TOF: m/z [M + H]$^+$ calcd for $C_{70}H_{48}N_4$: 945.388; found: 945.398. (Figure S1-S3)

Synthesis of TPE-4QL$^+$. Compound 3 (60 mg, 0.0636 mmol) was put into a 10 mL round-bottled flask and completely dissolved with DMF. Methane iodide (40 μL, 0.636 mmol) was slowly added and stirred overnight at room temperature, away from light. At the end of the reaction, anhydrous ether was added to precipitate and the red solid product compound TPE-4QL$^+$ (90.7 mg, 0.06 mmol) was purified with a yield of 90%. ^{1}H NMR (400 MHz, DMSO-d6) δ 9.37 (d, *J* = 6.4 Hz, 1H), 9.03 (d, *J* = 8.7 Hz, 1H), 8.48 (t, *J* = 7.6 Hz, 2H), 8.41 - 8.24 (m, 2H), 8.14 (d, *J* = 15.8 Hz, 1H), 8.04 (t, *J* = 7.8 Hz, 1H), 7.91 (d, *J* = 7.9 Hz, 2H), 7.28 (d, *J* = 7.7 Hz, 2H), 4.57 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d6) δ 152.91, 148.66, 145.50, 142.64, 141.89, 139.22, 135.52, 134.92, 132.16, 129.80, 129.21, 127.00, 126.85, 120.73, 119.91, 116.92, 45.30. ESI-MS: m/z calcd for $C_{74}H_{60}N_4^{4+}$, 251.12; found: 251.46. (Figure S4-S6)

3. Singlet-triplet Energy Gap Calculations

The calculation of excited state was performed at TD-DFT CAM-B3LYP/6-31G(d) level. The calculation of ground state and triplet state were performed at CAM-B3LYP/6-31G(d) level.

4. Theory Calculatation of the AIE Photosensitizer

Generally, the common way to improve the photosensitivity efficiency of PSs is to enhance the intersystem crossing (ISC). Therefore, how to improve the ISC is an important strategy for the design of efficient PSs. According to perturbation theory, the rate constant (k_{ISC}) of ISC can be calculated by the following formula:

$$k_{ISC} \propto <^1\Psi \mid \hat{H}_{SO} \mid ^3\Psi> / \exp(\Delta E_{ST}^2) \quad (1)$$

where, $<^1\Psi \mid \hat{H}_{SO} \mid ^3\Psi>$ is the spin-orbit coupling (SOC) matrix element，$\hat{H}_{SO}$ is the SOC hamiltonian, ΔE_{ST} represents the energy level difference between singlet S_n and triplet T_m. Formula (1) shows that larger SOC and smaller ΔE_{ST} can lead to

higher k_{ISC}.

The total ISC rate of the molecule is obtained from the sum of the rate at which the S_n state passes through the ISC to any T_m state：

$$k_{ISC}^{n} = \sum_{m} k_{ISC}^{nm} \tag{2}$$

Where, n represents singlet S_n, m represents triplet T_m. Formula (2) shows that the ISC rate constant k_{ISC} of S_n and T_m has the property of addition. Therefore, k_{ISC} can be greatly improved by rationally designing the molecular structure and reducing the energy level difference between S_n and T_m, thus resulting in the great enhancement of the photosensitivity efficiency of PSs.

5. Measurement of ROS Quantum Yield

ROS quantum efficiency of TPE-4QL$^+$ in water was measured under laser irradiation (430 nm from a fluorescence spectrophotometer, 0.25 mW·cm^{-2}) with

$$\Phi_{sample} = \Phi_{RB} \frac{K_{sample} \times A_{RB}}{K_{RB} \times A_{sample}}$$

ABDA as indicator and RB as standard reference. The concentration of ABDA was 100 μM, both the concentration of TPE-4QL$^+$ and RB were 5 μM. The absorbance at 378 nm was recorded under different illumination time. The calculation of ROS yield follows the following formula:

Where, K is the slope of absorbance and irradiation time, and A is the absorbance of TPE-4QL$^+$ at 430 nm and RB at 550 nm respectively. Φ_{RB} is the ROS quantum efficiency of standard reference Rose Bengal in water, which is 0.75. $K_{TPE\text{-}4QL+}$ and K_{RB} were 0.0082 and 0.0061, respectively (Fig. 2c). $A_{TPE\text{-}4QL+}$ at 430 nm were 0.351 and A_{RB} at 550 nm were 0.346 (Fig. S9). The quantum yield of TPE-4QL$^+$ in water is 99.4%.

6. Cell Culture and Fluorescence Imaging

An uterine neck cancer cell line (HeLa), a human neuroblastoma cell line (SH-SY5Y), a hepatic cancer cell line (HepG2), a human renal epithelial cell line (293T)

and a mouse fibroblast cell line (NIH-3T3) were provided by the Biomedical Engineering Center of Hunan University (Changsha, China). HeLa, SH-SY5Y, HepG2, 293T and NIH-3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37 ℃ in a moist atmosphere containing 5% CO_2. HeLa cells with a density of 1×10^4 cells /mL were placed in a cell culture dish and incubated at 37 ℃ with 5% CO_2 for 24 h. Then, remove the medium and wash it with PBS for three times, add the medium containing 5 μM TPE-4QL$^+$ and culture it at 37 ℃ for 2 h, remove the solution, add the culture base containing 200 nM mitochondrial green and 1 μM Hoechst 33342 for further incubation for 20 min, then wash it with PBS for three times to remove the dye in the solution. The cells were imaged with a Nikon A1R microscope. (TPE-4QL$^+$：λ_{ex} = 405 nm, λ_{em} = 650-700 nm；Mito-Tracker Green：λ_{ex} = 488 nm, λ_{em} = 500-550 nm; Hoechst 33342: λ_{ex} = 405 nm, λ_{em} = 410-450 nm).

7. Measurement of Singlet Oxygen in Living Cells

HeLa and NIH-3T3 cells with a density of 1×10^4 cells /mL were placed in a cell culture dish and incubated at 37 ^{o}C with 5% CO_2 for 24 h. The medium was removed and washed three times with PBS solution. The medium containing TPE-4QL$^+$ (10 μM) and DCFH-DA (10 μM) was added and cultured at 37 ^{o}C for 30 min. The cells were irradiated with white light (50 mW·cm^{-2}) for 30 s. The free dyes in the solution were removed by washing with PBS for three times. The dark group did not undergo any treatment before imaging (DCFH-DA: λ_{ex} = 488 nm, λ_{em} = 500-550 nm).

8. Cells Activity Imaging

Cell activity was detected by fluorescence imaging of Calcein-AM and pyridine iodide (PI). HeLa cells with a density of 1×10^4 cells /mL were placed in a cell culture dish and incubated at 37 ^{o}C with 5% CO_2 for 24 h. The medium containing TPE-4QL$^+$ (10 μM) was added and cultured at 37 ^{o}C for 2 h. Then the cells were irradiated with white light (50 mW·cm^{-2}) for 0 and 3 min, respectively, and then being incubated for

another 12 h. DMEM was replaced with 1 mL 1×Assay Buffer containing 3 μL calcein-AM and 1 μL PI, and being incubated for another 20 min. Images were obtained using confocal microscope (Calcein-AM: λ_{ex} = 488 nm, λ_{em} = 500-550 nm; PI: λ_{ex} = 561 nm, λ_{em} = 590-640 nm).

9. Cells Apoptosis Imaging

Cell apoptosis was detected by Annexin V-FITC and pyridinium iodide (PI) fluorescence imaging method. HeLa cells with a density of 1×10^4 cells /mL were placed in a cell culture dish and incubated at 37 °C with 5% CO_2 for 24 h. The medium was removed and washed three times with PBS. The medium containing TPE-4QL$^+$ (10 μM) was added and cultured at 37 °C for 2 h. Then the cells were irradiated with white light (50 mW·cm^{-2}) for 3 min and being incubated for another 20 min or 3 h. DMEM was replaced with 2 mL PBS containing 5 μL Annexin V-FITC and PI, and being incubated for 30 min. Annexin V-FITC was imaged with confocal microscope (Annexin V-FITC: λ_{ex} = 488 nm, λ_{em} = 500-550 nm; PI: λ_{ex} = 561 nm, λ_{em} = 590-640 nm).

10.Cell Cytotoxicity Assay

HeLa, SH-SY5Y, HepG-2, 239T and NIH-3T3 cells were cultured in 96-well plates, respectively. Each well was inoculated with a cell density of 1×10^5 cells /mL in 100 μL DMEM containing 10% FBS. After incubation for 24 h, the non-illumination group was incubated with 100 μL DMEM containing TPE-4QL$^+$ (50, 40, 35, 30, 25, 20, 15, 10, 5, and 0 μM, respectively). The illumination group was also incubated with 100 μL DMEM containing different concentrations of TPE-4QL$^+$ (10, 7.5, 5.0, 2.5, 1.25, 0.60, 0.30, 0.15, and 0 μM, respectively). After 2 h of incubation, the group was irradiated with 4 mW·cm^{-2} blue light for 10 min. Incubation was continued for 12 h. After being incubated for 12 h, wash with PBS for three times, add 10 μL Cell Counting Kit-8 (CCK-8) solution, continue to be incubated for another 2 h, and measure the absorbance of each well solution with multi-functional microplate reader at 450 nm.

11. 4T1 Tumor Model

Female Balb/c mice (about 4 week) were obtained from Hunan SJA Laboratory Animal Co. Ltd. (Changsha, China). The animal experiment part of the work was approved by the Ethics Committee of the College of Biology of Hunan University (Changsha, China, Certificate number// Ethics approval No. Is SYXK2018-0006). Female mice were subcutaneously inoculated with 4T1 cells.

12. *In Vivo* Fluorescence Imaging

TPE-4QL$^+$ (25 μL, 100 μM in PBS) was injected into the tumor site of nude mice bearing a subcutaneous 4T1 xenograft, or injected into the same place of normal nude mice, and imaging was performed at different time points with small animal multimodal imaging instrument.

13. *In Vivo* Tumor Inhibition with TPE-4QL$^+$

Twenty 18-20 g female Balb/c mice were randomly divided into four groups (n = 5). Four groups of mice were intratumorally injected with PBS (25 μL) and TPE-4QL$^+$ (25 μL, 100 μM in PBS), respectively. One control group and one experimental group were irradiated with blue LEDS for 20 min a day for 3 days, while the other control group and experimental group were shielded from light during the whole process. The length, width and weight of the tumor were measured every 3 days during the treatment period of 21 days. Tumor volume was calculated by the formula:

$$V(mm^3 = 0.5 \times length(mm) \times width(mm)^2$$

14. Supporting Figures

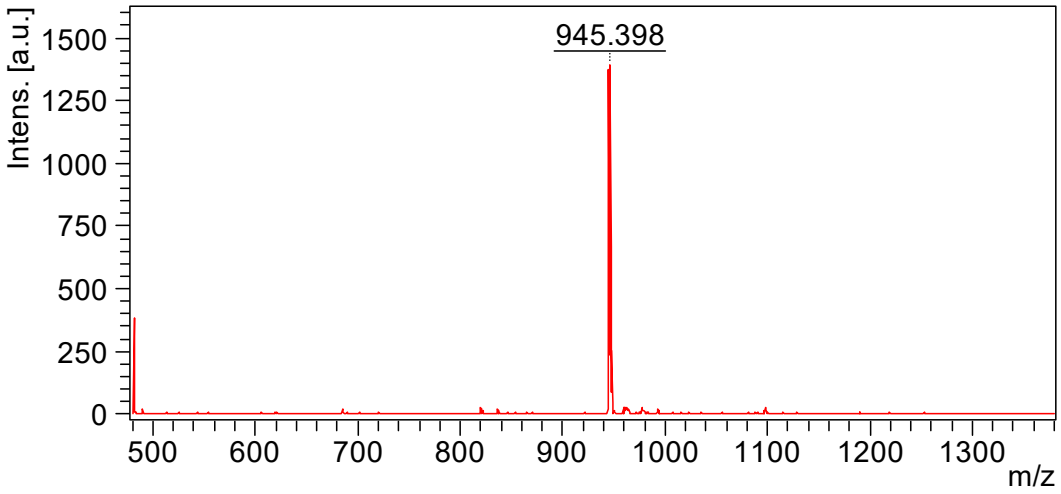


Figure S1. MALDI-TOF of TPE-4QL.

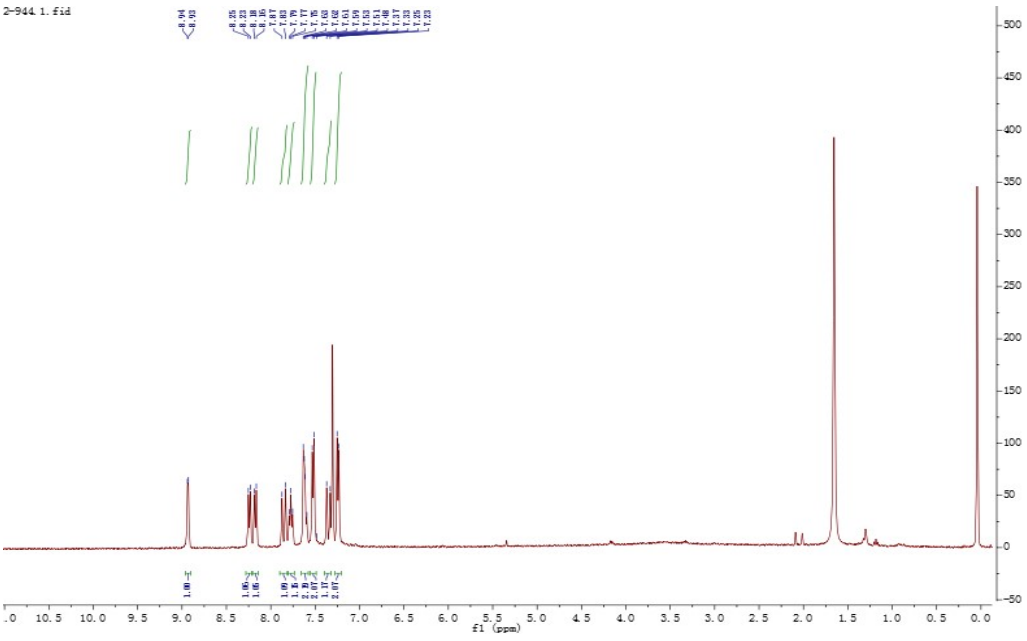


Figure S2. ^{1}H NMR of TPE-4QL.

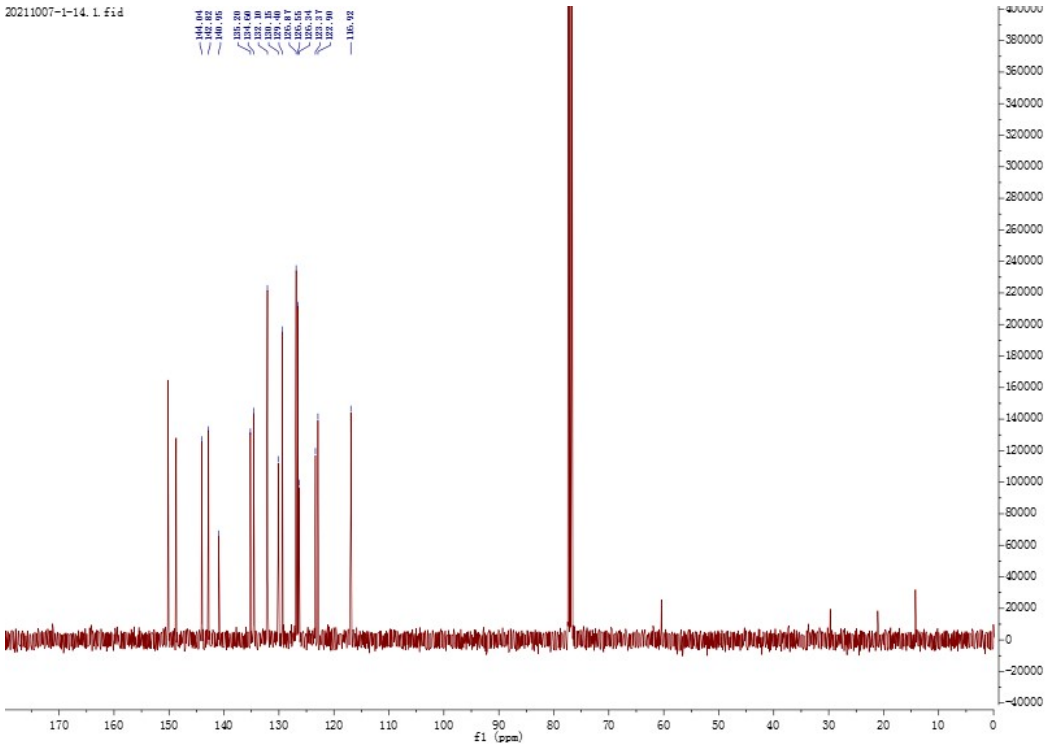


Figure S3. ^{13}C NMR of TPE-4QL.

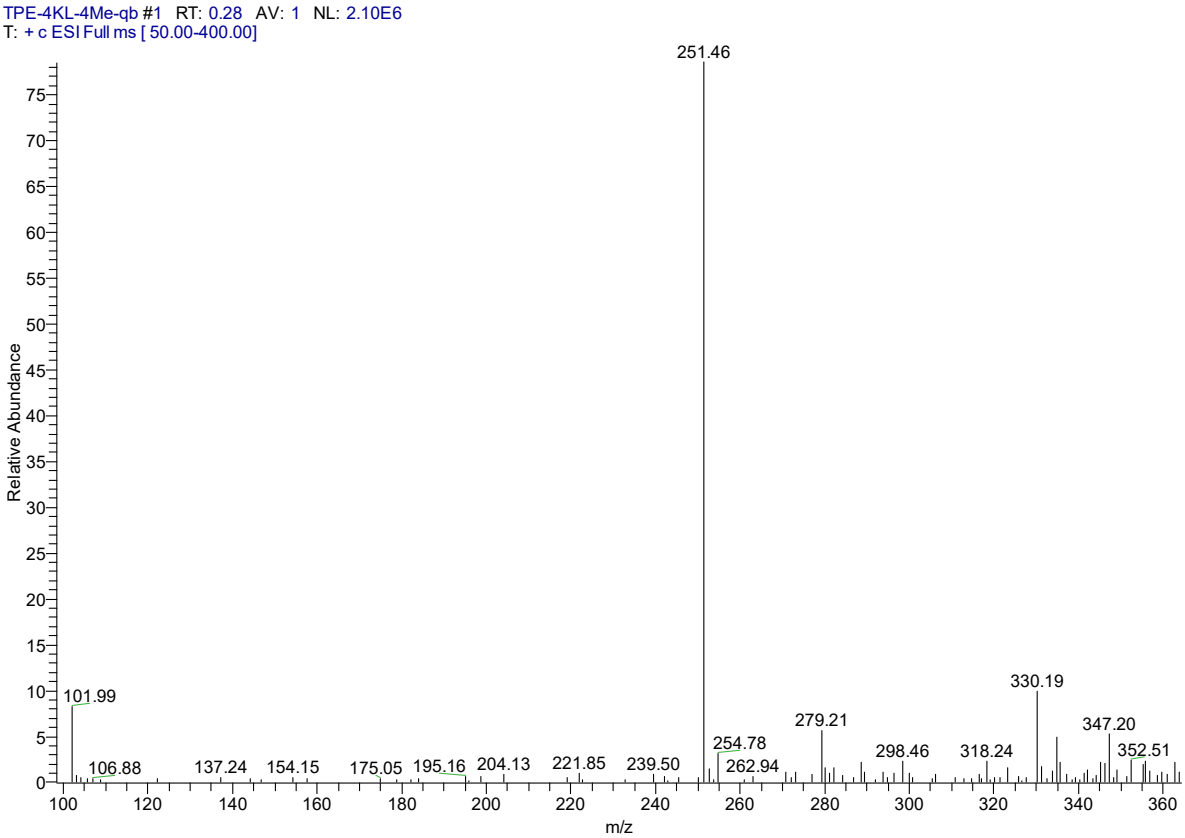


Figure S4. ESI-MS of TPE-4QL^{+}.

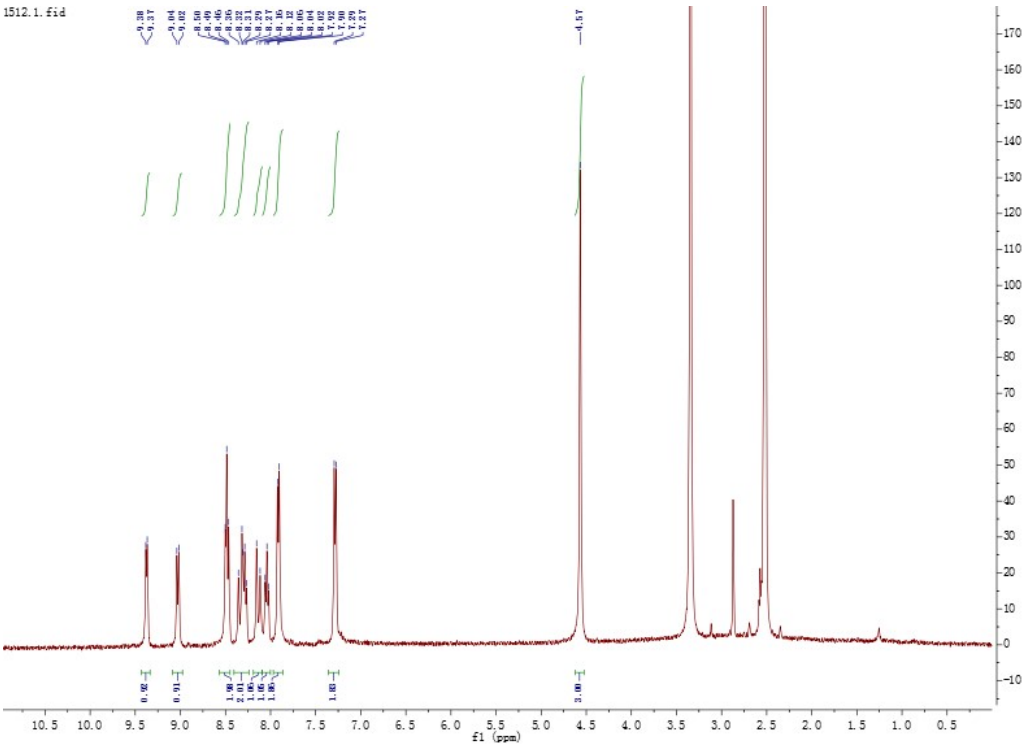


Figure S5. ^{1}H NMR of TPE-4QL^{+}.

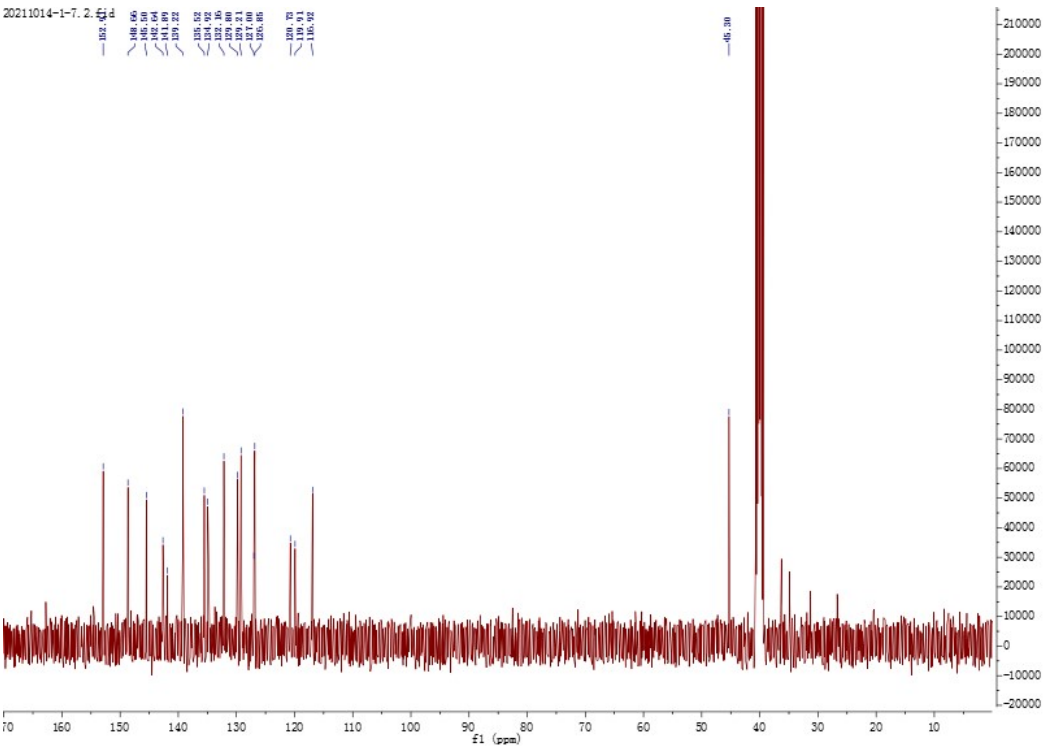


Figure S6. ^{13}C NMR of TPE-4QL^{+}.

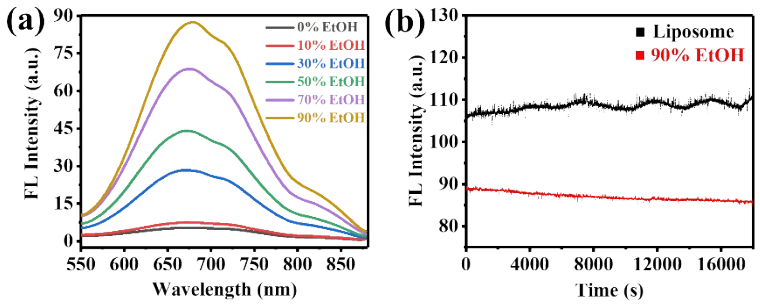


Figure S7. (a) Fluorescence emission spectra of TPE-4QL$^+$ (10 μM) in H_2O/EtOH mixture solution with different EtOH fractions (vol %). (b) The fluorescence intensity (λ_{ex} = 430 nm，λ_{em} = 680 nm) of TPE-4QL$^+$ (10 μM) changes with time in different solution.

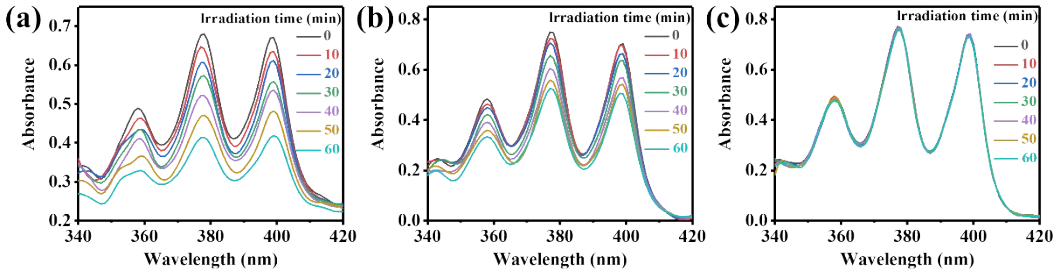


Figure S8. (a-c) UV-vis spectra of ABDA (50 μM) with (a) TPE-4QL$^+$ (5 μM) under 430 nm irradiation, (b) Rose Bengal (5 μM) under 550 nm irradiation, and (c) without PSs under irradiation, respectively, in aqueous solution.

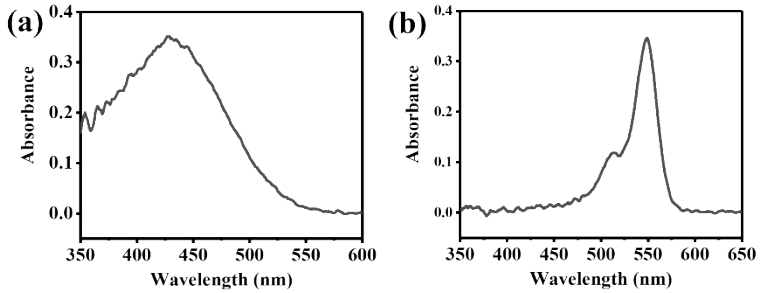


Figure S9. UV-vis spectra of (a) TPE-4QL$^+$ (5 μM) or (b) RB (5 μM) in water.

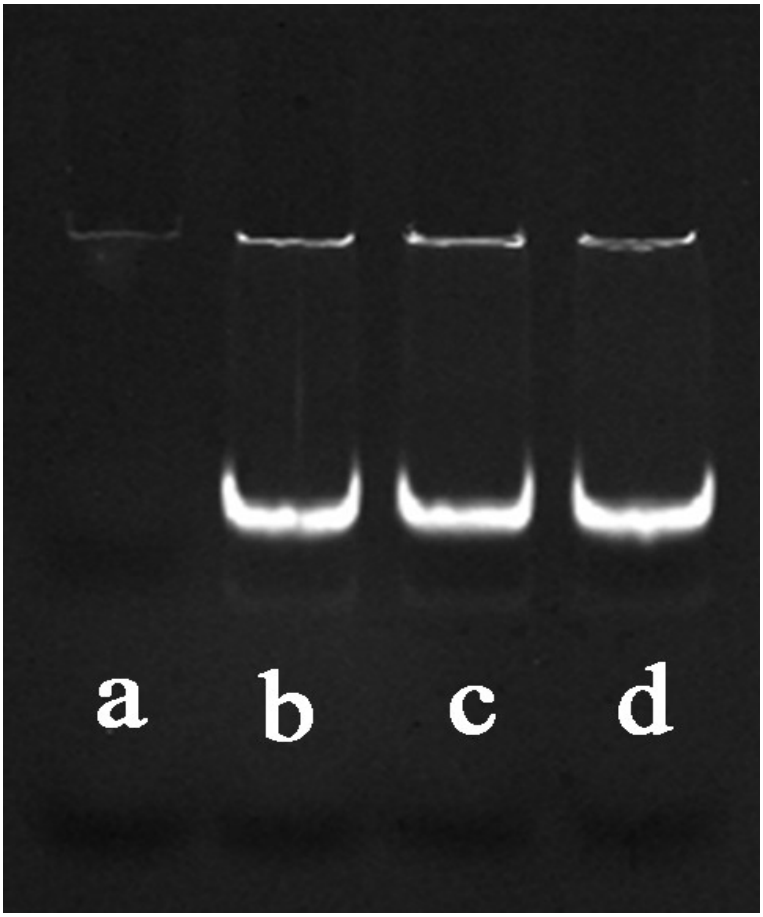


Figure S10. PAGE analysis of dsDNA treated with a) TPE-4QL$^+$(10 μM) under white light irradiation for 10 min or (b) TPE-4QL$^+$ (10 μM) in dark or (c) without TPE-4QL$^+$ under white light irradiation for 10 min or (d) without TPE-4QL$^+$ in dark.

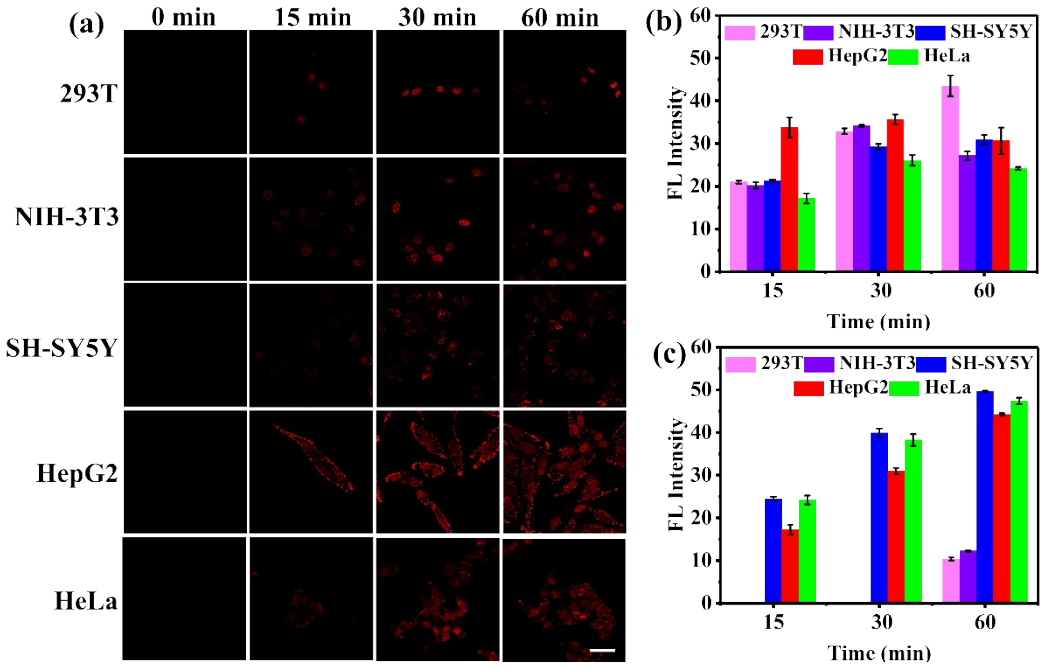


Figure S11. (a) Fluorescence images of various cells incubated with TPE-4QL (5 μM) for different times; (b) The corresponding average fluorescence intensity distribution of various cells incubated with TPE-4QL (5 μM) for different times; (c) The corresponding average fluorescence intensity distribution of various cells incubated with TPE-4QL$^+$ (5 μM) for different times. (TPE-4QL: λ_{ex} = 405 nm，λ_{em} = 580-630 nm; TPE-4QL$^+$: λ_{ex} = 405 nm，λ_{em} = 650-700 nm; Scale bar: 20 μm).

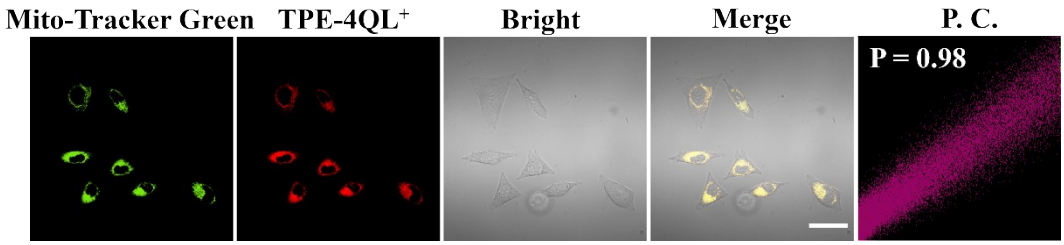


Figure S12. Colocalization images of TPE-4QL$^+$ in living HeLa cells after 45 min of incubation.

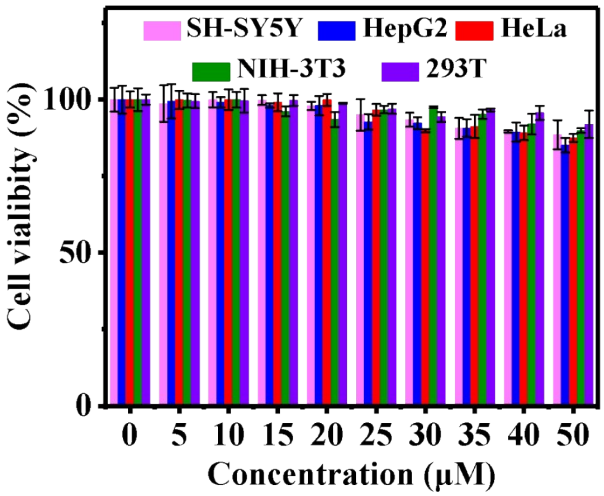


Figure S13. Cell viability of various cells treated with TPE-4QL$^+$ and without irradiation.

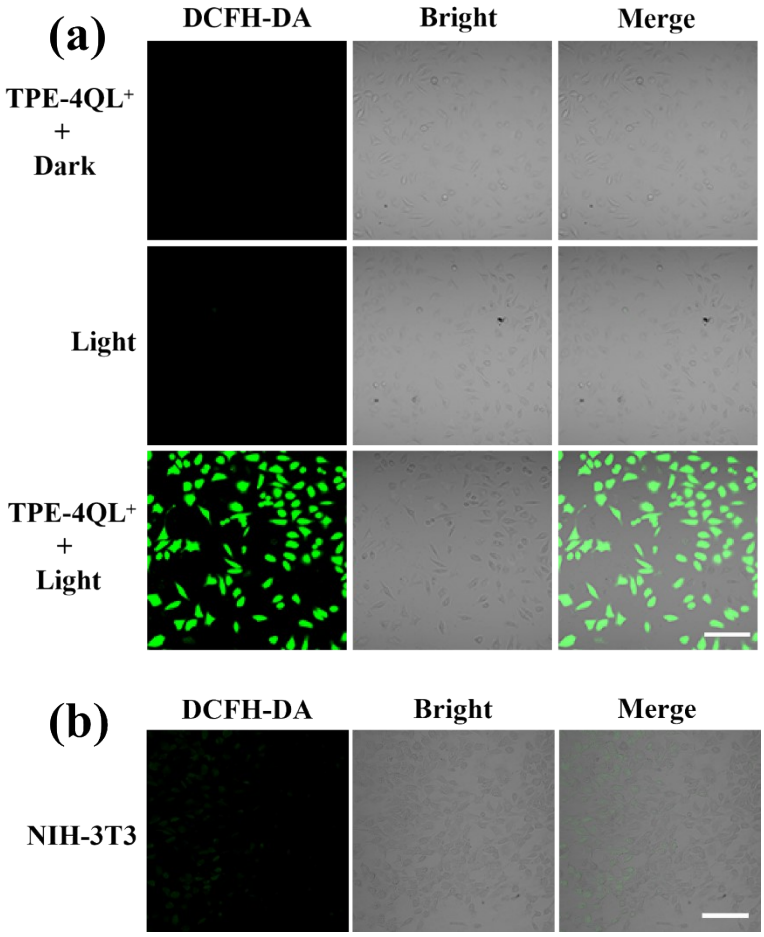


Figure S14. (a-b) Fluorescence images (λ_{ex} = 488 nm, λ_{em} = 500-550 nm) of DCFH-DA-treated (a) HeLa cells after incubation with TPE-4QL$^+$ and irradiation with (TPE-4QL$^+$ + Light) and without (TPE-4QL$^+$ + Dark) white light, or (b) NIH-3T3 cells after incubation with TPE-4QL$^+$ and irradiation with white light. Scale bar: 100 μm.

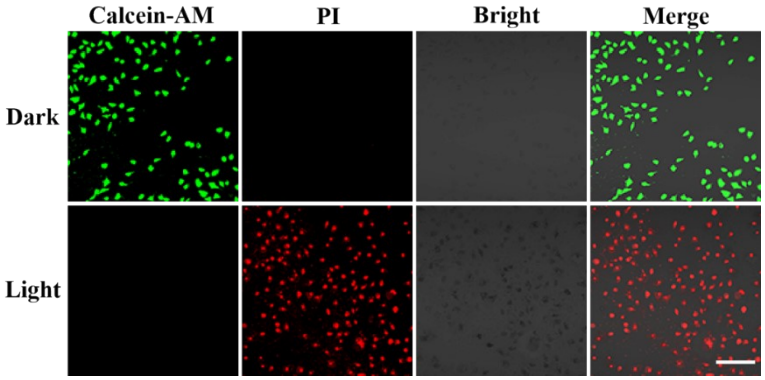


Figure S15. Fluorescence images of the Calcein-AM/PI-stained HeLa cells treated with TPE-4QL$^+$ (5 μM) and with (Light)/ without (Dark) irradiation. Calcein-AM: λ_{ex} = 488 nm, λ_{em} = 500-550 nm; PI: λ_{ex} = 561 nm, λ_{em} = 590-640 nm. Scale bars: 100 μm.

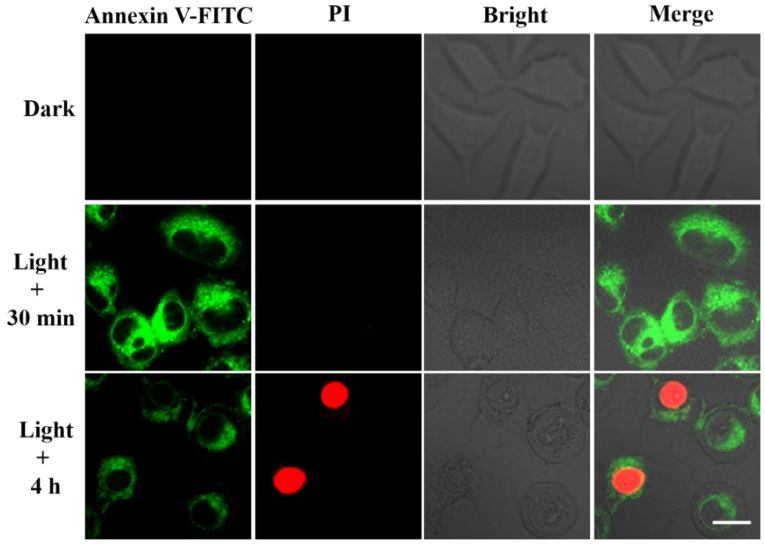


Figure S16. Fluorescence images of the TPE-4QL$^+$/Annexin V-FITC/PI-treated HeLa cells after irradiation with (Light) and without (Dark) white light and further incubation for 30 min or 5 h in the dark. Annexin V-FITC: λ_{ex} = 488 nm, λ_{em} = 500-550 nm; PI: λ_{ex} = 561 nm, λ_{em} = 590-640 nm. Scale bars: 20 μm.

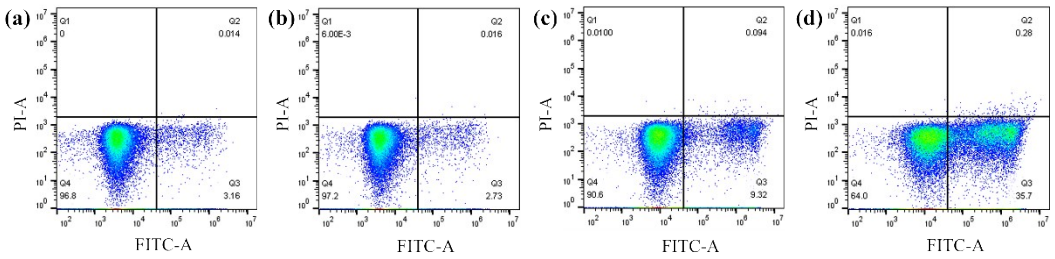


Figure S17. Flow cytometry assays of HeLa cells stained with Annexin V-FITC and PI. (a) PBS; (b) PBS + irradiation (white light, 50 mW·cm^{-2}, 1 min); (c) TPE-4QL$^+$; (d) TPE-4QL$^+$ + irradiation (white light, 50 mW·cm^{-2}, 1 min). Annexin V - FITC: λ_{ex} = 488 nm， λ_{em} = 500-550 nm. PI: λ_{ex} = 561 nm, λ_{em} = 590-640 nm.

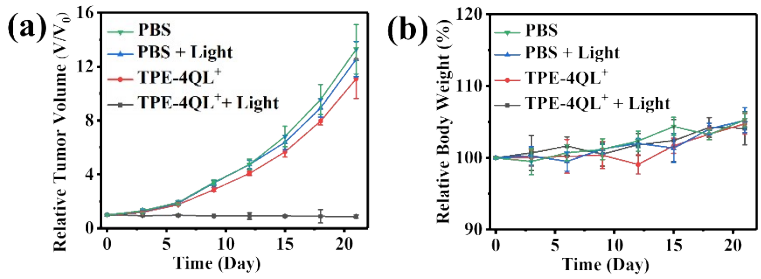


Figure S18. (a) Tumor growth curves (n = 3) of each mouse group injected with PBS (25 μL) and treated with (PBS+Light)/without (PBS) irradiation, and injected with TPE-4QL$^+$ (25 μL, 100 μM in PBS) and treated with (TPE-4QL$^+$ + Light)/without (TPE-4QL$^+$) irradiation. (b) Body weight changes in each group mice during the treatment period.

14. References

1. T. Zhang, Y. Li, Z. Zheng, R. Ye, Y. Zhang, R. T. Kwok, J. W. Lam, B. Z. Tang, J. Am. Chem. Soc. 2019, **141**, 5612-5616.

2. Y. Li, F. Guo, Z. Zha, Z. Wang, Chem. - Asian J. 2013, **8**, 534-537.