Electronic Supplementary Material (ESI) for ChemComm.

Supplementary Information

A protein-conjugated photosensitizer with mitochondrial targeting for enhanced photodynamic therapy

Mingwan Shi, Wei Pan, Peng Gao, Yuanyuan Chen, Kaiye Wang, Na Li,* and Bo Tang**

College of Chemistry, Chemical Engineering and Materials Science, Key Laboratory of Molecular and Nano Probes, Ministry of Education, Institute of Molecular and Nano Science, Collaborative Innovation Center of Functionalized Probes for Chemical Imaging in Universities of Shandong, Shandong Normal University, Jinan 250014, P. R. China.

E-mail: panwei@sdnu.edu.cn; lina@sdnu.edu.cn; tangb@sdnu.edu.cn

Experimental Procedures

Materials and Reagents. 2, 3, 3-trimethyl-3H-indole, 6-bromohexanoic and 2-(Boc-amino)-1-ethanol were obtained from Tianjin Heowns Biochemical Technology Co., Ltd. N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) was obtained from Shanghai Macklin Biochemical Co., Ltd. Triphenylphosphine (TPP) and 4-Dimethylaminopyridine (DMAP) were obtained from Adamas Reagent, Ltd. Methanol, dichloromethane (DCM), toluene, dimethyl formamide (DMF) and acetic anhydride dimethyl sulfoxide (DMSO) were purchased from China National Pharmaceutical (Shanghai, China). Reactive Oxygen Species Assay Kit was obtained from Beyotime (Shanghai, China). 2, 7-dichlorofluorescein diacetate (DCFH-DA) was obtained from Beyotime. Biotechnology (China) 3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), 1, 3-diphenylisobenzofuran (DPBF), propidium iodide (PI) and calcein acetoxymethyl ester (Calcein AM) were purchased from Sigma-Aldrich (USA). The mouse breast cancer cell line (4T1 cells) were purchased from KeyGEN biotechnology (Nanjing, China). Analytical grade reagents were used with no further purification. All aqueous solution were prepared using distilled-deionized water of 18.2 $M\Omega \cdot cm^{-1}$.

Instruments. Fluorescence spectra were obtained with FLS-980 Edinburgh fluorescence spectrometer with a xenon lamp. UV-Vis absorption spectra were measured on a pharmaspec UV-1700 UV-Visible spectrophotometer (Shimadzu, Japan). Confocal fluorescence imaging studies were performed with a TCS SP8 confocal laser scanning microscopy (Leica, Germany). The absorbance of formozan was measured with a microplate reader (Synergy 2, Biotek, USA) in the MTT assay. All the NMR spectra were recorded on a Bruker NMR spectrometers. Chemical shifts (δ) for ^1H NMR (400 Hz) were given in ppm. Data were reported as follows: chemical shift, intergration, multiplicity (s=single, d=doublet, t=triplet, br=broad, m=multiplet) and coupling constants (Hz). High resolution mass spectra (HRMS) were recorded on a Bruker Daltonics maXis UHR-TOF MS.

Synthesis of compound 1. The compound **1** was prepared according to the previous report.[1] Under the protection of argon, 2-chloro-1-formyl-3-(hydroxymethylene) cyclohex-1-ene (525 mg, 3 mmol), 1-(5- carboxypentyl)-2,3,3-trimethyl-3H-indol-1-ium bromide (2.13 g, 6 mmol), anhydrous sodium acetate (525 mg, 3 mmol) and absolute ethanol (10 mL) were added to a 100 ml reaction flask, the mixture heated to 70 °C for 1 h. The reaction was stopped, and ethanol was evaporated under reduced pressure to give the crude product. Then the crude product was purified by column chromatography over silica gel using CH_2Cl_2/MeOH (v/v, 10:1) as the eluent (Yield: 85%).

Synthesis of compound 2. The compound **2** was prepared according to the previous report.[2] 3-Bromopropanamide (1 g, 4.56 mmol) was dissolved in 20 mL of n-butanol, then triphenylphosphine (1.19 g, 4.56 mmol) was added, the reactants were heated to reflux at 120°C for 12 h. After the reaction was completed, the solution was cooled to room temperature for 15 min. The solution was then poured into a mixed solution of

methyl tert-butyl ether (30 mL) and toluene (20 mL). The mixture was stirred at room temperature to become the slurry, the slurry was filtered through the sand core funnel, and the filter cake was washed twice with methyl tert-butyl ether (30 mL) to obtain a white solid (Yield: 76%).

Synthesis of compound 3 (Mito-PS). The mixture of **1** (228 mg, 0.3 mmol), **2** (180.45 mg, 0.45 mmol), EDC (118 mg, 0.45 mmol) and DMAP (11 mg, 0.45 mmol) in DMF (8 mL) were stirred at room temperature for 5 h. After removal of the DMF solvent under vacuum, the crude product was purified by column chromatography over silica gel using CH_2Cl_2/MeOH (v/v, 20:1) as the eluent, (Yield: 35%). ^{1}H NMR (400 MHz, MeOD-d4): δ 8.44 (t, J = 12 Hz, 2H), 7.91-7.87 (m, 3H), 7.80-7.74 (m, 12H), 7.53 (t, J = 8 Hz, 2H), 7.46-7.26 (m, 6H), 6.33 (dd, J = 20 Hz, J = 16 Hz, 2H), 4.22-4.16 (m, 4H), 3.42-3.35 (m, 4H), 2.73 (br, 4H), 2.32 (t, J = 8 Hz, 2H), 2.20 (t, J = 8 Hz, 2H), 1.95 (br, 2H), 1.86-1.83 (m, 6H), 1.74 (s, 6H), 1.72 (s, 6H), 1.70-1.65 (m, 4H), 1.52-1.43 (m, 4H). HRMS (ESI) m/z calcd for $C_{63}H_{73}ClN_3O_3P^{2+}$ [M^{2+}]: 492.7534, found: 492.7504.

Synthesis of compound 4. The compound **4** was prepared according to the previous report.[3,4] N-tert-butoxycarbonyl-1, 2-ethylenediamine (0.64 g, 4 mmol) was dissolved in DCM (20 mL) and triethylamine (1.7 mL, 12 mmol) was added. Then chloroacetyl chloride (380 μL, 2.4 mmol) was slowly added at 0°C. The mixture were stirred at room temperature for 6 h. Then, Then the mixture were washed successively with water (20 mL), saturated aqueous $NaHCO_3$ (20 mL) and saline (20 mL), and dried over $NaSO_4$. The final product was filtered and concentrated to obtain the white powder.

The above product was dissolved in a solution of ethyl acetate containing hydrogen chloride and stirred at room temperature for 1 h. The solvent were distilled and concentrated, then ethyl acetate was added, and the hydrogen chloride in the mixture was removed to leave a residue. Ether was added to the residue, the residue was shaken to obtain a brownish-gray precipitate, and the precipitate was centrifuged to remove the ether-containing supernatant. This step was repeated 2-3 times, and the final product was vacuum dried to obtain a brownish-gray solid (Yield: 54%).

Synthesis of compound Pro-PS. The mixture of **1** (228 mg, 0.3 mmol), **4** (82 mg, 0.6 mmol), EDC (118 mg, 0.45 mmol) and DMAP (11 mg, 0.45 mmol) in DMF (8 mL) were stirred at room temperature for 5 h. After removal of the DMF solvent under vacuum, the crude product was purified by column chromatography over silica gel using CH_2Cl_2/MeOH (v/v, 20:1) as the eluent, (Yield: 24%). ^{1}H NMR (400 MHz, DMSO-d6): δ 8.27-8.24 (m, 3H), 7.90 (br, 1H), 7.65 (d, J = 8 Hz, 2H), 7.47-7.41 (m, 4H), 7.29 (t, J = 8 Hz, 2H), 6.34 (d, J = 12 Hz, 2H), 4.21 (br, 4H), 4.04 (s, 2H), 3.09 (br, 4H), 2.71 (br, 4H), 3.29-3.26 (m, 4H), 2.72 (br, 4H), 2.21 (t, J = 8 Hz, 2H), 2.05 (t, J = 8 Hz, 2H), 1.86 (br, 2H), 1.73 (br, 4H), 1.67 (s, 12H), 1.56 (br, 4H), 1.41-1.34 (m, 4H). HRMS (ESI) m/z calcd for $C_{46}H_{59}ClN_4O_4^+$ [M^+]: 801.3908, found: 801.3926.

Synthesis of compound Mito-Pro-PS. The mixture of **3** (345 mg, 0.3 mmol), **4** (408 mg, 3 mmol), EDC (287.5 mg, 1.5 mmol) and DMAP (18 mg, 0.15 mmol) in DMF (8 mL) were stirred at room temperature for 5 h. After removal of the DMF solvent under vacuum, the crude product was purified by column chromatography over silica gel using CH_2Cl_2/MeOH (v/v, 20:1) as the eluent (Yield: 31%). ^{1}H NMR (400 MHz, MeOD-d4): δ 8.44 (br, 2H), 7.91-7.86 (m, 3H), 7.81-7.72 (m, 13H), 7.53 (t, J = 8 Hz,

2H), 7.46-7.28 (m, 6H), 6.29 (t, *J* = 16 Hz, 2H), 4.19 (br, 4H), 4.03 (s, 2H), 3.44-3.38 (m, 2H), 3.29-3.26 (m, 4H), 2.72 (br, 4H), 2.20 (t, *J* = 8 Hz, 4H), 1.95 (br, 2H), 1.88-1.81 (m, 6H), 1.73 (s, 6H), 1.72 (s, 6H), 1.71-1.63 (m, 4H), 1.47-1.45 (m, 4H). HRMS (ESI) m/z calcd for $C_{67}H_{80}ClN_5O_3P^{2+}$ [M^{2+}]: 551.7682, found: 551.7761.

Detection of UV-Vis absorbance and fluorescence spectrum. Mito-PS, **Pro-PS** and **Mito-Pro-PS** solutions with same concentration were prepared. The absorbance spectra from 650-850 nm were recorded. And the fluorescence spectra from 790-850 nm were recorded. λ_{ex} = 780 nm.

Fluorescence quantum yield (Φ_f). The fluorescence quantum yield was detected according to the literature method.[5] Fluorescence quantum yields were measured by comparing the fluorescence intensity of the sample with that of a fluorescence reference according to the following equation:

$$\Phi f_{sample} = \Phi f_{ref} \frac{F_{sample} A_{ref}}{F_{ref} A_{sample}}$$

F_{sample} and F_{ref} are the integrated area under the fluorescence spectra for the sample and reference. A_{sample} and A_{ref} are the absorbance of the sample and reference. **ICG** in DMSO, which has a known fluorescence quantum yield of 0.12, was used as the reference.

Singlet oxygen quantum yield ($\Phi_{\square}$). The Singlet oxygen quantum yield was detected according to the literature method.[6,7] The ROS generation efficiency of **PS**s was evaluated by singlet oxygen capture agent, DPBF. Briefly, the absorbance of DPBF at 415 nm was adjusted to about 1.0, and then **PS**s or **ICG** was added to the cuvette and the absorbance was adjusted to 0.3 at 802 or 795 nm, respectively. The mixture was then placed in a cuvette and irradiated with an 808 nm light source for different time (0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110 and 120 s), and the corresponding absorption spectra was measured immediately. The Singlet oxygen quantum yield were calculated by a relative method comparing the quantum yield of photosensitizer to the **ICG** ($\Phi_{\square, ICG}$=0.008 in DMSO) as the reference.[6] $\Phi_{\square}$ were calculated according to the following equation:

$$\Phi\Delta_{sam} = \Phi\Delta_{ICG} \frac{m_{sam} F_{ICG}}{m_{ICG} F_{sam}}$$

where sam designate the PSs. m is the slope of absorbance attenuation curve of DPBF at 415 nm, and F is the absorbance correction factor, which is obtained by $F = 1\text{-}10^{-O.D.}$ (O.D. is the absorbance of the solution at 808 nm).

Gel electrophoresis. Mito-Pro-PS, **Mito-PS** and **Pro-PS** were reacted with bovine serum albumin (BSA) in PBS buffer solution and stirred in a 37 °C water bath for 6 h. The reaction mixture was dialyzed against the dialysate using a dialysis bag (MWCO 3500). The BSA in the three groups was then separated by using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Finally, the fluorescence bands of the photosensitizers (PSs) and BSA in the three groups were displayed by using the ChemiDoc™ Touch Imaging System (Bio-Rad).

Evaluating the activity of protein following ROS generated by Mito-Pro-PS as

follows: (1) To reduce the disulfide bond, HRP (250 μL, 1 mg/mL) was mixed with dithiothreitol (DTT) (100 mM) for 15 min. The reduced HRP was then dialyzed through an ultrafiltration centrifuge tube. **Mito-PS** (0.05 mM) and **Mito-Pro-PS** (0.05 mM) were then dissolved in tetrahydrofuran (THF) (800 μL, 0.5 mg/mL) and mixed with reduced HRP (100 μL, 0.5 mg/mL). Then 20 L of triethylamine (5 mg/mL) was added to promote the thiol-ene click reaction. The reaction mixture was incubated for 2.5 h at room temperature. After the incubation, **Mito-PS**+HRP and **Mito-Pro-PS**+HRP were washed until no material color was observed in the supernatant. Finally, **Mito-PS**+HRP and **Mito-Pro-PS**+HRP were redispersed in water. (2) Tetramethylbenzidine (TMB) (100 μL, 0.004 moL/L) and H_2O_2 (100 μL, 0.01 moL/L) were dissolved in 2 mL of PBS and added to deionized water in a volume of 5 mL. After 3 min, the color did not change significantly. HRP was then added and the absorbance at different time points was measured at a wavelength of 380 nm. The experimental methods for detecting the protein activity of **Mito-Pro-PS**+HRP and **Mito-PS**+HRP are the same as those described above. (3) **Mito-Pro-PS**+HRP, **Pro-PS**+HRP, **Mito-PS**+HRP and HRP were irradiated with an 808 nm laser (1.0 W/cm^2) for different time points (0, 2, 4, 6, 8 and 10 min), and then added to the reaction solution of TMB and H_2O_2 to observe the absorbance and indirectly reflect protein activity.

Verification of the generation of ROS *in vitro*. The generation of ROS was verified using DPBF and analyzing the UV-vis spectra. **Mito-PS**, **Pro-PS**, or **Mito-Pro-PS** (0.5 mM) was added to DPBF (0.05 mM) ethanol solution and irradiated with an 808 nm laser (1.5 W/cm^2) for different time points (0, 1, 2, 4, 6, 8, 10 min).

Cell culture. 4T1 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium. All cells were supplemented with 10% fetal bovine serum (BI) and 100 units/mL of 1% antibiotics penicillin/streptomycin (Gibco) to maintain at 37 C in a 100% humidified atmosphere containing 5% CO_2.

MTT assays. [8] (1) Verify the toxicity of the **Mito-Pro-PS**: The cytotoxicity of **Mito-Pro-PS** was detected with 4T1 cells by a MTT assay. The 4T1 cells were seeded into a 96-well plates and cultured for 24 h. The culture medium was replaced with 200 μL of freshly complete medium containing **Mito-Pro-PS** (0, 2.5, 5, 7.5 and 10 μM). The cells were further cultured for 12 h. The excess unbound materials were washed for three times with PBS and 150 μL of MTT solution (0.5 mg/mL) were then added to each well. After 4 h of treatment, the MTT solution was discarded, and 150 μL of dimethyl sulfoxide (DMSO) was added to dissolve crystals. Finally, the absorbance was measured at 490 nm using a microplate reader. (2) Laser parameter optimizing: 4T1 cells were incubated in 96-well plates for 24 h. The old culture solution was discarded, and 1640 medium containing **Mito-Pro-PS** (7.5 μM) was added to the cells. After 2 h, residual **Mito-Pro-PS** was removed by PBS buffer. Then, different laser powers (0.5, 1, 1.5 and 2 W/cm^2, respectively) and different irradiation times (5, 15, 30, 60 and 90 s, respectively) were irradiated to treat the cells. The control group did not receive any treatment. The cell viability was assessed by MTT assay. (3) *In vitro* therapy: MTT tests were also carried out to evaluate the therapy effectiveness of different materials. The cells were cultured by the same steps as above: 4T1 cells were seeded in 96-well plates. Six groups of cells were performed as follows: PBS, Laser, **Mito-Pro-PS**, **Mito-**

PS+Laser, **Pro-PS**+Laser and **Mito-Pro-PS**+Laser groups. The concentration of the PSs was 7.5 μM, the cells were further incubated with materials for 2 h, and the Laser groups of cells were treated irradiation with the 808 nm laser at 1.0 W/cm^2 for 30 s. And the cells were cultured for another 6 h. The cell viability was evaluated with MTT assay.

To evaluate the immobilization of intracellular proteins by Mito-Pro-PS. 4T1 cells were cultured for 24 h in a confocal dish. **Mito-PS**, **Pro-PS** or **Mito-Pro-PS** (7.5 μM) was added to the culture medium and incubated with cells. After 2 h, the culture solution was discarded, and cells were fixed by adding paraformaldehyde for 1 h. Then added 0.1% Triton to incubate the cells for 10 minutes, the purpose was to punch the cell membrane. The mixture was then washed three times with PBS and imaged with confocal fluorescence imaging.

Real-time monitoring the fluorescence of intracellular produced ROS. 4T1 cells were incubated with **Mito-PS**, **Pro-PS** or **Mito-Pro-PS** (7.5 μM) for 2 h at 37 °C. Then, 4T1 cells were washed with PBS buffer. Fresh culture medium was added to further culture the cells. DCFH-DA (10 μM) was then added and co-cultured with 4T1 cells for 20 min. 4T1 cells were irradiated with an 808 nm NIR laser for 30 s. Subsequently, 4T1 cells were examined with confocal laser scanning microscopy (CLSM) with 488nm excitation for 60 min. Confocal images were obtained at 10 min interval.

Detection of ROS in cells.[9] To evaluate the capacity to produce ROS by each preparation, 4T1 cells incubated in confocal dishes were divided into six groups (PBS, Laser, **Mito-Pro-PS**, **Mito-PS**+Laser, **Pro-PS**+Laser, **Mito-Pro-PS**+Laser). For each group, they were incubated with the relative preparations (7.5 μM) for 2 h at 37 °C. Fresh culture medium was added to further culture the cells. DCFH-DA (10 μM) was added and co-cultured with 4T1 cells for 20 min. For the laser irradiation groups, 4T1 cells were irradiated with an 808 nm NIR laser for 30 s. The confocal images were immediately obtained by confocal fluorescence imaging with 488 nm excitation.

Live/dead cell staining assays. The 4T1 cells incubated in confocal dishes were divided into six groups (PBS, Laser, **Mito-Pro-PS**, **Mito-PS**+Laser, **Pro-PS**+Laser, **Mito-Pro-PS**+Laser). For each group, the 4T1 cells incubated with different materials (7.5 μM) were irradiated or not with 808 nm laser at 1.0 W/cm^2 for 30 s. After 6 h, the cells were washed twice with PBS. Then, the calcein-AM (20 nM) and PI (4 μM) solution were stained in PBS buffer for 20 min. Finally, the cells were washed three times with PBS and imaged by confocal fluorescence imaging.

Flow cytometry analysis of apoptosis. Cell apoptosis was performed by flow cytometry using 4T1 cells. The 4T1 cells were seeded in culture dishes with RPMI 1640 and were divided into six groups (PBS, Laser, **Mito-Pro-PS**, **Mito-PS**+Laser, **Pro-PS**+Laser, **Mito-Pro-PS**+Laser). For each group, 4T1 cells incubated with different materials (7.5 μM) were irradiated or not with an 808 nm laser at 1.0 W/cm^2 for 30 s. After further incubation with fresh medium for another 6 h, the cells were washed with PBS several times and then collected. Subsequently, cells were washed with PBS three times and stained with Annexin V-FITC/PI for 20 min. The cells were then analysed by flow cytometry.

Co-localization analysis of Mito-Pro-PS in 4T1 cells by CLSM and imaging flow

cytometer. 4T1 cells were first cultured in a confocal dish for 24 h. Then **Mito-Pro-PS** (7.5 μM) dispersed in 1640 medium was added to the confocal plates. After incubation for 2 h, cells were washed three times with PBS. The Petri dish was then washed three times with PBS. 4TI cells were incubated with **Mito-Pro-PS** (7.5 μM) for 2 h. Then the cells were washed three times with PBS buffer, and incubated with Mito-Tracker Green (25 nM) at 37°C for 15 min. Finally, cells in all groups were washed three times with PBS and analysed with a confocal laser scanning microscope (Mito-Tracker Green: λex = 488 nm, **Mito-Pro-PS**: λex = 642 nm).

Detection of mitochondrial membrane potential (ΔΨm). 4T1 cells were first cultured in a confocal plate for 24 h. Then **Mito-Pro-PS** (7.5 μM) was dispersed in 1640 medium and added to the confocal plates. After incubation for 2 h, the cells were washed twice with PBS buffer to remove residual **Mito-Pro-PS** and 2 mL of fresh 1640 was added. Under 808 nm laser irradiation for 30 s, the cells were incubated for another 6 h. Rhodamine 123 (5 μg/mL) was then used to stain the cells for 15 min. Confocal images were acquired by CLSM with 488 nm excitation and collected with a bandpass filter with a range of 500-550 nm.

Caspase-3 activation. 4T1 cells were first cultured in confocal plates for 24 h. Cells were treated with different conditions (PBS, Laser, **Mito-Pro-PS**, **Mito-PS**+Laser, **Pro-PS**+Laser, **Mito-Pro-PS**+Laser), where the concentration of 7.5 μM, and laser light of 1.0 W/cm^2 808 nm was irradiated for 30 s. The cells were incubated for 12 h and washed twice with PBS buffer. Cells were incubated with primary antibody anti-caspase-3 after being fixed with paraformaldehyde (4%) for 10 min. Cells were exposed with an enhanced secondary antibody for 100 min at room temperature. Confocal images were acquired by confocal laser scanning microscopy with 633 nm excitation and collected in the range from 640 to 700 nm. In addition, cytochrome C was also verified using an immunofluorescence staining method. The cells were incubated with different treatment. After incubation for 12 h, cells in different group were performed as described above.

Tumor model establishment. Animal experiments were reviewed and approved by the Ethics Committee of Shandong Normal University, Jinan, P. R. China (approval number AEECSDNU 2021065). All animal experiments were conducted and obeyed the Principles of Laboratory Animal Care (People's Republic of China) and the Guidelines of the Animal Investigation Committee, Biology Institute of Shandong Academy of Science, China. Balb / C mice (4-6 weeks old, female, ~18g) were fed for 12 h under normal conditions of light and dark circulation, and mice were provided plenty of food and water. In this experiment, the 4T1 breast cancer model was selected to evaluate the therapeutic effect. Therefore, 1×10^7 4T1 cells in 100 μL of serum-free RPMI 1640 medium were subcutaneously injected into the right hip region of Balb/c mice. After the tumor size reached approximately 75-100 mm^3, the mice were used in subsequent experiments.

***In vivo* therapeutic effect.** 4T1 tumor-bearing mice were divided into six groups (PBS, Laser, **Mito-Pro-PS**, **Mito-PS**+Laser, **Pro-PS**+Laser, **Mito-Pro-PS**+Laser). Physiological saline of different materials（100 μL, 200 μM）was administered to each mouse by intratumor injection. After 15 min, the tumor site was irradiated with a

1.0 W/cm^2 808 nm NIR laser for 30s. Tumor size and body weight were measured every other day for 16 days after treatment (tumor volume = L×W^2/2, W=width, L=length). The relative tumor volume of each mouse was calculated as V/V_0 (V_0 is the tumor volume at the start of treatment).

***In vivo* biosafety experiment.** The mice were divided into six groups and treated with different materials. After 12 h, the tumors of the mice were dissected and placed in a fixative for hematoxylin and eosin (H&E) staining. Similarly, six groups of mice were treated with different materials, and five major organs (liver, lung, spleen, kidney, and heart) were dissected after 7 days for H&E staining. At the same time, the mice were subjected to eyeball blood collection and divided into two batches. One batch was immediately placed at 4 °C storage for blood routine testing, the other batch was allowed to stand at room temperature for 1 h and then centrifuged at 4500 rpm in 4°C, the supernatant was taken and stored at -80° C for biochemical analysis.

Supplementary figures

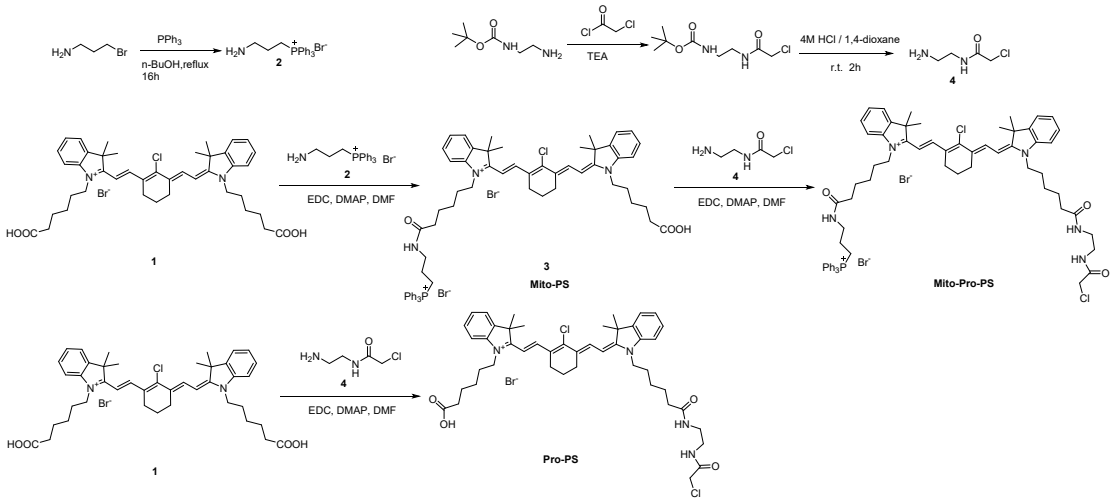


Fig. S1 Synthetic routes of **Mito-Pro-PS**.

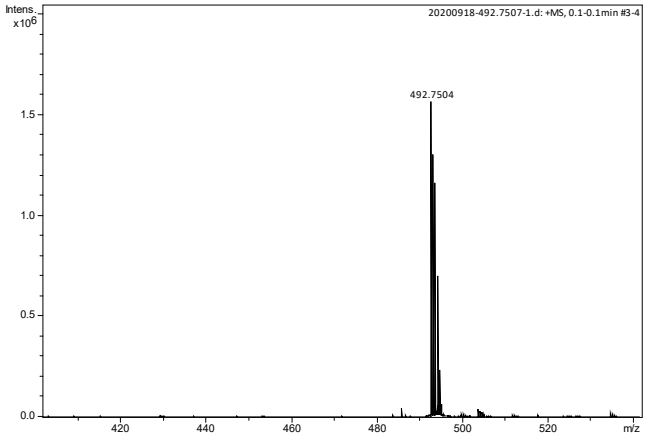


Fig. S2 HRMS spectrum of **Mito-PS**.

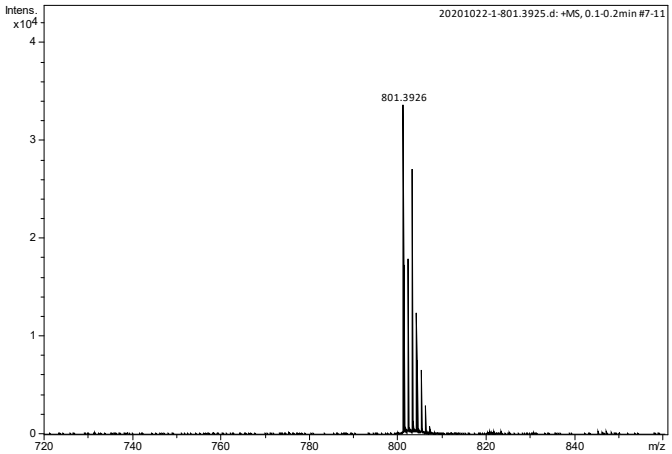


Fig. S3 HRMS spectrum of **Pro-PS**.

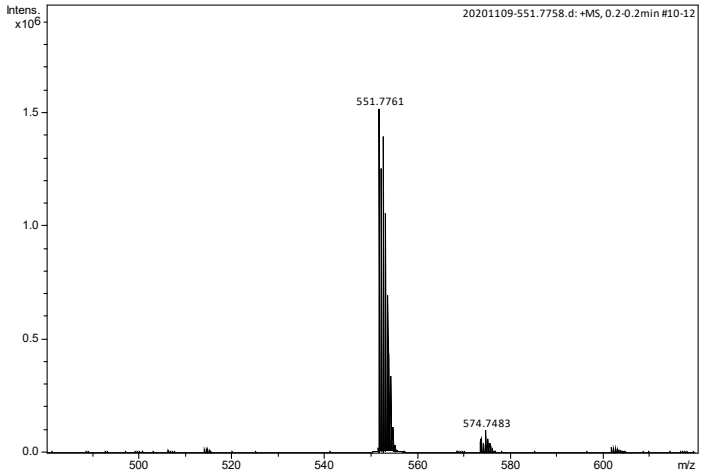


Fig. S4 HRMS spectrum of **Mito-Pro-PS**.

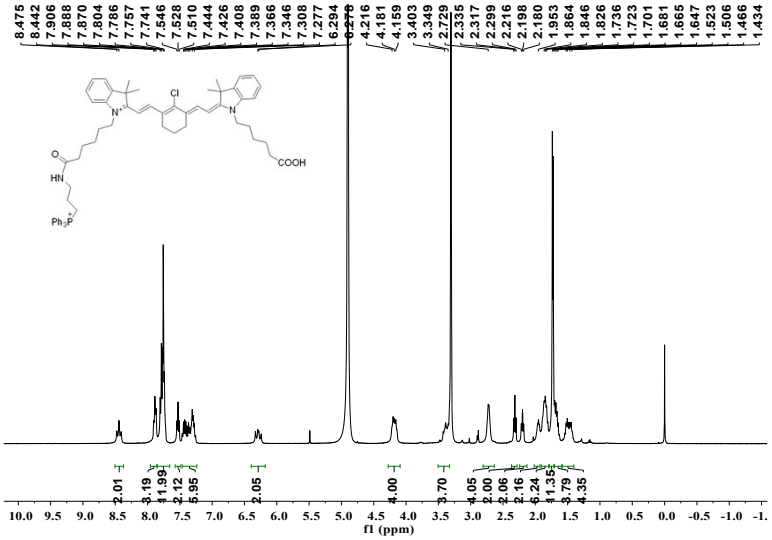


Fig. S5 ^{1}H-NMR (400 MHz, MeOD-d4) spectrum of **Mito-PS**.

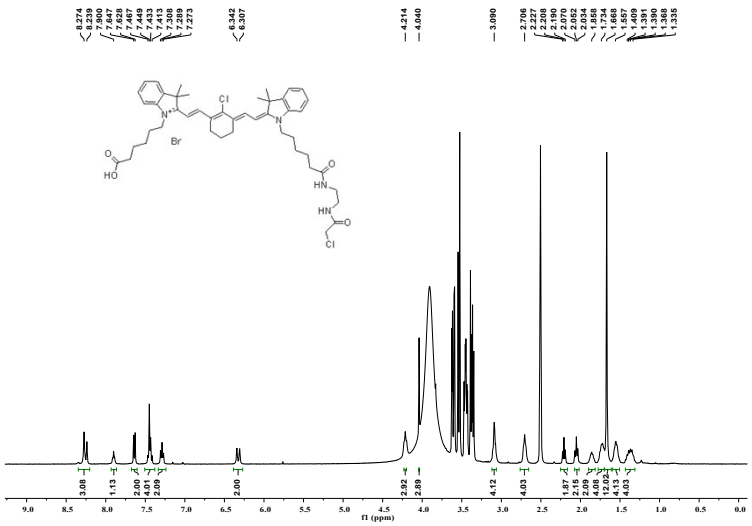


Fig. S6 ^{1}H-NMR (400 MHz, DMSO-d6) spectrum of **Pro-PS**.

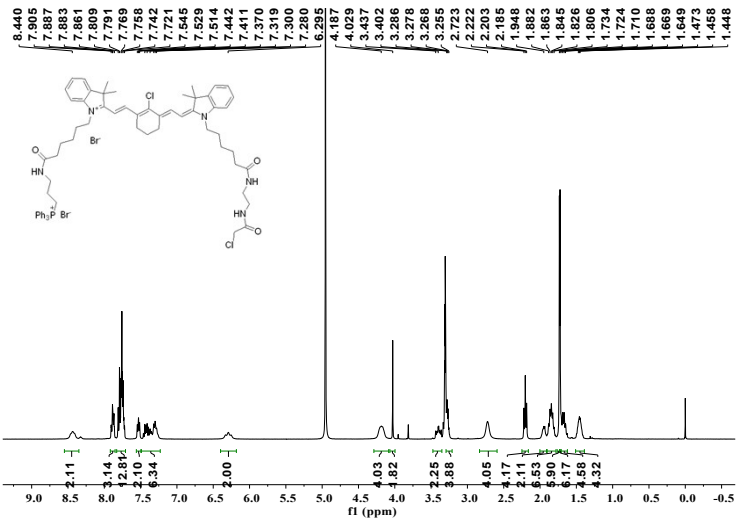


Fig. S7 ^{1}H-NMR (400 MHz, MeOD-d4) spectrum of **Mito-Pro-PS**.

HPLC Analysis Report

Sample : TP-CH-1701

Sequeece:分子 1

Solvent ASE :0.1%Trifluoroacetic in 100% Acetonirile

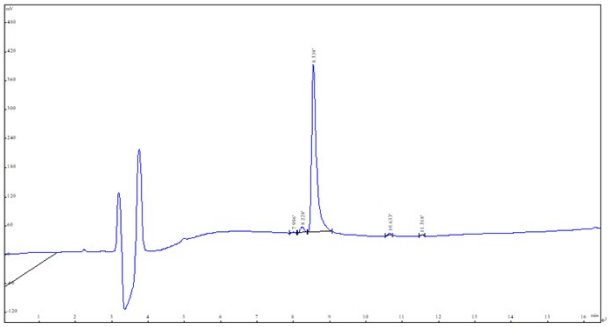
Solvent B :0.1%Trifluoroacetic in 100% Water

Gradient	:	A	B
	0.01min	5%	95%
	25.0min	70%	30%

Flow rate :1.0ml/min

Wavelength :214nm

Volume :10ul

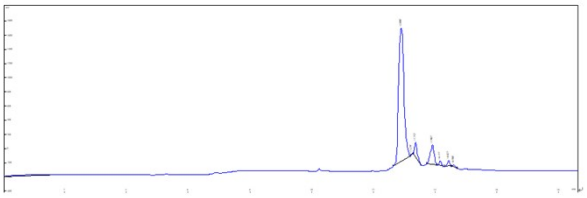


Rank	Time	Name	Conc.	Area
1	7.996		0.3372	11258
2	8.228		2.547	85031
3	8.538		96.14	3209543
4	10.633		0.7526	25125
5	11.518		0.2253	7522

Fig. S8 HPLC spectrum of **Mito-PS**.

HPLC Analysis Report

Sample ID: TP-CH-1702
Sequeece: 分子 2
Columan: 4.6*150mm,kromasil C18-5
Solvent A: 0.1%Trifluoroacetic in 100% Acetonirile
Solvent B: 0.1%Trifluoroacetic in 100% Water
Gradient: A B
0.01min 5% 95%
25.0min 70% 30%
Flow rate: 1.0ml/min
Wavelength: 214nm
Volume: 10ul

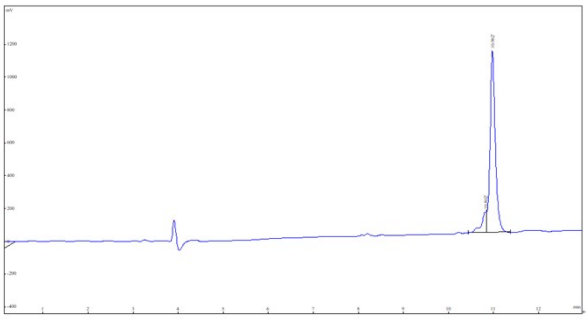


Rank	Time	Name	Conc.	Area
1	12.888		83.86	22731789
2	13.239		0.1987	53863
3	13.352		4.195	1139853
4	13.903		8.671	2350600
5	14.153		1.078	292309
6	14.423		1.458	395361
7	14.588		0.5384	145964

Fig. S9 HPLC spectrum of **Pro-PS**.

HPLC Analysis Report

Sample ： TP-CH-1703
Sequeece:分子 3
Solvent ASE: 0.1%Trifluoroacetic in 100% Acetonirile
Solvent B: 0.1%Trifluoroacetic in 100% Water
Gradient: A B
0.01min 5% 95%
25.0min 70% 30%
Flow rate: 1.0ml/min
Wavelength: 214nm
Volume: 10ul



Rank	Time	Name	Conc.	Area
1	10.802		10.71	1062679
2	10.962		89.29	8855717

Fig. S10 HPLC spectrum of **Mito-Pro-PS**.

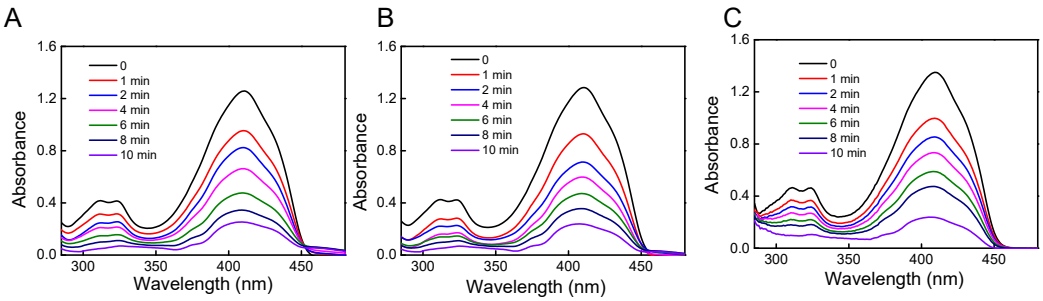


Fig. S11 UV-Vis absorption spectra of DPBF with **Mito-PS** (A), **Pro-PS** (B) and **Mito-Pro-PS** (C) under different times.

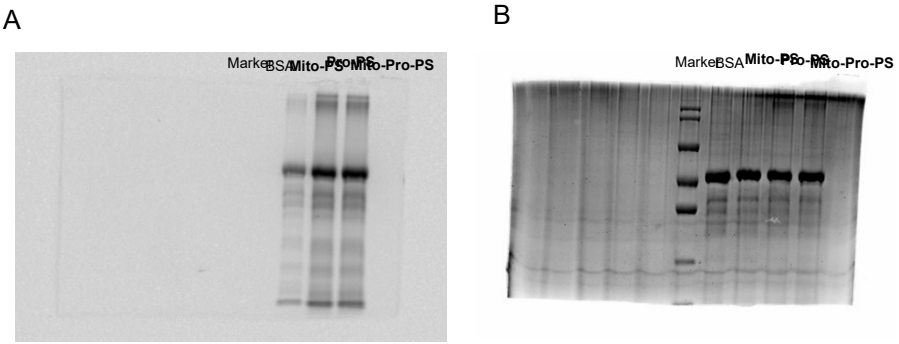


Fig. S12 The original pictures of Gel electrophoresis after different treatments. (A) The fluorescence images of three PSs. (B) The fluorescence images of BSA stained with Coomassie brilliant blue.

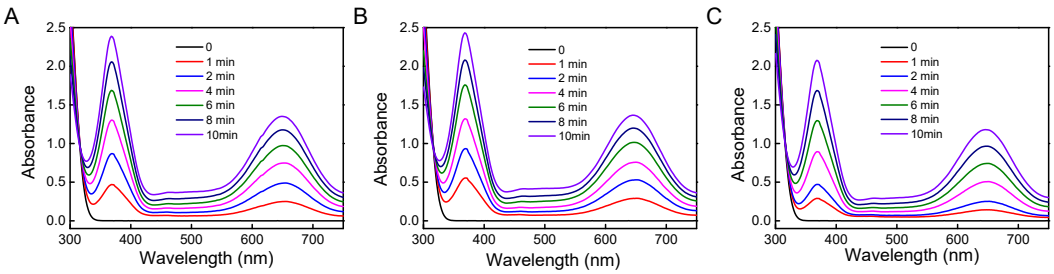


Fig. S13 The UV-Vis spectra of azo compounds with HRP (A), **Mito-PS**+HRP (B) and **Mito-Pro-PS**+HRP (C).

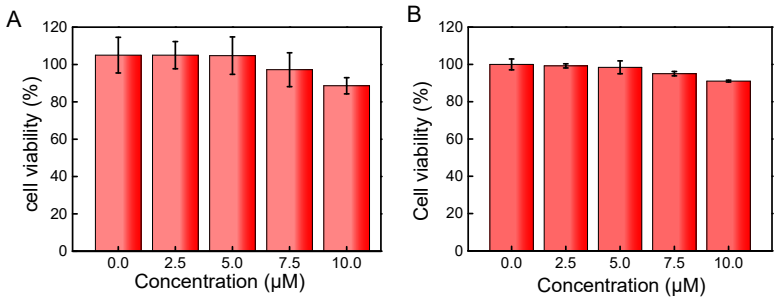


Fig. S14 MTT experiment of 4T1 cells (A) and normal TC-1 cells (B) after incubated by different concentrations of **Mito-Pro-PS**.

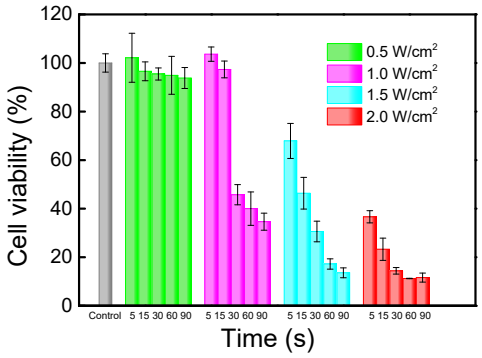
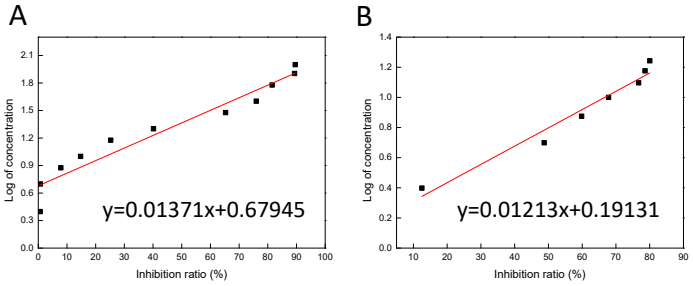


Fig. S15 Cell viability of 4T1 cells incubated with **Mito-Pro-PS** (7.5 μM) under various parameters (irradiation time and power).



Sample	IC50 dark	IC50 laser	$PI = \frac{IC50\ dark}{IC50\ laser}$
Mito-Pro-PS	23.17 μM	6.28 μM	3.7
Mito-PS	46.18 μM	21.33 μM	2.2
Pro-PS	32.80 μM	10.76 μM	3.0

Fig. S16 Cytotoxicity of **Mito-Pro-PS** under dark (A) and laser irradiation (B) conditions.

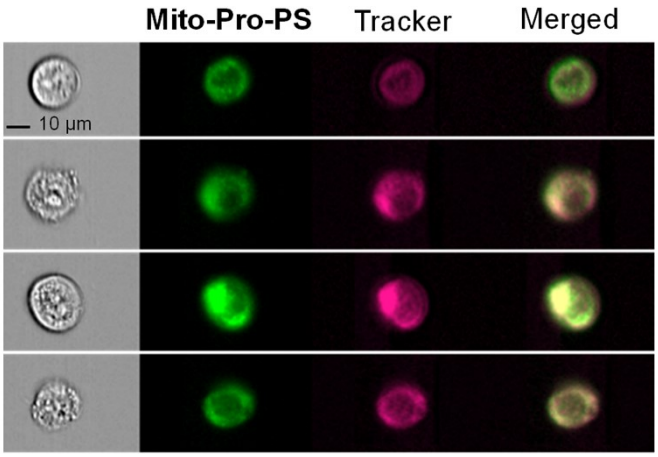


Fig. S17 The co-localization images of **Mito-Pro-PS** (7.5 μM) with the mitochondria using flow cytometry (Scale bar are 10 μm).

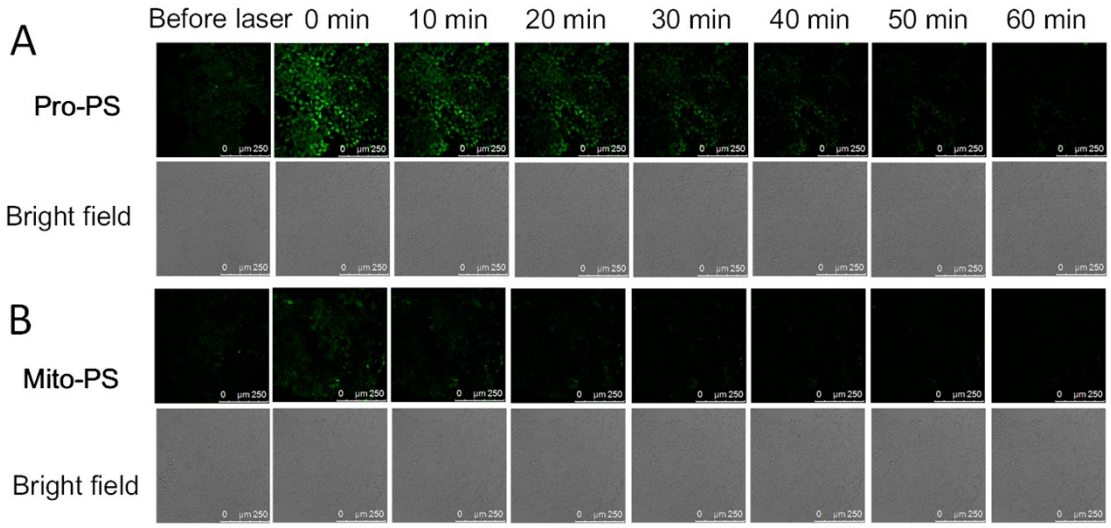


Fig. S18 Real-time monitoring of intracellular produced ROS with DCFH-DA under laser irradiation for 30 s. 4T1 cells were treated with (A) **Pro-PS** or (B) **Mito-PS** (7.5 μM). Following laser irradiation, cells images were obtained at 10 min intervals for up to 60 min (scale bar=250 μm).

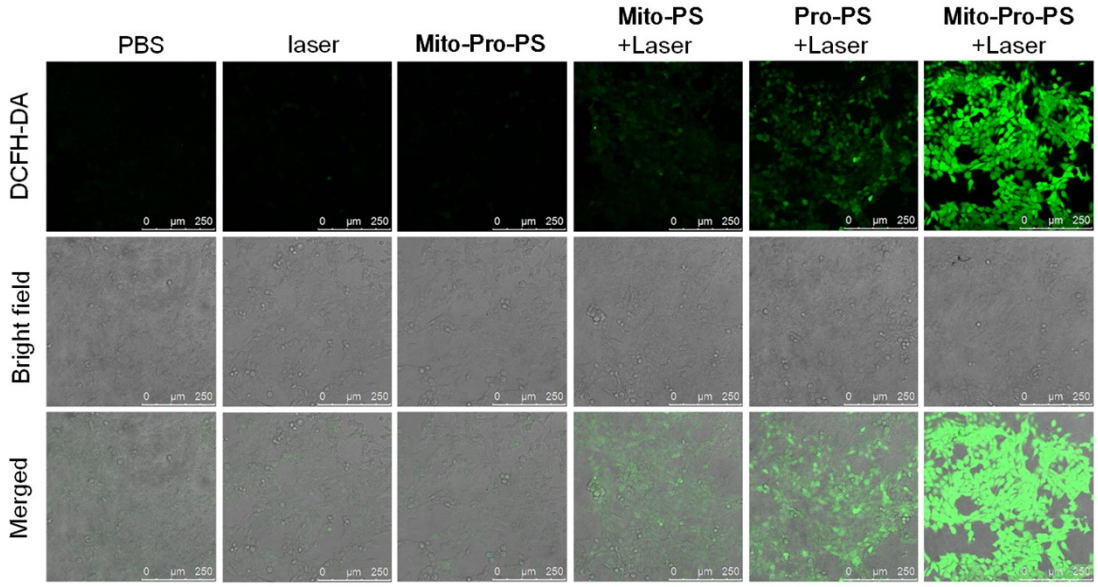


Fig. S19 Evaluation of ROS production based on DCFH-DA (10 μM) with different treatments. (λex: 488 nm, λem : 500 - 550 nm). Scale bar are 250 μm.

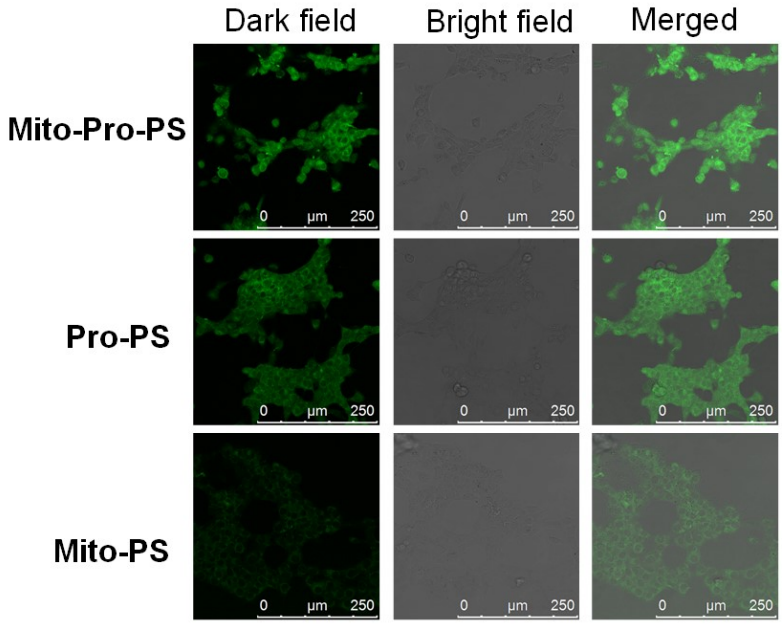


Fig. S20 Fluorescence confocal imaging of the 4T1 cells fixed with paraformaldehyde after incubated with **Mito-PS**, **Pro-PS** and **Mito-Pro-PS** (7.5 μM). Scale bar are 250 μm.

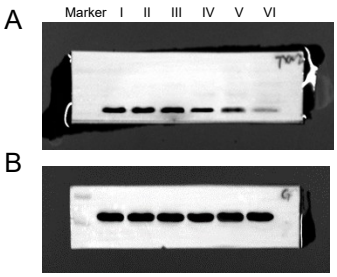


Fig. S21 The original pictures of the western blot after different treatments. (A) TXN2 and (B) GAPDH. (I: PBS, II: Laser, III: **Mito-Pro-PS**, IV: **Mito-PS**+Laser, V: **Pro-PS**+Laser, VI: **Mito-Pro-PS**+Laser).

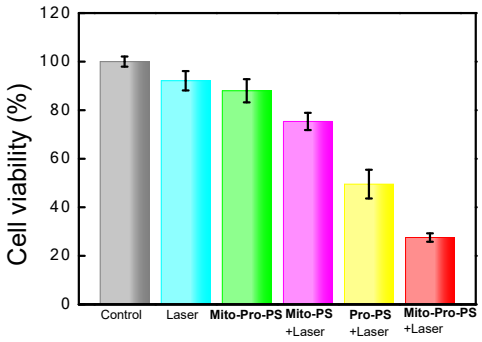


Fig. S22 Cell viability of 4T1 cells with different treatments (1 W/cm^2, 30 s).

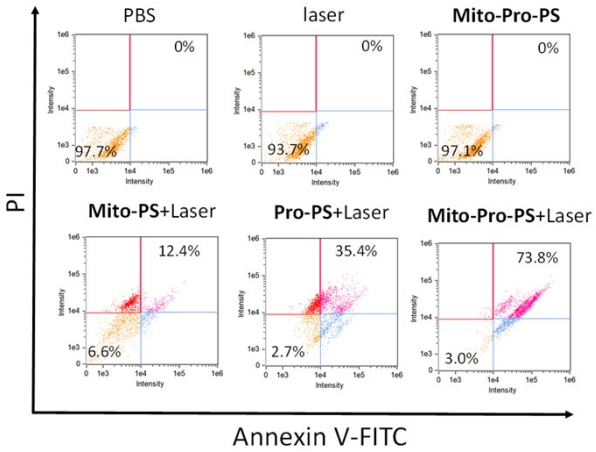


Fig. S23 Apoptosis and necrosis of the 4T1 cells with different treatments were analyzed by flow cytometry. The concentrations of the meterials was 7.5 μM.

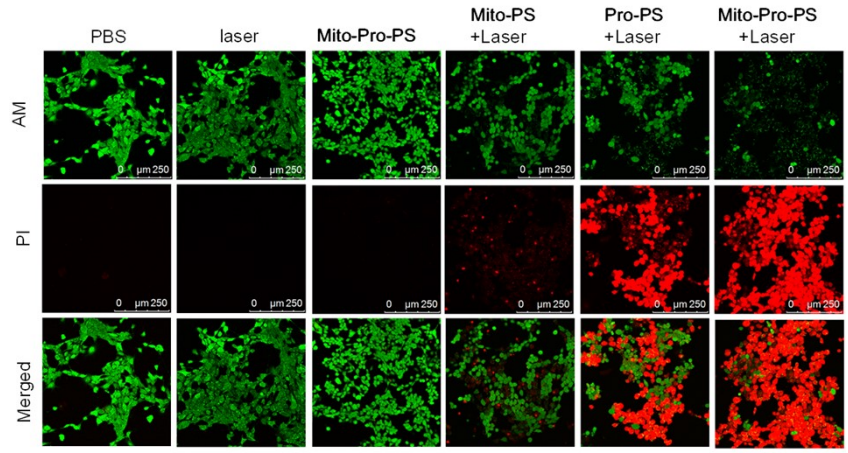


Fig. S24 The live/dead experiment of 4T1 cells by different treat methods (AM (20 nM): λex = 488 nm, λem = 500 - 550 nm; PI (4 μM): λex = 561 nm, λem = 600 - 650 nm). Scale bar are 250 μm.

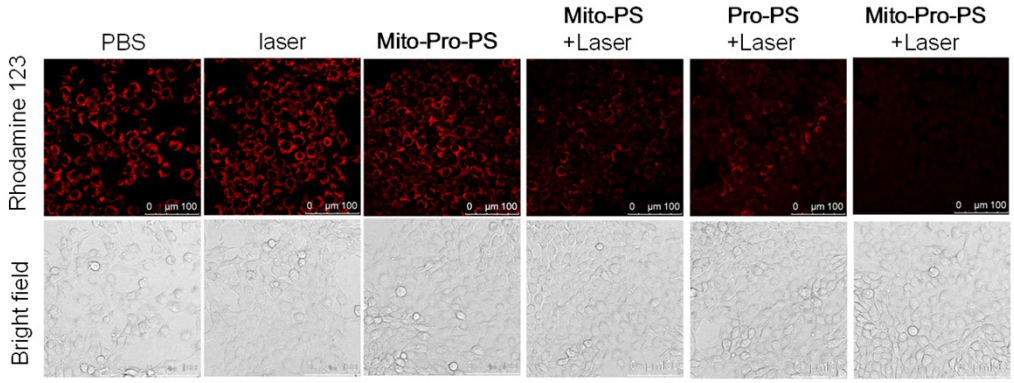


Fig. S25 Confocal images of Δψm by CLSM after the cells were stained with Rhodamine 123 (λex = 488 nm, λem = 500 - 550 nm). Scale bars are 100 μm.

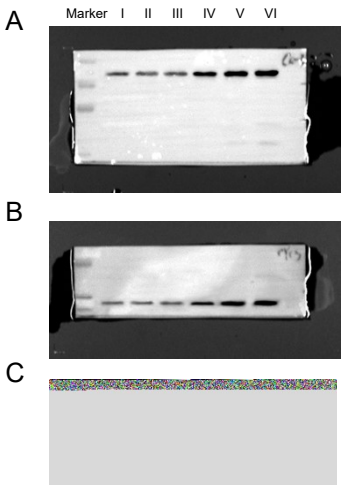


Fig. S26 The original pictures of the western blot after different treatments. (A) caspase-3, (B) cytochrome Cand (C) GAPDH. (I: PBS, II: Laser, III: **Mito-Pro-PS**, IV: **Mito-PS**+Laser, V: **Pro-PS**+Laser, VI: **Mito-Pro-PS**+Laser).

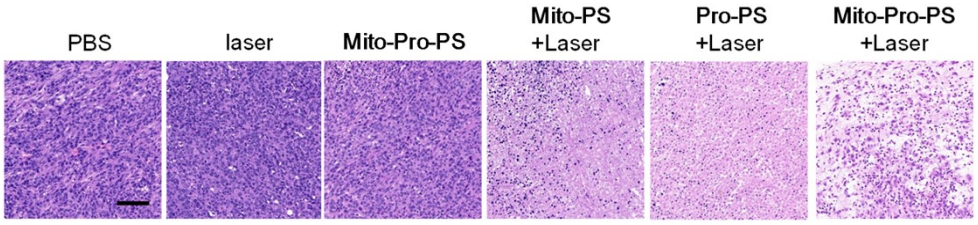


Fig. S27 H&E staining of the tumor tissues with different treatments. Scale bar are 100 μm.

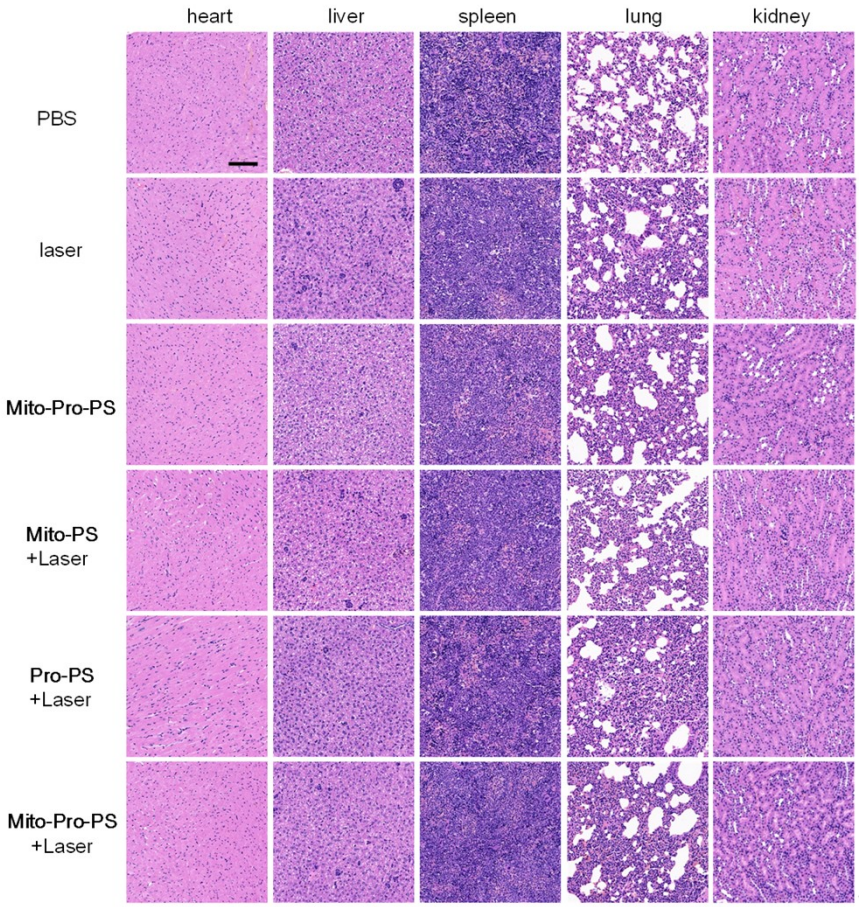


Fig. S28 H&E staining of the five major organs (heart, liver, spleen, lung and kidney) with different treatments. Scale bar are 100 μm.

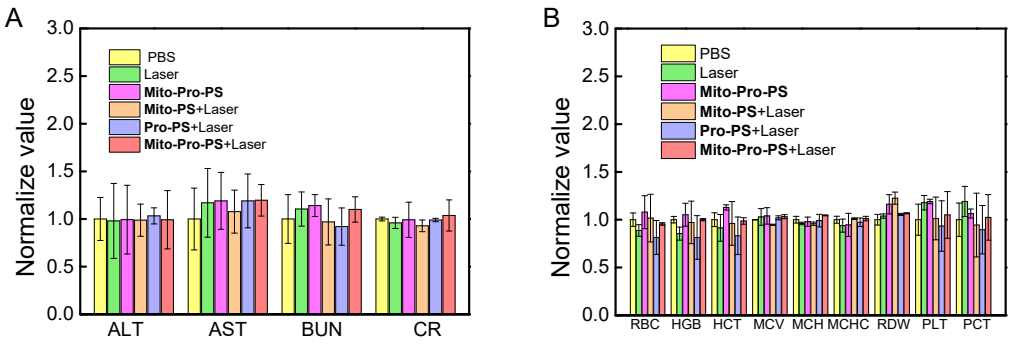


Fig. S29 Blood routine (A) and blood biochemistry (B) of analysis with different groups.

Table S1. Photophysical properties of different PSs.

Sample	Solvent	λ_{ab}/nm	λ_{em}/nm	ε/ $L \cdot mol^{-1} \cdot cm^{-1}$	Φ*f*	$\Phi_{\square}$
ICG	DMSO	795	812	186000	0.12	0.0080
PS	DMSO	802	810	190000	0.0912	0.0083
Mito-PS	DMSO	802	810	184900	0.0879	0.0081
Pro-PS	DMSO	802	810	186400	0.08842	0.0079
Mito-Pro-PS	DMSO	802	810	185600	0.08818	0.0079

ICG as a reference for singlet oxygen quantum yield measured. **ICG** was used as a standard for quantum yield measure. Where $\Phi_{\square}$ represent Singlet Oxygen quantum yield, Φ*f* represent quantum yield, ε represent molar extinction coefficient.

Reference

1 X. Tan, S. Luo, D. Wang, Y. Su, T. Cheng and C. Shi, *Biomaterials*, 2012, **33**, 2230.
2 Q. Zeng, Q. Guo, Y. Yuan, Y. Yang, B. Zhang, L. Ren, X. Zhang, Q. Luo, M. Liu, L. S. Bouchard and X. Zhou, *Anal. Chem.*, 2017, **89**, 2288.
3 C. Sun, H. Lin, X. Gong, Z. Yang, Y. Mo, X. Chen and J. Gao, *J. Am. Chem. Soc.*, 2020, **142**, 198.
4 Q. He , Y. Qi , C. Liu , J. Li and H. Hu, *Synlett*, 2017; **28**, 1939.
5 R. C. Bwnson and H. A. Kues, *Phys. Med. Biol.*, 1978, **23**,159.
6 M. M. Leitão, D. Melo-Diogo, C. G. Alves, R. Lima-Sousa and I. J. Correia, *Adv. Healthcare Mater.*, 2020, 1901665.
7 L. Yang, B. Huang, S. Hu, Y. An, J. Sheng, Y. Li, Y. Wang, and N. Gu, *Nano Res.*, 2022, **15**, 4285.
8 X. Wan, H. Zhong, W. Pan, Y. Li, Y. Chen, N. Li and B. Tang, *Angew. Chem. Int. Ed.*, 2019, **58**, 14134.
9 Z. Yu, Q. Sun, W. Pan, N. Li and B. Tang, *ACS Nano*, 2015, **9**, 11064.