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A ratiometric theranostic nanoprobe for pH imaging-guided photodynamic therapy

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Experimental

Materials and methods. Rink amine resin (0.38 mmol/g) and N-fluorenyl-9-methoxycarbonyl (Fmoc)-protected D-amino acid of Fmoc-Lys(Mtt)-OH were obtained from GL Biochem Ltd. (Shanghai, China). Fmoc-PEG_8-CH_2CH_2COOH was purchased from Zhejiang Bomei Biological Technology Co., Ltd. (China). 2′,7′-dichloroflorescein diacetate (DCFH-DA), methylthiazolyldiphenyl-tetrazolium bromide (MTT), Annexin V-FITC, Calcein-AM, propidium iodide (PI) and Hoechst 33342 were obtained from R&D-SYSTEMS. Trifloroacetic acid (TFA), triisopropylsilane (TIS), protoporphyrin IX (PpIX), diisopropylethylamine (DIEA), Rhodamine B and o-benzotriazole-N,N,N′,N′-tetramethyluroniumhexaflorophosphate (HBTU) were provided by Aladdin Reagent Co. Ltd. (China). Nigericin (sodium salt) was obtained from Cayman. Dimethyl sulfoxide (DMSO), methanol, dichloromethane (DCM), hydrochloric acid (HCl), sodium hydroxide (NaOH) and N,N′-dimethylformamide (DMF) were obtained from Shanghai Chemical Co. (China). Singlet oxygen sensor green (SOSG), LysoTracker Green, Dulbecco's modified Eagle's medium (DMEM), minimum essential medium (MEM), trypsin, MitoTracker Green, fetal bovine serum (FBS), Roswell Park Memorial Institute 1640 (RMPI 1640), Dulbecco's phosphate buffered saline (PBS) and penicillin-streptomycin were purchased from Invitrogen Corp.

The molecular weight was characterized by ESI-MS (ThermoFisher Scientific). The particle size and zeta potential were analyzed by Nano-ZS ZEN 3600 (Malvern) and the morphology was observed by TEM (JEOL-1400 PLUS). The UV-vis

absorbance was recorded by Lambda 35 (Perkin-Elmer) and the fluorescence was measured by LS55 luminescence spectrometer (Perkin-Elmer). The pH value was measured by pH meter (Leici Corp., PHS-3C). Confocal laser scanning microscope (CLSM) images were obtained and analyzed by LSM 880 (Carl Zeiss). Cell viability was measured by MTT assay using microplate reader (Mithras2 LB 943, BERTHOLD) and the light toxicity was conducted using 630 nm LED light (light intensity: 29.8 mW/cm^2). Hematoxylin-eosin staining (H&E) staining images were observed by inverted microscope (Revolve FL, ECO Laboratories). *In vivo* fluorescence images were measured and analyzed by *In-Vivo* FX Pro (Bruker). Flow cytometry (Amnis, Merck millipore) was employed to analyze the cell death and cellular uptake behaviors. 630 nm He-Ne laser (laser intensity: 250 mW/cm^2) was used to investigate the anti-tumor studies *in vivo*.

Synthesis of Rhodamine B-PEG$_8$-K(PpIX). Rhodamine B-PEG$_8$-K(PpIX) was synthesized manually using Rink amine resin by standard SPPS method. Briefly, Rink amine resin was soaked in distilled DMF for 1 h. After removing the Fmoc protecting group by piperdine/DMF (20%, V/V), Fmoc-Lys(Mtt)-OH was coupled onto the resin by using HBTU and DIEA as coupling agents. Subsequently, Fmoc-PEG$_8$-CH_2CH_2COOH and Rhodamine B were coupled onto the resin using the same methods. Then, TFA/DCM (1%, V/V) was employed to remove the Mtt protecting group, and PpIX was coupled onto the ε-amine of Lys. Finally, Rhodamine B-PEG$_8$-K(PpIX) was cleaved from the resin by using TFA/H_2O (95%/5%, V/V) and the

obtained solid powder was stocked at -20 °C in the dark. The molecular weight of Rhodamine B-PEG8-K(PpIX) was characterized by ESI-MS.

Preparation and characterizations of the nanoprobe. Rhodamine B-PEG_8-K(PpIX) (100 mg/L) was disolved in distilled water under ultrasonication to obtain the nanoprobe (designated as RPP). The particle size and zeta potential of RPP were characterized by Nano-ZS ZEN360. The morphology of RPP was observed by TEM. The UV-vis absorbance of RPP (25 mg/L) and the equivalent concentration of PpIX (containing 0.1% DMSO) and Rhodamine B were measured by UV-vis spectrophotometer.

pH responsibility of RPP. PBS solutions with various pH were firstly prepared by dropping HCl or NaOH solution. Subsequently, RPP (25 mg/L) was added and the fluorescence spectrum was recorded by using luminescence spectrometer. Besides, the ratiometric fluorescence changes of RPP at various pH values and slit widths were also calculated. The excitation wavelength was 514 nm or 543 nm. As the controls, the fluorescence spectra of RPP, Rhodamine B as well as the mixture of Rhodamine B and PpIX at various pH values were recorded using the exciation wavelength at 405 nm and 514 nm respectively. The UV-vis absorbance and the fluorescence spectra of PpIX at various pH values were also detected using the excitation wavelength at 514 nm.

Cell culture. Murine mammary carcinoma (4T1) cells were cultured in RPMI-1640 medium containing 10% FBS and 1% antibiotics at 37 °C. African green monkey

kidney (COS7) cells and mouse embryo fibroblast (NIH3T3) cells were cultured in DMEM medium containing 10% FBS and 1% antibiotics at 37 °C.

$^1O_2$ production and measurement. The production of $^1O_2$ was measured by using SOSG as the sensor. Briefly, SOSG (5 μM) was mixed with RPP (25 mg/L) or the equivalent concentration of PpIX (containing 0.1% DMSO) in PBS at the pH of 4.0, 5.0, 6.8 or 7.4. Subsequently, the mixed solution was irradiated with He-Ne laser (laser intensity: 80 mW/cm^2) at the time intervals or incubated in the shield of light. The fluorescence of the solution was recorded by using excitation wavelength at 488 nm and collecting the emission wavelength at 524 nm. As the control, the fluorescence of SOSG (5 μM) in PBS at various pH values with various illumination time was monitored using the similar method. The intracellular $^1O_2$ at pH 7.4, 6.0 or 5.0 was measured by using DCFH-DA as the indicator. In brief, RPP (100 mg/L) or the equivalent concentration of PpIX (containing 0.1% DMSO) was incubated with 4T1 cells for 4 h at pH 7.4, 6.0 or 5.0. Subsequently, the cells were washed and incubated with DCFH-DA for 20 min. Then the cells were irradiated with LED light for 3 min (light intensity: 29.8 mW/cm^2) or incubated in the shield of light. The intracellular fluorescence was observed and analyzed by CLSM. 4T1 cells without any treatments or treated with DCFH-DA only at pH 7.4, 6.0 or 5.0 were used as the controls.

Subcellular locations of RPP. The subcellular localization of RPP was evaluated by co-staining strategies and observed by CLSM. 4T1 cells were seeded and cultured

for 24 h. After that, the cells were co-incubated with RPP (100 mg/L) for 24 h. Subsequently, the cells were washed thrice by PBS and stained by LysoTracker Green, MitoTracker Green or Hoechst 33342, respectively. Finally, the subcellular localization of RPP was observed and analyzed by CLSM.

Fluorescence imaging of pH in 4T1 cells by RPP. Fluorescent images of 4T1 cells at pH 7.4, 6.0 or 5.0 were observed by CLSM. Firstly, 4T1 cells were seeded and cultured for 24 h. Then the 4T1 cells were co-cultured with RPP (100 mg/L) for 4 h. Subsequently, the cells were washed by PBS and further incubated with H^+/K^+ ionophore nigericin (5 mg/L) at pH 7.4, 6.0 or 5.0 for 0.5 h, which was a standard approach to equilibrate the intracellular and extracelluar pH. Finally, the cells were washed and the intracellular fluorescence was observed by CLSM.

PDT therapeutic effect of RPP *in vitro*. The PDT therapeutic effect of RPP was investigated by MTT assay, flow cytometry and CLSM. For MTT assay, 4T1 cells were seeded in 96-well plates and cultured for 24 h. After which, gradient concentrations of RPP were added into each well and incubated for 4 h at pH 7.4 or 6.0. For PDT-treated group, the cells were exposed to the LED light (light intensity: 29.8 mW/cm^2) for 5 min. For control group, the cells were incubated in the dark. After 20 h, 20 μL of MTT (5 mg/mL in PBS) was added into each well for another 4 h. Then the supernatant was removed and 150 μL of DMSO was added into each well. The optical density (OD) was detected at 570 nm by a microplate reader. The relative cell viability was calculated as follows: cell viability (%) = OD (sample)×100/OD

(control), in which OD (control) was obtained in the absence of RPP and OD (sample) was obtained in the presence of RPP. The cytotoxicity of RPP against COS7 cells and NIH3T3 cells at pH 7.4 in the presence or absence of light irradiation was measured as the control using the similar method.

Furthermore, the PDT therapeutic effect of RPP was evaluated by detecting the cell apoptosis against 4T1 cells using flow cytometry. Firstly, 4T1 cells were seeded in 6-well plates and cultured for 24 h. After that, the cells were treated with RPP (100 mg/L) for 4 h at pH 7.4 or 6.0 and then irradiated for 3 min. 4T1 cells without any treatment or treated with RPP (100 mg/L) while without light irradiation were used as the controls. Finally, the cells were collected, stained with Annexin V-FITC and PI for flow cytometry analysis. The cell apoptosis assay of RPP against NIH3T3 cells at pH 7.4 in the presence or absence of light irradiation was measured as the control using the similar method.

Besides, the PDT therapeutic effect of RPP was also evaluated using live/dead cell staining assay by CLSM. Briefly, after treated with RPP (100 mg/L) for 4 h at pH 7.4 or 6.0, 4T1 cells were exposed to light irradiation for 3 min. Similarly, 4T1 cells without any treatment or treated with RPP (100 mg/L) while without light irradiation were used as the controls. Finally, the cells were collected, stained with Calcein-AM and PI for CLSM observations. The live/dead cell staining assay of RPP against NIH3T3 cells at pH 7.4 in the presence or absence of light irradiation was measured as the control using the similar method.

***In vivo* optical imaging.** All of the animal experiments were operated in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Animal Experiment Center of Guangzhou Medical University (Guangzhou, China), as well as the Regulations for the Administration of Affairs Concerning Experimental Animals. 4T1 tumor-bearing mice were obtained by subcutaneously injecting 4T1 cells into the left back of hind leg region of female BALB/c mice. When 4T1 tumor volume reached about 250-300 mm^3, the tumor-bearing mice were intravenously injected with RPP at an equivalent PpIX concentration of 6 mg/kg. At the predetermined time, 4T1 tumor-bearing mice were imaged by using *In-Vivo* FX Pro (Bruker) imaging system. After 32 h post-administration, the mice were sacrificed and the main organs and tumors were harvested and imaged. And the fluorescence imaging of the corresponding tissues was analyzed by the instrument software.

***In vivo* anti-tumor study.** 4T1 tumor-bearing mice model was established as described above. When the tumor volume reached about 100 mm^3, the mice were randomly divided into three groups (5 mice in each group) including PBS group, RPP group as well as RPP and light group. 200 μL of PBS and RPP at an equivalent PpIX concentration of 6 mg/kg were intravenously injected into the mice. After 24 h, the mice in RPP and light group were irradiated with 630 nm He-Ne laser (250 mW/cm^2) for 5 min. During the treatment, the body weight and tumor volume of the mice in various groups were monitored every other day. The relative tumor volume was defined as V/V_0, where V_0 represented the tumor volume at the first day without any treatment and V was calculated as follows: $V= width^2 \times length/2$. After 13 days, the

4T1 tumor-bearing mice were sacrificed and the tumors were harvested, imaged, and weighted. Moreover, the obtained tumors and main organs were peeled off for histological analysis *via* H&E staining. The blood of the mice in various groups was also collected on the 13th day for blood biochemistry and blood routine analysis.

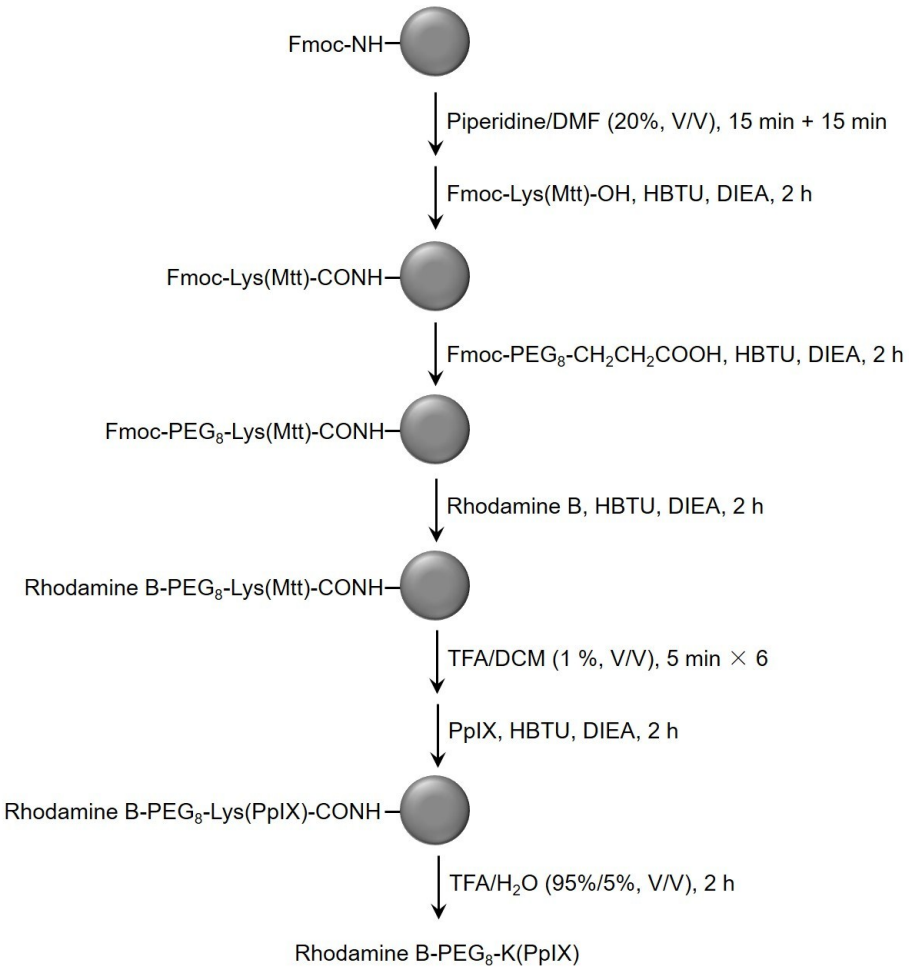

Fig. S1 Detailed synthetic procedure of Rhodamine B-PEG_8-K(PpIX).

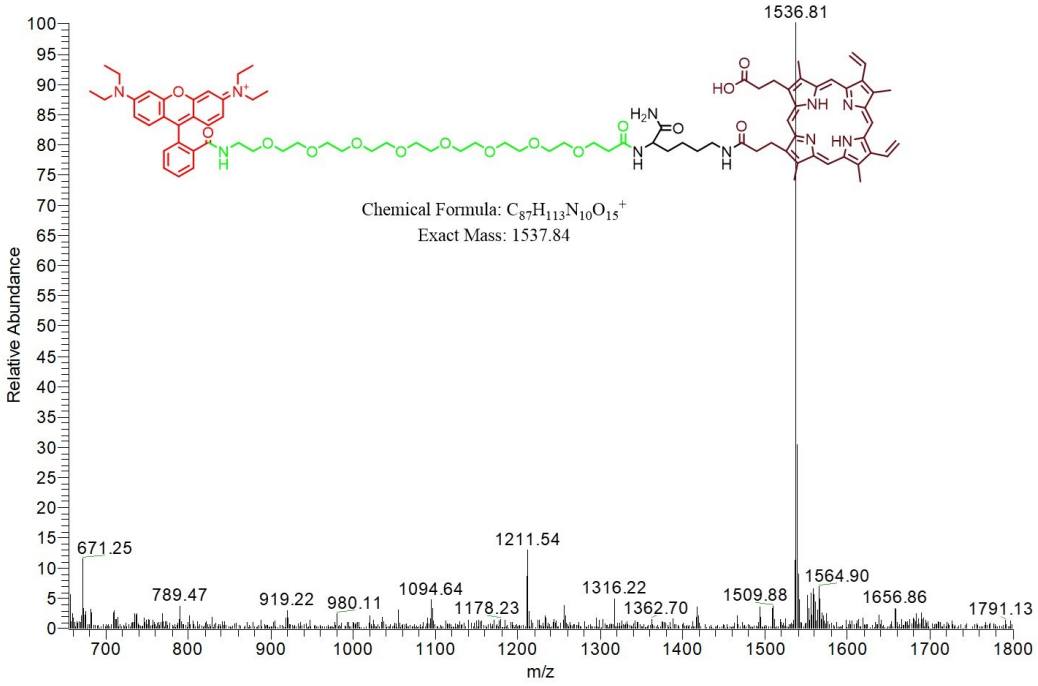

Fig. S2 ESI-MS of Rhodamine B-PEG$_8$-K(PpIX).

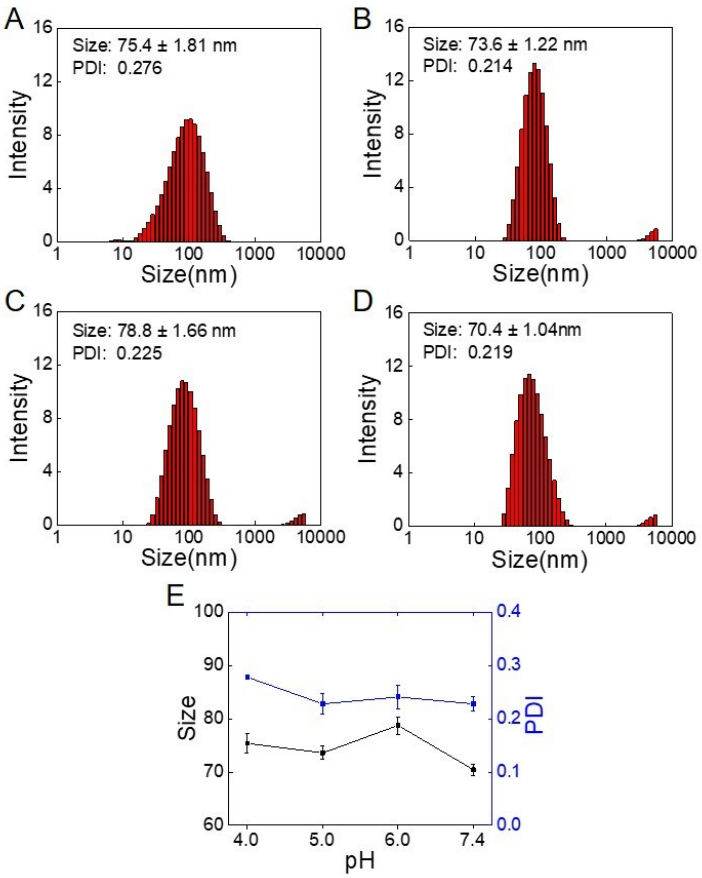

Fig. S3 Hydrodynamic diameter of RPP at A) pH 4.0, B) pH 5.0, C) pH 6.0 and D) pH 7.4. E) The hydrodynamic diameter and PDI changes of RPP at various pH values.

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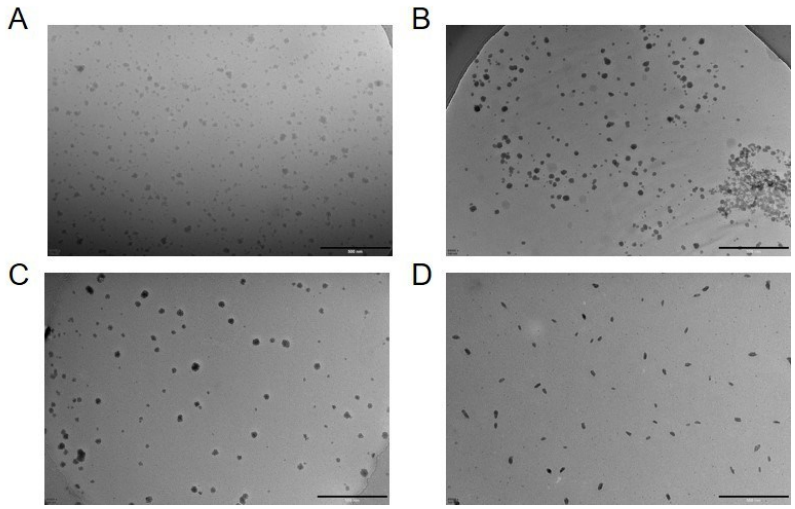

Fig. S4 TEM images of RPP at A) pH 4.0, B) pH 5.0, C) pH 6.0 and D) pH 7.4. Scale bar: 500 nm.

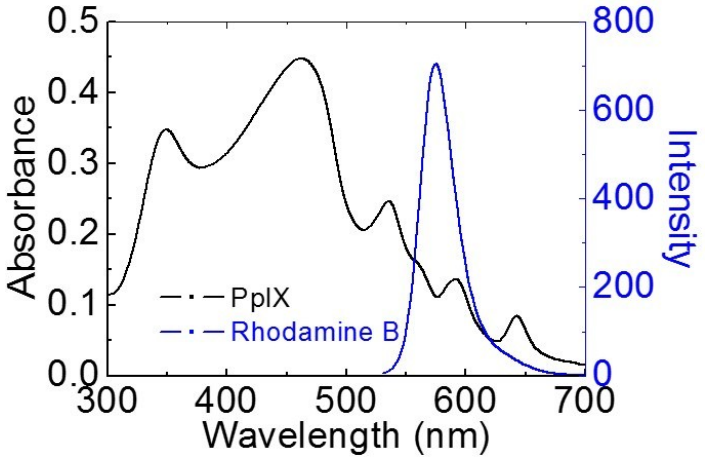

Fig. S5 The UV-vis absorbance of PpIX and the fluorescence spectrum of Rhodamine B using the exciation wavelength at 514 nm.

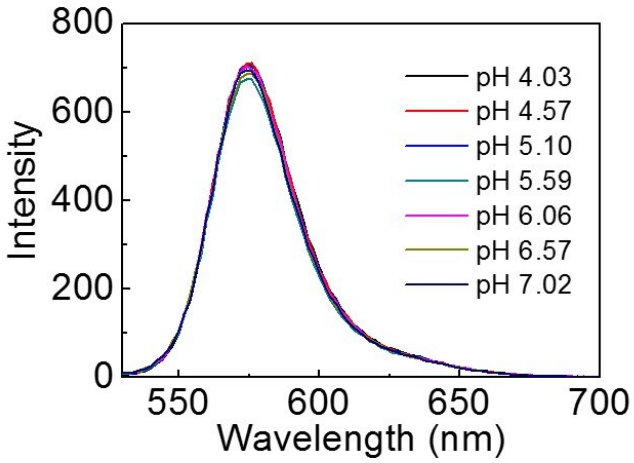

Fig. S6 Fluorescence spectra of Rhodamine B at various pH values. Excitation wavelength: 514 nm.

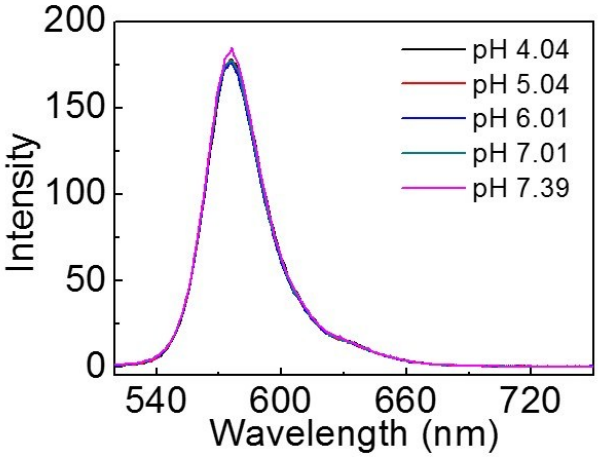

Fig. S7 Fluorescence spectra of the mixture of Rhodamine B and PpIX at various pH values. Excitation wavelength: 514 nm.

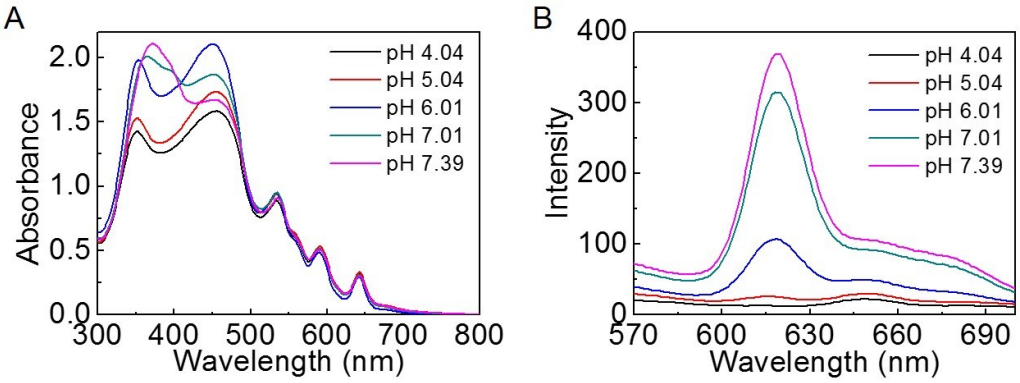

Fig. S8 A) UV-vis absorbance and B) fluorescence spectra of PpIX at various pH values. Excitation wavelength: 514 nm.

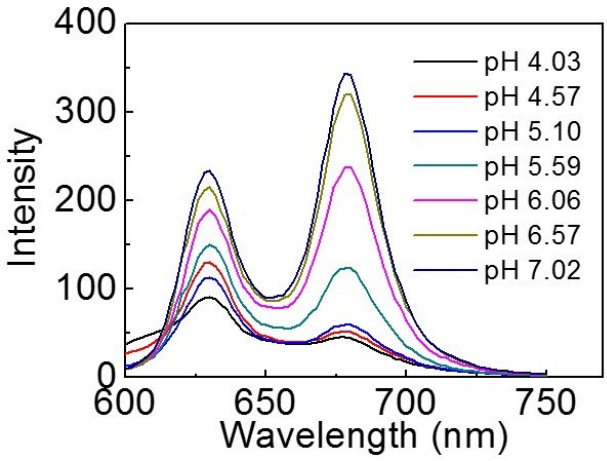

Fig. S9 Fluorescence spectrum of RPP at various pH values. Excitation wavelength: 405 nm.

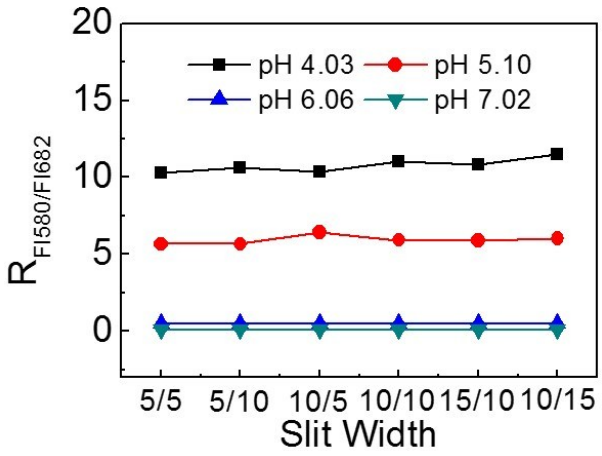

Fig. S10 Ratiometric fluorescence changes of RPP at various pH values and various slit widths. Excitation wavelength: 514 nm.

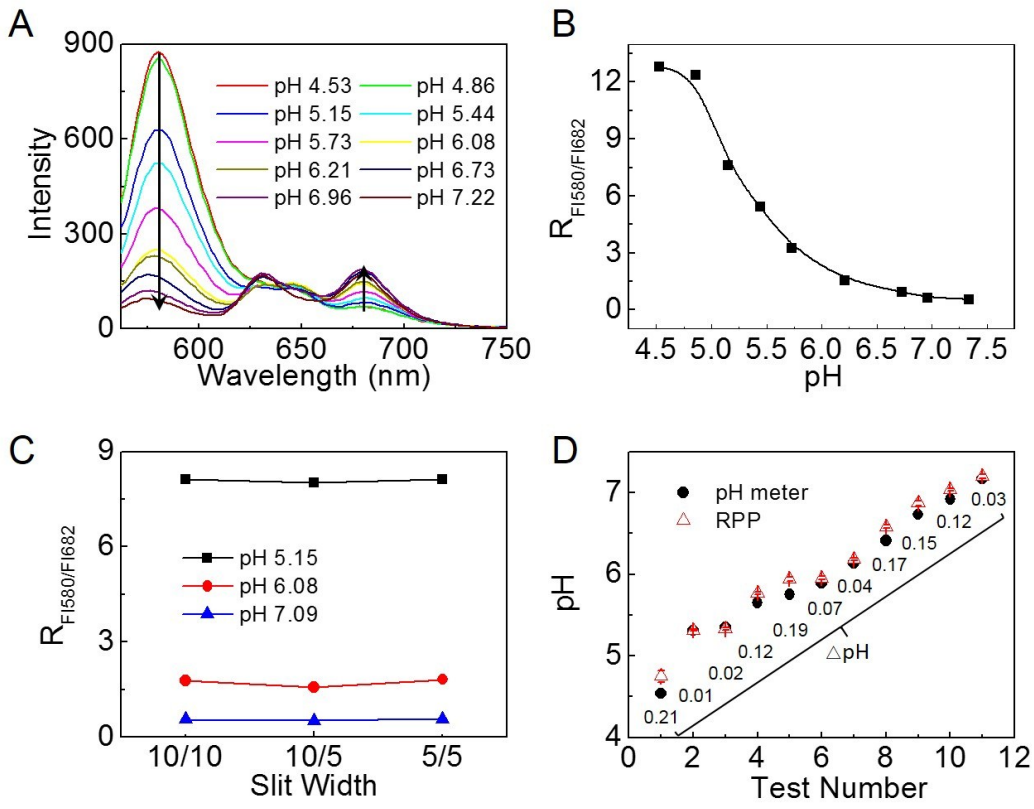

Fig. S11 A) Fluorescence spectrum and B) ratiometric fluorescence changes of RPP at various pH values. C) Ratiometric fluorescence changes of RPP at various pH values and various slit widths. D) The pH values measured by pH meter or calculated by ratiometric fluorescence of RPP. Excitation wavelength: 543 nm.

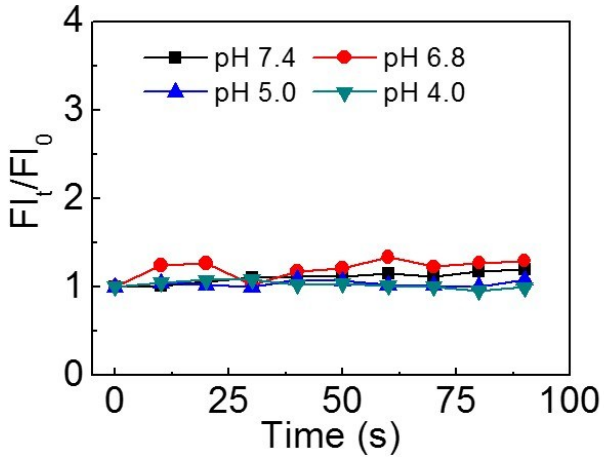

Fig. S12 Time related SOSG fluorescence changes at various pH values with various illumination time.

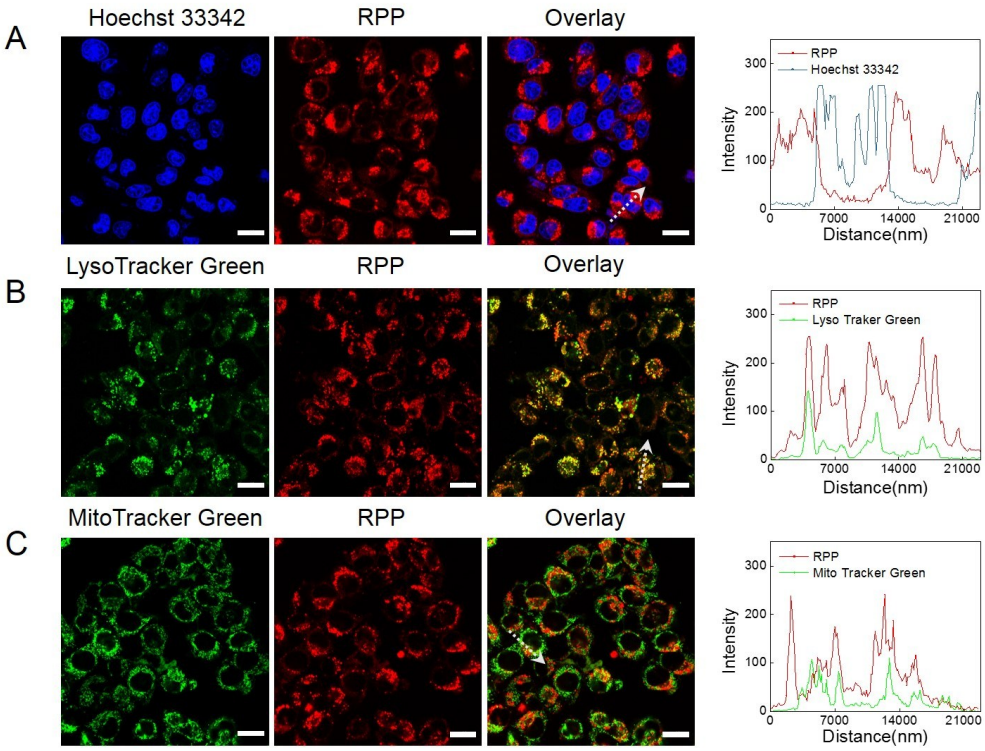

Fig. S13 The subcellular locations of RPP. CLSM images and the corresponding fluorescence profile analysis of 4T1 cells after treatment with RPP and stained by A) Hoechst 33342, B) LysoTracker Green and C) MitoTracker Green . Scale bar: 10 μm.

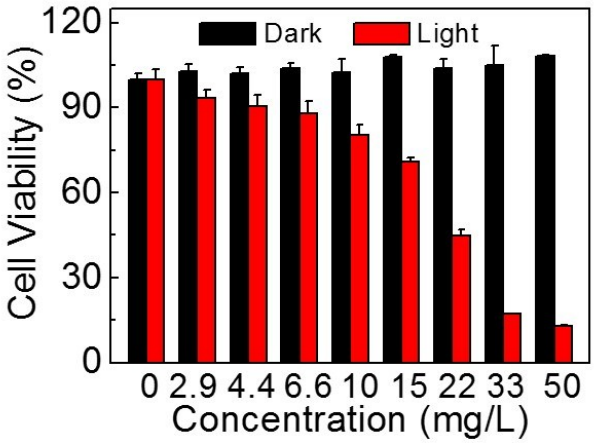

Fig. S14 The cytotoxicity of RPP against NIH3T3 cells at pH 7.4 by MTT assay in the presence or absence of light irradiation.

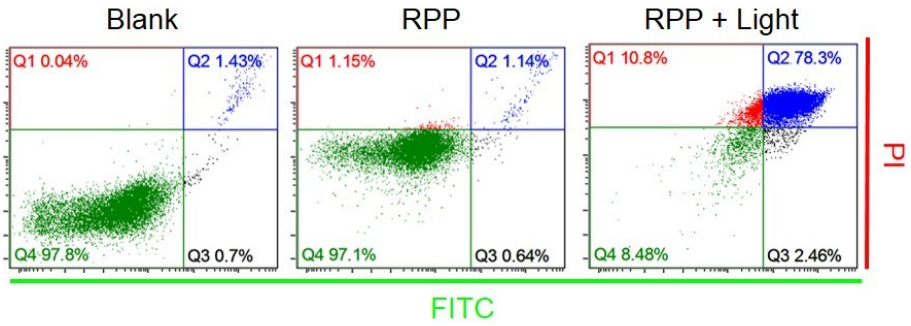

Fig. S15 Flow cytometry analysis of NIH3T3 cells treated by RPP with or without light irradiation at pH 7.4.

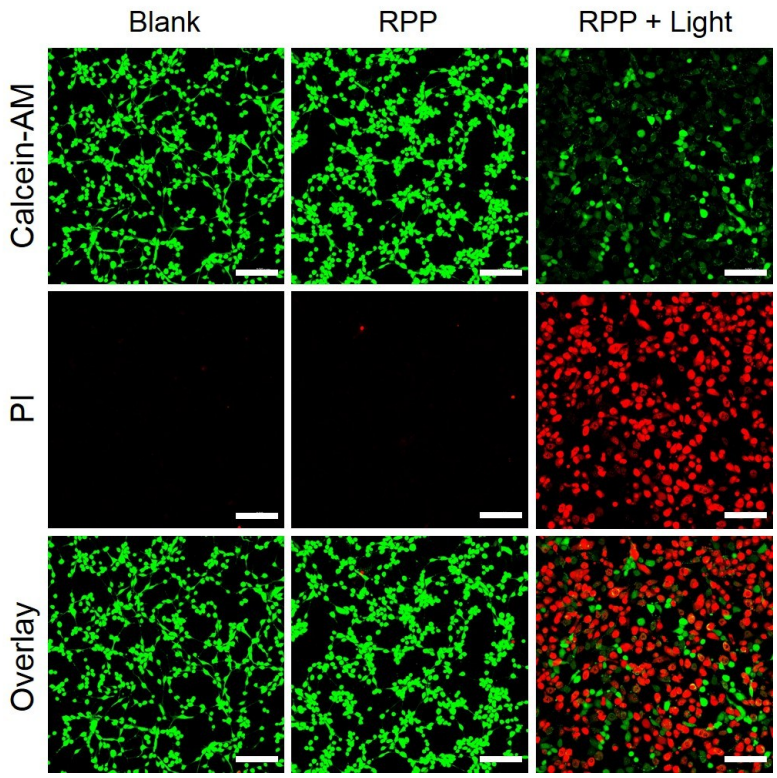

Fig. S16 CLSM images of NIH3T3 cells treated by RPP with or without light irradiation at pH 7.4 and stained by Calcein-AM/PI. Scale bar: 100 μm.

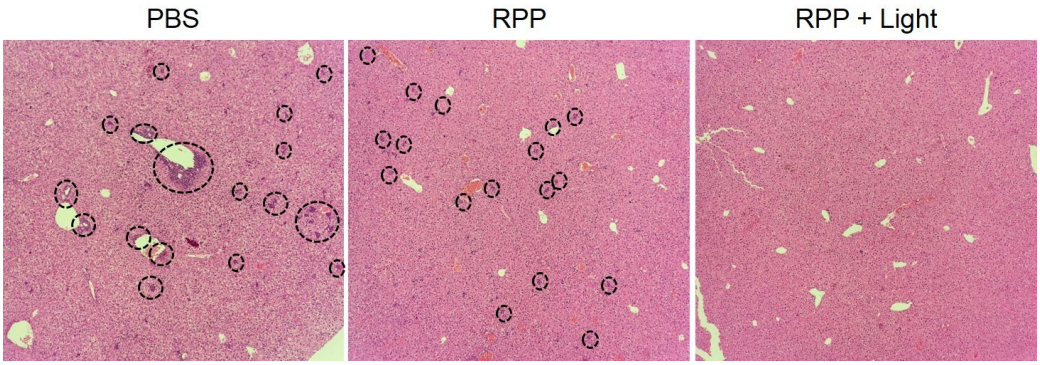

Fig. S17 H&E staining of liver sacrificed from the mice after treatment with PBS, RPP, RPP and light. The circle dots represented the metastatic tumors. 4 × magnification.