Electronic Supplementary Information

Smart Drug: A pH-responsive photothermal ablation agent for Golgi apparatus activated cancer therapy

Fengfeng Xue,[a] Ying Wen,[a] Peng Wei,[a] Yilin Gao,[a] Zhiguo Zhou,[b] Shuzhang Xiao[c] and Tao Yi* [a]

[a] Department of Chemistry and Collaborative Innovation Center of Chemistry for Energy Materials, Fudan University, 220 Handan Road, Shanghai 200433, China; Email: yitao@fudan.edu.cn
[b] The Education Ministry Key Lab of Resource Chemistry, Shanghai Key Laboratory of Rare Earth Functional Materials, Shanghai Normal University, Shanghai 200234, China
[c] Hubei Key Laboratory of Natural Products Research and Development, College of Chemistry and Life Science, China Three Gorges University, Hubei 443002, China

1. Experimental Procedures

1.1 Materials and characterization

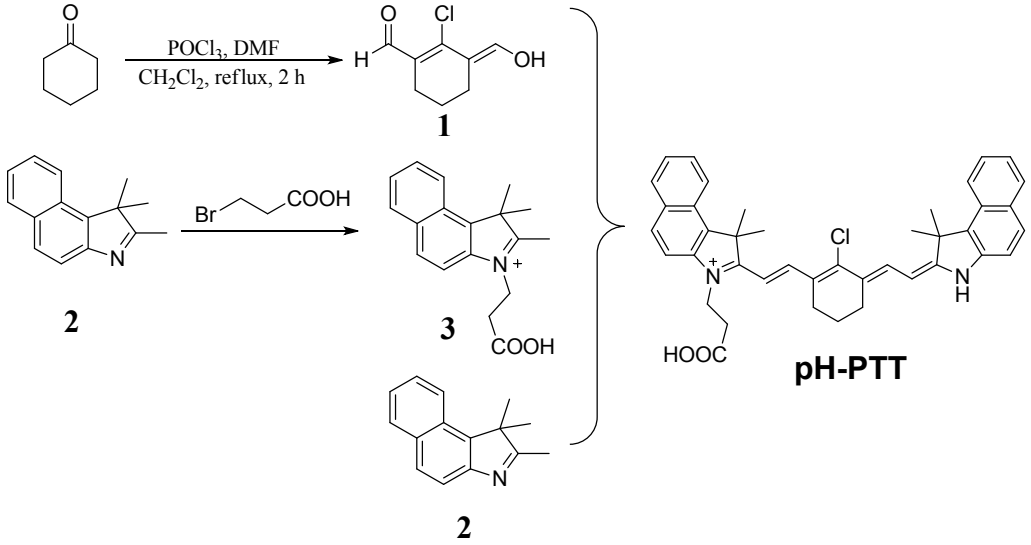
All the starting materials were obtained from commercial suppliers and used as received. Cyclohexanone, 1,2-dichlorobenzene and 3-bromopropionic acid were purchased from J&K Chemical Technology. $POCl_3$ was purchased from Energy Chemical Company. 1,1,2-trimethyl-1H-benz[e]indole was purchased from Beijing InnoChem Science & Technology Co., Ltd. All organic solvents were supplied from Strong Chemical Company (Shanghai, China). MTT, Calcien AM (AM), Propidium iodide (PI) and cell culture reagents were purchased from Invitrogen. (E)-2-chloro-3-(hydroxymethylene) cyclohex-1-enecarbaldhyde and 1-(2-carboxuethyl)-2,3,3-trimethyl-3H-indol-1-ium bromide were synthesized according to literature. [1]

1.2 Instruments

^1H NMR and ^{13}C NMR spectra were recorded with a Bruker DRX 500 spectrometer at 400 and 100 MHz, respectively. Proton chemical shifts are reported in parts per million downfield from tetramethylsilane (TMS). The high-resolution mass spectra (HR-MS) were measured on a Bruker Micro TOF II 10257 instrument. UV-visible spectra were recorded on a Shimadzu UV-2550 spectrometer. Steady emission experiments at room temperature were measured on an Edinburgh instrument FLS-920 spectrometer with a Xe lamp as an excitation source. The dynamic diameter was determined by a Zetasizer Nano-ZS (Malvern Instruments, UK). Confocal laser scanning microscopy (CLSM) was performed on an Olympus FV1000 confocal fluorescence microscope with a 60× oil-immersion objective lens.

1.3 The synthesis of pH-PTT and BSA-pH-PTT

1.3.1 The synthesis of pH-PTT



Scheme S1. The synthesis route of **pH-PTT**.

To a mixture of compound **1** (1.72 g, 10 mmol) in ethanol, compound **2** (2.09 g, 10 mmol) and compound **3** (2.82 g, 10 mmol) were added, then the resulting solution was stirred at 50ºC for 5 h under N_2 atmosphere. After cooling to room temperature, the reaction mixture was poured into brine (100 mL) and extracted with dichloromethane (3×30 mL). The organic phases were combined, dried over $MgSO_4$, filtered and concentrated to afford a red solid. The crude product was purified by flash chromatography with gradient elution (petroleum ether/ ethyl acetate of 10/1 to 2/1, v/v) to give **pH-PTT** as a red solid (2.00 g, 31.89 %). ^{1}H NMR (DMSO-*d6*, 400 MHz): δ 8.42 (d, 1H, J = 14.4), 8.29-8.21 (m, 3H), 8.05 (t, 4H, J = 6.4), 7.72 (d, 1H, J = 8.8), 7.62 (m, 3H), 7.54-7.47 (m, 3H), 6.44 (d, 1H, J = 14.4), 6.30 (d, 1H, J = 13.6), 4.50 (t, 2H, J = 7.2), 2.79-2.73 (m, 6H), 1.95 (s, 6H), 1.90-1.86 (m, 2H), 1.78 (s, 6H). ^{13}C NMR (DMSO-*d6*, 100 MHz) δ (ppm): 178.76, 172.16, 170.86, 158.88, 158.51, 158.14, 157.76, 146.62, 142.86, 139.88, 139.54, 133.47, 132.29, 131.40, 131.06, 130.26, 130.16, 129.81, 128.32, 127.61, 126.92, 126.10, 124.91, 124.42, 122.44, 122.05, 116.78, 113.90, 113.36, 111.41, 103.13, 99.54, 51.96, 49.99, 31.74, 27.08, 26.46, 25.84, 24.69, 23.60, 20.59. HR-MS (ESI Positive) calc. for $C_{41}H_{40}ClN_2O_2^+$, 627.2773 [M^+], found 627.2772.

1.3.2 The synthesis of pH-PTT

The synthesis of **BAS-pH-PTT** was according to the literature.[2] Briefly, 5 mg **pH-PTT** was firstly dispersed in 1 mL DMSO. 300 μL above **pH-PTT** solution was added into 100 mg BSA dispersed in 10 mL water and stirred for 1 h. **BSA-pH-PTT** nanoparticles were obtained after dialyzing the solution for 8 h in a dialysis bag (MWCO 3500 Da). The encapsulation efficiency of **pH-PTT** by BSA was nearly 100%, as evidenced by the fact that the filtrate was completely colorless even after a long period of dialysis.

1.4 Photo-thermal experiment in solution

The PBS solution (1 mL) containing **BSA-pH-PTT** with different concentrations (20, 40 μM) at pH 5.60 and 7.46 was put in a quartz cuvette with an optical path length of 1.0 cm. The cuvette was illuminated by 808 nm laser (GG-808-1500 MW) with a power density of 1.45 W/cm^2 for 300 seconds. The increase of the temperature was monitored by a digital thermocouple device. The photothermal conversion efficiency of **BSA-pH-PTT** was determined according to previous method as following equation.[3]

$$\eta = \frac{hA\Delta T_{\max} - Q_{Dis}}{I(1-10^{-A\lambda})}$$

Where η is the photothermal conversion efficiency, h is heat transfer coefficient, A is the surface area of the container, ΔT is ambient temperature of surroundings, I is incident laser power, A_λ is the absorbance of pH-PTT, Q_{Dis} is the baseline energy inputted by the sample cell.

1.5 Cell culture

The cancer cells (HeLa, KB and HePG-2) and normal cells (HGF-1, DC and HL-7702) were provided by the Institute of Biochemistry SIBS, CAS (China) and Cell Biology and Wuhan Procell life science & Technology Co. Ltd. HGF-1 were cultured in phenol-red-free Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% Pen-Strep and other cells were cultured in phenol-red-free Dulbecco's modified essential medium (RPMI 1640) supplemented with 10% fetal bovine serum (FBS) and 1% Pen-Strep. Cells were incubated at 37°C under 5% CO_2 and split with trypsin/EDTA solution (0.25%) as recommended by the manufacturer.

1.6 MTT assay

The methyl thiazolyl tetrazolium (MTT) assay was used to detect the cytotoxicity of **BSA-pH-PTT**. Cells were seeded in 96-well plates at a density of 1×10^4 cells per well, then cultured in 5% CO_2 at 37°C for 24 h. After the cells were incubated with **BSA-pH-PTT** at different concentrations (0, 1, 2.5, 5, 10, 12.5 and 25 μM in DMSO/cell culture medium with 10% FBS = 1: 49) for 12 and 24 h, respectively, MTT (20 μL, 5 mg/mL) was added to each well of the 96-well assay plate for 4 h at 37°C. After dimethyl sulfoxide (DMSO, 200 mL/well) was added, the absorbance was measured at 490 nm using a microplate reader. All samples were analyzed in triplicate.

1.7 Flow cytometry

To determine the uptake content of **BSA-pH-PTT**, HePG-2 and HL-7702 cells were incubated with **BSA-pH-PTT** (25 μM) in serum-free medium for 2 h after seeded in 6-well plates at 1×10^6 cells/well for 24 h. After rinsing with PBS for three times, cells were harvested by trypsinization. Then the cells were spun-down, re-suspended with PBS, and determined by flow cytometry (collecting by 755 nm filter, Ex: 633 nm). The data were analyzed by using Flwojo 7.6.1 software.

1.8 CLSM imaging

HePG-2 and HL-7702 cells (5×10^8 /L) were plated on 14 mm glass coverslips and allowed to adhere for 24 h. Then the cells were incubated with **BSA-pH-PTT** (25 μM) for 2 h at 37°C. The **BSA-pH-PTT** loaded cells were incubated with commercial dyes Lyso Tracker Green (200 nM), Mito Tracker Green (500 nM) or Golgi Green (200 nm) for 30 min. After incubation, the cells were washed three times with PBS. CLSM imaging was performed on an Olympus FV1000 confocal scanning system with a 60× oil-immersion objective lens. Red channel: 650 ± 25 nm for **pH-PTT** (25 μM), λ_{ex} = 543 nm; green channel: 530 ± 25 nm for Lyso tracker, Mito tracker, or Golgi green, λ_{ex} = 488 nm.

1.9 Cytotoxicity and photo-thermal cytotoxicity of BSA-pH-PTT

The photo-thermal cytotoxicity of **BSA-pH-PTT** was first measured by MTT method. The experiment was divided into seven groups: Control (C), laser only (L), **BSA-pH-**

PTT without laser (0), and with laser for 15, 30, 45 and 60 min. Before adding MTT, cells were first seeded in 96-well plates at a density of 1×10^4 cells per well, then cultured in 5% CO_2 at 37°C for 24 h. Cells were incubated with **pH-PTT** 25 μM for 2 h, then washed with cell culture medium. After that, cells were exposed to 808 nm laser (1.45 W/cm^2) for 0, 15, 30, 45 and 60 min and incubated for another 24 h.

The photo-thermal cytotoxicity of **BSA-pH-PTT** was also measured by AM-PI assay via CLSM imaging as the same method. The experiment was divided into seven groups: Control (C), laser only (L), **BSA-pH-PTT** without laser (0), and with laser for 15, 30, 45 and 60 min. HePG-2 and HL-7702 cells (5×10^8 /L) were plated on 14 mm glass coverslips and allowed to adhere for 24 h. Then the cells were incubated with **BSA-pH-PTT** (25 μM) for 2 h at 37 °C, washed with cell culture medium. After that, cells were exposed to 808 nm laser (1.45 W/cm^2) for 0, 15, 30, 45 and 60 min and incubated for another 24 h. The treated cell groups were further co-stained with 200 nM Calcine AM (AM) and 200 nM propidium iodide (PI) to distinguish live (green) and dead (red) cells for 8 min. CLSM imaging was performed on an Olympus FV1000 confocal scanning system with a 20× oil-immersion objective lens. Red channel: 617 ± 25 nm for PI (200 nM), λ_{ex} = 543 nm; green channel: 515 ± 25 nm for AM (200 nM), λ_{ex} = 488 nm

1.10 In vivo photothermal therapy on a HePG-2 Xenograft Tumor Model

HePG-2 cells were incubated to the nude mice (n = 4−6 weeks old). When the tumor volume reached 50-100 mm^3, the mice were intratumorally injected with **BSA-pH-PTT** (30 mg kg^{-1} body weight on dissolution in cell medium/DMSO 49/1) and then irradiated by 808 nm laser (1.45 W/cm^2) for 10 min. Control groups were as follows: control group 1, mice injected with the saline solution (50 μL); control group 2, mice injected with pH-PTT with the same dose; control group 3, mice irradiated by 808 nm laser (1.45 W/cm^2) for 10 min. After treatment for 20 days, the tumors were taken out, fixed with 4% paraformaldehyde, and embedded in paraffin for H&E and TUNEL staining according to the standard procedure.

2. Additional spectra and images

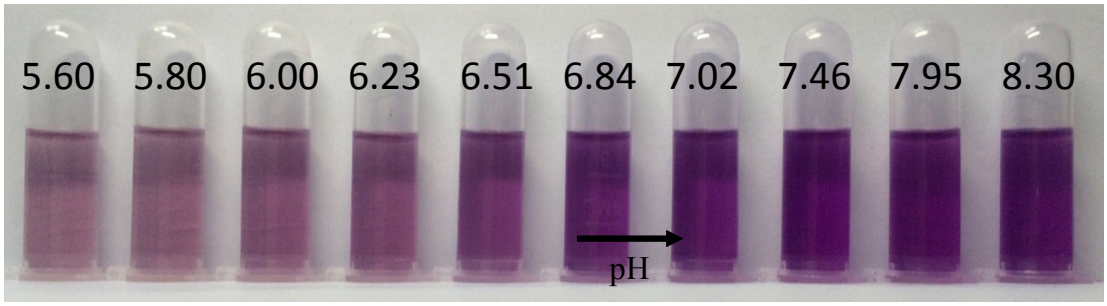


Fig. S1. The picture of **pH-PTT** (25 μM) in different pH from 5.60 to 8.30.

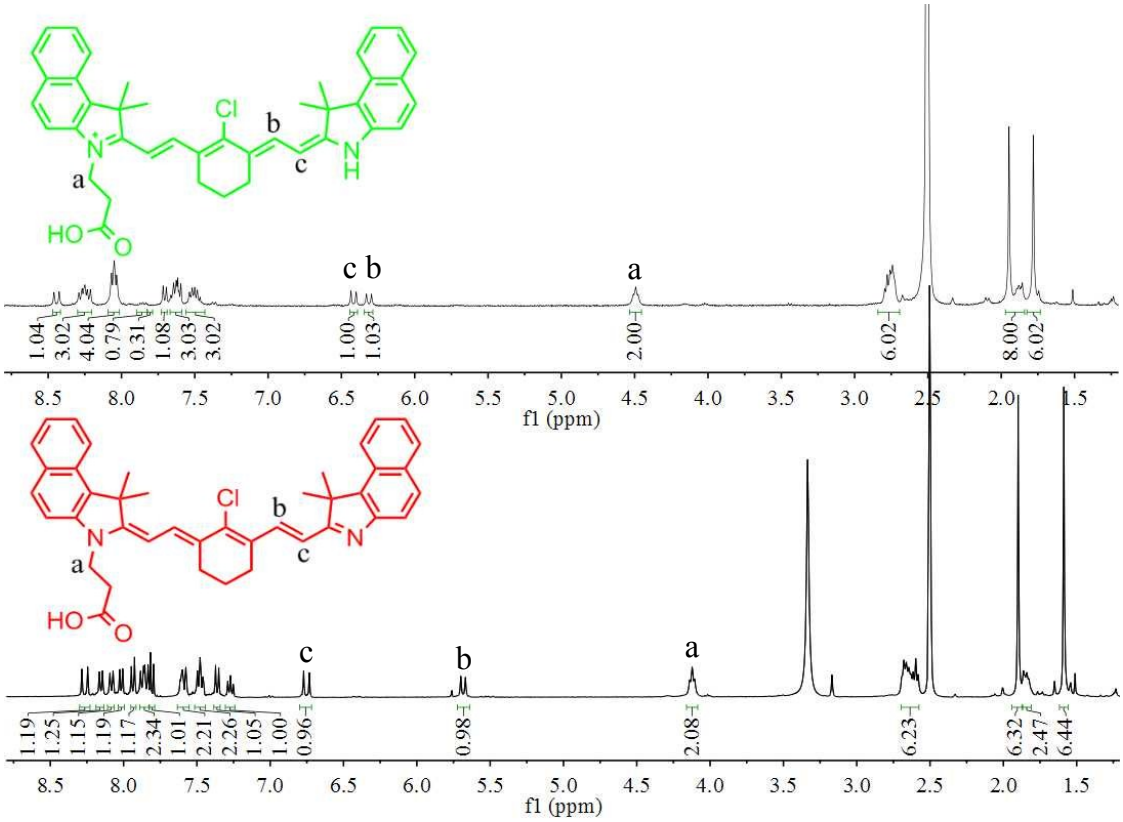


Fig. S2. The ^{1}H NMR of **pH-PTT** in acidic (upper) and alkaline (bottom) soluton. In acidic condition, H_a was shift to low field due to the formation of large conjugated structure that the N atom was trasformed to be electron deficiency and H_b, H_c trend to be consistent because of the large conjugated structure which uniformed the electron cloud density.

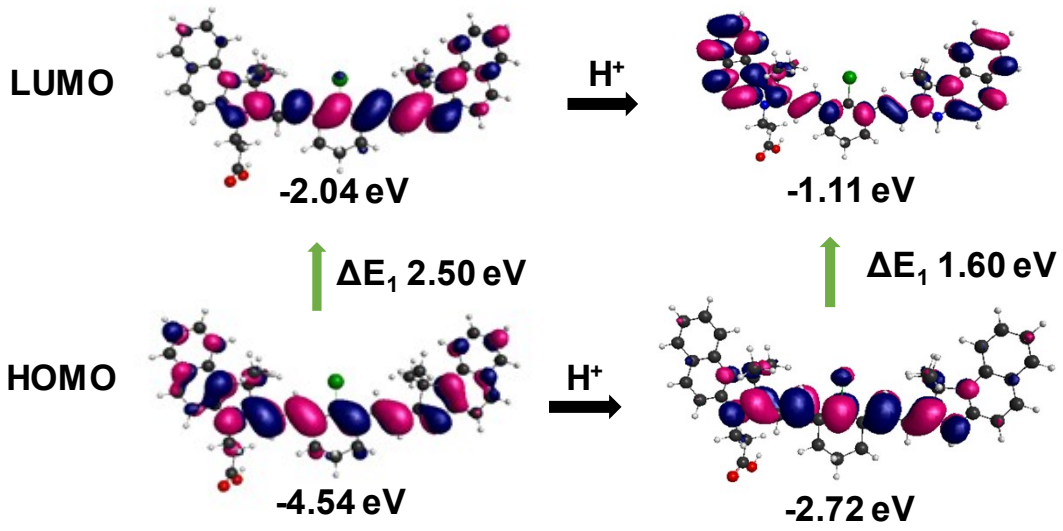


Fig. S3. Frontier molecular orbital plots in deprotonated and protonated forms of **pH-PTT**. The corresponding HOMO and LOMO energy levels and HOMO-LUMO energy gaps are indicated.

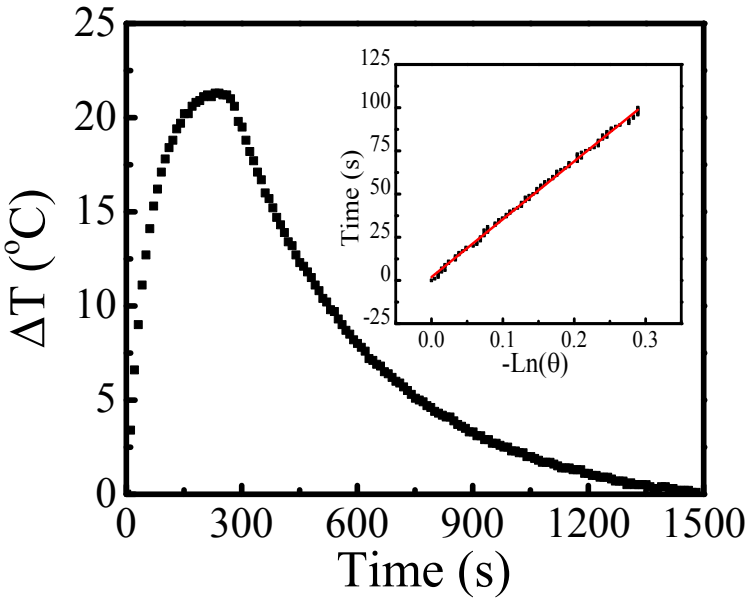


Fig. S4. The photo-thermal effect upon the irradiation of **BSA-pH-PTT** (20 μM) with NIR laser, in which the irradiation lasted for 5 min, and then the laser was shut off. Inset: time constant for the heat transfer from the system is determined to be hA = 334 s by applying the linear time data from the cooling period versus negative natural logarithm of driving force temperature, which is obtained from the cooling stage of panel.

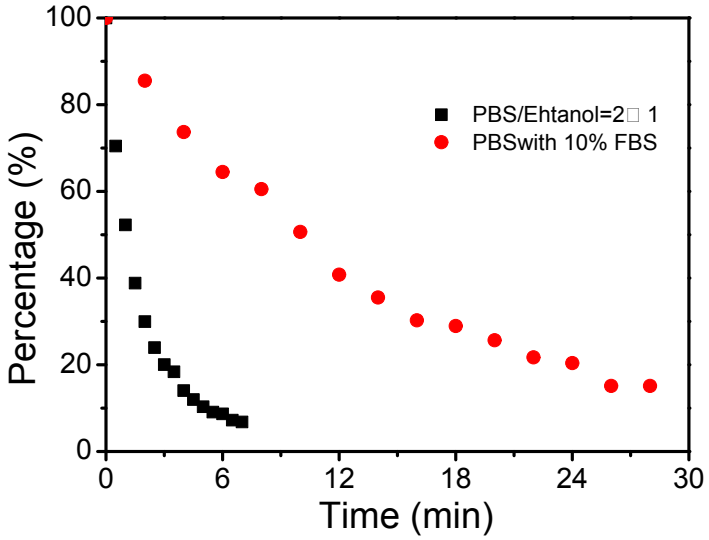


Fig. S5. Photo-stability of **pH-PTT** (25 μM) in PBS/ethanol and **BSA-pH-PTT** in PBS irradiated by 808 nm laser (2 W/cm^2) for different time. a) The percentage of absorbance decrease at 808 nm with irradiation time, which show enhanced photo-stability of **pH-PTT** in FBS medium.

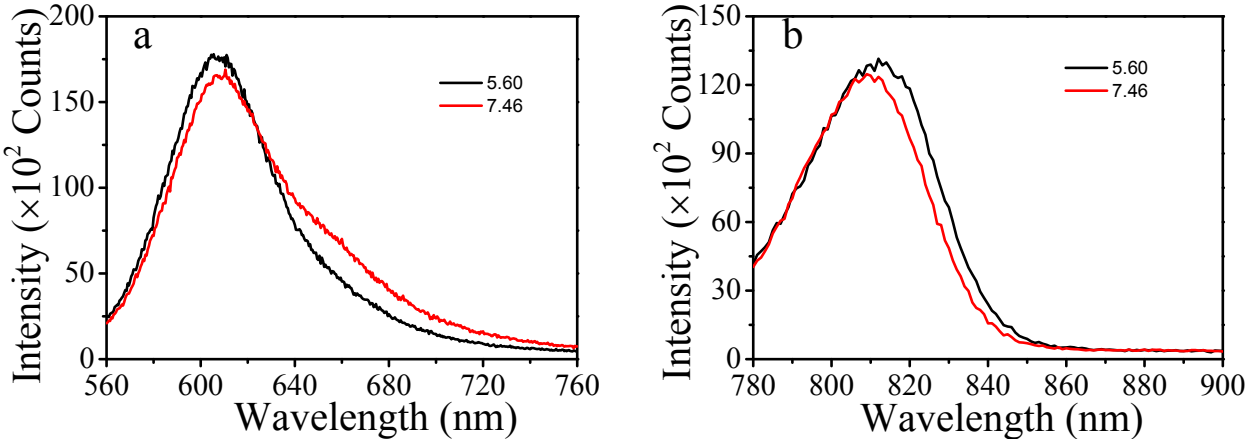


Fig. S6. The fluorescent spectra of 20 μM **BSA-pH-PTT** in PBS of different pH (5.60, 6.00, 7.46), excited by 540 nm (a) and 760 nm (b).

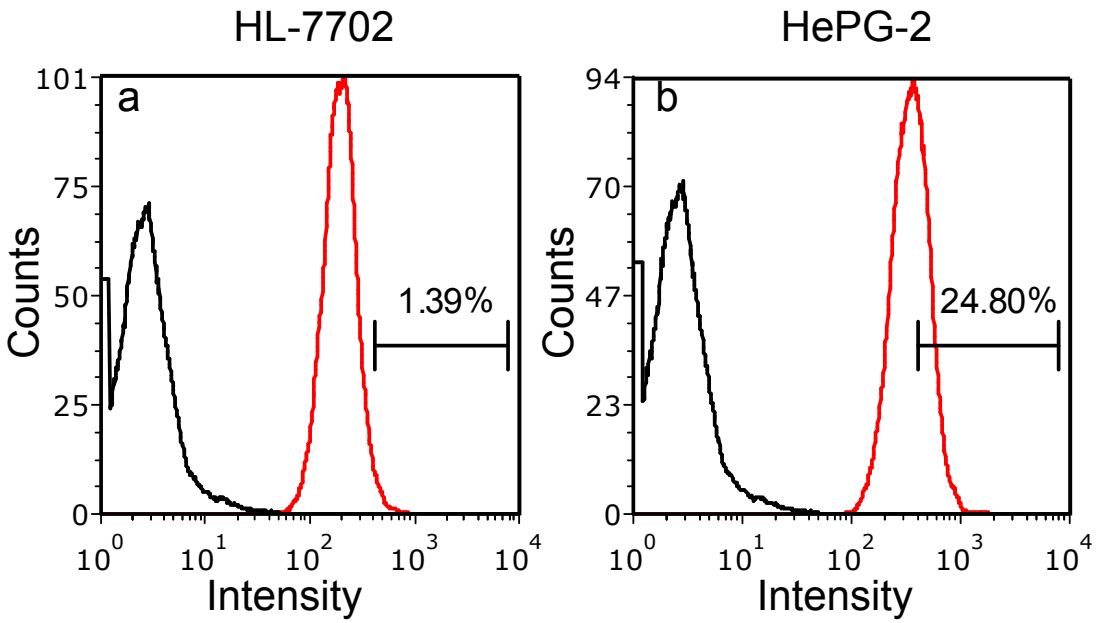


Fig. S7. Flow cytometry analysis of **BSA-pH-PTT** (25 μm) in HL-7702 cells (a) and HePG-2 cells (b) for 120 min

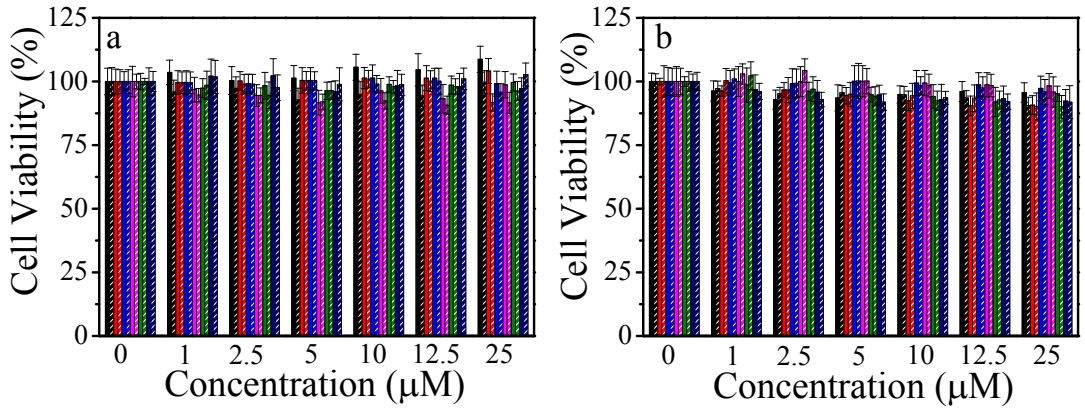


Fig. S8. a) The cell viability of **BSA-pH-PTT** at different concentration of **BSA-pH-PTT** (0, 1, 2.5, 5, 10, 12.5, 25 μM) in different cancer cells and normal cells for 12 h (no pattern) and 24 h (white pattern) measured by MTT assay. Cancer cells: HeLa (red), KB (magenta) and HePG-2 (navy); normal cells: HGF-1 (black), DC (blue) and HL-7702 (olive). b) The 12 h (no pattern) and 24 h (white pattern) cell viability of decomposition product of **BSA-pH-PTT** at different concentration (0, 1, 2.5, 5, 10, 12.5, 25 μM) in cancer cells: HeLa (red), KB (magenta) and HePG-2 (navy) and normal cells: HGF-1 (black), DC (blue) and HL-7702 (olive) measured by MTT assay.

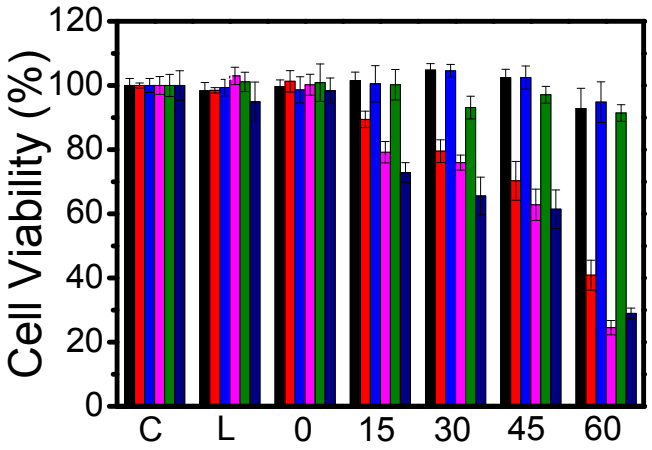


Fig. S9. Cell viability of cancer cells: HeLa (red), KB (magenta) and HePG-2 (navy) and normal cells: HGF-1 (black), DC (blue) and HL-7702 (olive) treated with **BSA-pH-PTT** (25 μM) irradiated by 808 nm laser (1.45 W/cm^2), the experiment was divided into seven groups: Control (C), laser only (L), **BSA-pH-PTT** without irradiation (0) and irradiated with laser for 15, 30, 45 and 60 min.

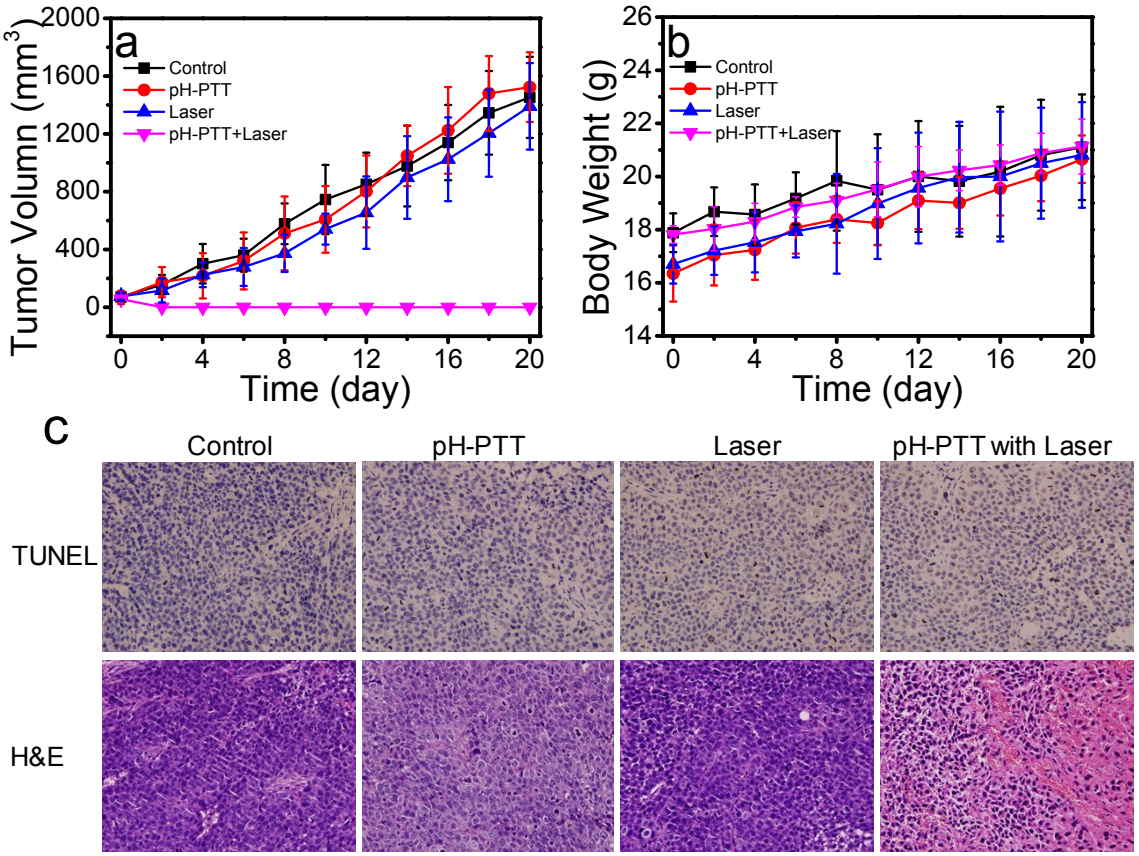


Fig. S10. In vivo measurement of BSA-pH-PTT. (a) The changes of tumor volume after 20-day treatment, (b) The changes of mouse body weight after 20 days treatments, (c) TUNEL and H&E staining images of representative specimens at 400× magnification after PTT for 2 days.

References

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