Electronic Supplementary Information (ESI)

GSH-triggered Release of Sulfur Dioxide Gas to Regulate Redox Balance for Enhanced Photodynamic Therapy

Yu Zhang[a], Wei Shen[b], Peng Zhang[b,c], Li Chen[a*],Chunsheng Xiao[b,c*]

[a.] Department of Chemistry, Northeast Normal University, Changchun 130024, P. R. China. E-mail: chenl686@nenu.edu.cn.

[b.] Key Laboratory of Polymer Ecomaterials, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun 130022, P. R. China. E-mail: xiaocs@ciac.ac.cn.

[c.]Jilin Biomedical Polymers Engineering Laboratory, Changchun, 130022, P. R. China

Experimental

1. Materials and methods

1.1 Materials

L-Glutamic acid was purchased from Shanghai Huishi Biochemical co., Ltd. Triphosgene was obtained from Nanjing Xie Zun Medicine co., Ltd. Propargyl alcohol and sodium azide were obtained from Nanjing Duodian Chemical Co., Ltd. Methoxy poly(ethylene glycol) (mPEG, M_n=5000 g mol^{-1}), 9,10-anthracenediylbis(methylene)dimalonic acid (AMDA), 4′, 6-diamidino-2-phenylindole dihydrochloride (DAPI), reduced L-glutathione (GSH) and 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Aldrich. γ-Propargyl-L-glutamate N-carboxy anhydride (PLG-NCA)[1] and the amino-terminal mPEG (mPEG-NH_2)[2] were synthesized as described in our previous works.[3, 4] Pyrrole was obtained from Aladdin Reagent (Shanghai) Co., Ltd. Trifluoroacetate and L-Cysteine (Cys) were purchased from Shanghai Macklin Biochemical Co., Ltd. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Thermo Fisher Scientific (Shanghai, China). N-(3-azidopropyl)-2,4-dinitrobenzenesulfonamide (AP-DNs) was synthesized according to our previous work.[5] Tin dichloride and copper (II) sulfate pentahydrate was obtained from Xilong Scientific Co., Ltd. (Guangzhou). Tetrahydrofuran (THF) and *N*, *N*-Dimethylformamide (DMF) were purified by a solvent purification system (MB SPS-800, MBRAUN, Germany). The deionized water was prepared by the Milli-Q system (Millipore Co., Billerica, MA, USA). The SO_2 probe 7-diethylaminocoumarin-3-aldehyde (DEACA) was prepared according to the procedure reported before.[6] The other reagents and solvents were purchased from Sinopharm Chemical Reagent Co, Ltd. and used as obtained.

1.2 Characterization

^{1}H nuclear magnetic resonance (^{1}H NMR) spectra were recorded on Bruker AV-300 or Bruker AV-500 NMR spectrometer in deuterated dimethyl sulfoxide (DMSO-d_6), deuterated methanol (CD_3OD), deuterated water (D_2O), or deuterated chloroform ($CDCl_3$). The molecular weights and polydispersities ($Đ$) of the obtained polymers were determined by gel permeation chromatography (GPC) equipped with a Waters 515 HPLC pump and a Waters 2414 Refractive Index Detector. Dynamic light scattering (DLS) measurement was performed on a WyattQELS instrument with a vertically polarized He−Ne laser (DAWN EOS, Wyatt Technology Co., USA). The scattering angle was fixed at 90°. Transmission electron microscopy (TEM) measurement was performed on a JEOL JEM-1011 transmission electron microscope (Tokyo, Japan) with an accelerating voltage of 100 kV. The fluorescence intensity measurement was performed on a Fluorescence Master System (Photon Technology International, USA). The absorbance spectra were measured with an ultraviolet-visible (UV-Vis) spectrophotometer (Lambda 365, PerkinElmer). The *ex vivo* fluorescence imaging was obtained by Maestro 500FL *in vivo* optical imaging system.

2. Synthesis and methods

2.1 Synthesis of mPEG-PPLG

The synthesis of methoxy poly(ethyleneglycol)-*block*-poly(γ-propargyl-L-glutamate) (mPEG-PPLG) was realized by ring-opening polymerization of γ-propargyl-L-glutamate *N*-carboxy anhydride (PLG-NCA) with mPEG-NH_2 as macroinitiator. First, 80 mL of anhydrous DMF was added to the bottle with dried mPEG-NH_2 (4.1 g, 0.82 mmol amino groups), to which PLG-NCA (4.3 g, 20.36 mmol) dissolved in 30 mL of anhydrous DMF was added. The polymerization was performed at room temperature for 3 days. The solution was purified by dialyzing in a dialysis bag (MWCO 3500 Da) against deionized water for 3 days. The final product mPEG-PPLG was obtained

after lyophilization (yield: 60 %). ^{1}H NMR (500 MHz, DMSO-d_6) δ 4.70 (s, 32H), 4.36-4.18 (m, 16H), 3.53 (s, 452H), 2.48-2.35 (m, 32H), 1.99-1.77 (m, 32H).

2.2 Synthesis of mPEG-PLG(DNs) via click reaction

The mPEG-PPLG (0.77 g, containing 1.6 mmol alkyne pendants) and AP-DNs (1.06 g, 3.2 mmol) was dissolved in 30 mL of DMSO. Bubbling the solution with nitrogen for 20 min. Sodium ascorbate (0.16 g, 0.82 mmol) was added to the flask under continuous nitrogen (N_2) flushing and copper sulfate pentahydrate (0.09 g, 0.36 mmol) was added after five minutes. Then reaction mixture was stirred under nitrogen (N_2) atmosphere for 72 h at 40 °C. After that, the solution was dialyzed (MWCO 3500 Da) against DMSO for 2 days and subsequently deionized water for 3 days. After lyophilization, the mPEG-PLG(DNs) was obtained (yield: 56.7 %). ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.88 (s, 16H), 8.60 (s, 32H), 8.20 (d, J = 9.0 Hz, 16H), 8.07 (s, 16H), 5.07 (s, 32H), 4.35 (s, 32H), 4.21 (s, 16H), 3.49 (s, 452H), 2.96 (s, 32H), 2.33 (d, J = 23.5 Hz, 32H), 1.98 (s, 32H), 1.92-1.74 (m, 32H).

2.3 Synthesis of 5-(4-aminophenyl)-10,15,20-triphenylporphyrin (Por-NH_2)

The synthetic route is shown in Scheme 2. Firstly, *meso*-tetraphenylporphyrin (TPP) was synthesized. Freshly distilled pyrrole (200 mM, 14 mL) was added to a mixture of 20 mL benzaldehyde and 300 mL propionic acid, and heat up to 135 °C. After refluxing for 30 min, it was cooled to room temperature, filtered and washed with methanol and hot water, and vacuum dried to remove excess solvent. Finally, the crude product was purified by recrystallization in dichloromethane and methanol. Next, 5-(4-nitrophenyl)-10,15,20-triphenylporphyrin (Por-NO_2) was synthesized. TPP (0.5 g, 0.816 mmol), sodium nitrite (100 mg, 1.45 mmol), and TFA (50 mL) were stirred at room temperature for 3.5 min. The reaction was then quenched with water and the

Por-NO_2 was extracted with dichloromethane. After evaporation of the solvent, the crude product was purified by column chromatography on silica gel. Finally, the 5-(4-aminophenyl)-10,15,20-triphenylporphyrin (Por-NH_2) was synthesized. Under a nitrogen atmosphere, Por-NO_2 was mixed with concentrated hydrochloric acid and tin dichloride overnight. After that, the reaction solution is neutralized by aqueous ammonia and extracted with dichloromethane. The solvent is removed by rotary distillation and the solid was purified by column chromatography on silica gel to give Por-NH_2 (yield: 39%). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.94 (d, *J* = 4.7 Hz, 2H), 8.83 (s, 6H), 8.21 (m, 6H), 7.99 – 7.97 (d, *J* = 7.7 Hz, 2H), 7.78 – 7.70 (m, 9H), 7.04 – 7.02 (d, *J* = 8.25, 2H), 3.98 (s, 2H), -2.75 (s, 2H).

1.4 Preparation of NP-DN, NP-Por, and NP-DN-Por

The blank mPEG-PLG(DNs) micelles (NP-DN) were prepared by a membrane dialysis method. Briefly, 50 mg of mPEG-PLG(DNs) was dissolved in 2 mL of DMF, which was then added dropwise into 20 mL of deionized water under stirring. The mixture was stirred for 2 h at room temperature, and then DMF was removed by dialysis (MWCO 3500 Da) against deionized water for 12 h (the water was replaced every 2 h). After lyophilization, the NP-DN were obtained.

The Por-NH_2-loaded mPEG-PPLG micelles (NP-Por) and Por-NH_2-loaded mPEG-PLG(DNs) micelles (NP-DN-Por) was prepared by a similar method as described above. The loading content (LC) and loading efficiency (LE) of Por-NH_2 were determined by UV-Vis spectrophotometer and calculated according to the following formulas:

LC (%) = (weight of loaded Por-NH_2 / weight of NP-DN-Por) × 100%

LE (%) = (weight of loaded Por-NH_2 / weight of feeding Por-NH_2) × 100%

1.5 Determination of SO_2 production (characterized by HSO_3^-) in the thiol-triggered SO_2

release process

The generation of SO_2 was detected by using 7-diethylaminocoumarin-3-aldehyde (DEACA) as the probe. First, NP-DN with different concentrations were dissolved in phosphate buffered saline PBS (pH = 5.8), and then 20 molar equivalents of Cys and 5 μM of DEACA were added to the above solution. After further reaction for 5 min, the fluorescence intensity of all solution was measured by fluorescence spectrophotometer at different time points (λ_{ex}: 390 nm).

1.6 Determination of primary amines in the thiol-triggered SO_2 release process

The primary amines generation property of NP-DN was identified using the fluoresceamine by fluorescence spectrometer according to the reported literature.[7]

1.7 Detection of ROS in solution

9,10-anthracenediylbis(methylene)dimalonic acid (ABDA) was used as an indicator to investigate the ROS generation of Por-NH_2. 40 μL of the ABDA solution (40 μg/mL) was added to 30 μg/mL NP-DN-Por solution, and 30 μg/mL NP-DN solution. Then, 1 mL of the mixed solution in a 2 mL Centrifuge tube was irradiated with light (40 mW/cm^2, 400–700 nm). The absorbance of ABDA at 380 nm under different illumination times was recorded on ultraviolet-visible (UV-Vis) spectrophotometer.

1.8 Cellular uptake

The 4T1 cells were purchased from from Shanghai Bogoo Biotechnology Co. Ltd. (China). The cells were seeded in a 6-well plates at a density of 1.0×10^5 cells per well, and cultured in 1.8 mL DMEM containing 10% FBS at 37 °C, 5% CO_2 atmosphere for 24 h. Then, NP-DN-Por dissolved in 200 μL of PBS were added and cultured for another 1 h or 4 h. After being washed for five times with PBS, cells were fixed in 4% formaldehyde for 20 min. Subsequently, the cell nuclei stained

with DAPI of cells were observed via confocal laser scanning microscopy (CLSM).

1.9 Intracellular singlet oxygen generation

Intracellular singlet oxygen generation was conducted by confocal laser scanning microscopy (CLSM) and flow cytometry analysis (FCA) through using DCFH-DA as the probe.

For FCA, the 4T1 cells were seeded in a 6-well plates at a density of 3.0×10^5 cells per well, and cultured in 1.8 mL DMEM containing 10% FBS at 37 °C, 5% CO_2 atmosphere for 24 h. Then, NP-DN, NP-Por, NP-DN-Por dissolved in 200 μL of PBS were added and cultured for another 4 h. DCFH-DA (final concentration 10 μM) was added and the cells were cultured for 20 min. Following a 10 min of light irradiation (40 mW/cm^2, 400–700 nm), the cells were washed five times with PBS. The FCA was performed on a Guava EasyCyte™ 12 Flow Cytometer (Millipore, Billerica, MA, USA). Cells without drug treatment were set as control.

For CLSM observation, the 4T1 cells were seeded in a glass-bottomed dish at a density of 5.0×10^4 cells, and cultured in 1.8 mL DMEM containing 10% FBS at 37 °C, 5% CO_2 atmosphere for 24 h. Then, NP-DN, NP-Por, NP-DN-Por dissolved in 200 μL of PBS were added and cultured for another 4 h. DCFH-DA (final concentration 10 μM) was added and the cells were cultured for 20 min. Following a 10 min of light irradiation (40 mW/cm^2, 400–700 nm), the cells were washed for five times with PBS and observed by CLSM as soon as possible.

1.10 Cytotoxicity measurements

The cytotoxicities of NP-DN, Por-NH_2, NP-Por, NP-DN-Por were evaluated by MTT assay. The 4T1 tumor cells and L929 normal cells were seeded in a 96-well plate at a density of 5000 cells per well, and cultured in 180 μL DMEM containing 10% FBS at 37 °C, 5% CO_2 atmosphere for 24 h. Then, NP-DN, Por-NH_2 (containing 1% DMSO), NP-Por, NP-DN-Por dissolved in 20 μL of PBS

were added to 4T1 cells and NP-DN-Por dissolved in 20 μL of PBS were added to L929 cells. For determination of the toxicity in dark, the plates were incubated for another 72 h. For determination of the toxicity under light irradiation, the cells were subjected to a 10 min of light irradiation (40 mW/cm^2, 400-700 nm) followed by a 4 h of incubation and then incubated for another 72 h. The cell viability was evaluated by using the MTT assay. The results at 490 nm were measured by using a microplate reader (Bio-Rad 680 microplate reader). Cells treated with PBS were set as control.

1.11 Animal model.

Female Balb/C mice (6–8 weeks) were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). All animals were treated according to the guidelines outlined in the Guidelines for the Care and Use of Laboratory Animals and all animal experimental procedures were approved by the Animal Care and Use Committee of Jilin University. 4T1 cells (2.0×10^6) suspended in 0.1 mL of PBS were injected into the mammary gland of mice to establish 4T1 tumor-bearing mouse model in this study.

1.12 *Ex vivo* fluorescence imaging of Por-NH_2

When the tumors of mice grew to about 70 mm^3, 4T1 tumor bearing mice were intravenously injected with NP-DN-Por (containing 8.0 mg kg^{-1} Por-NH_2). After 1, 2, 4, 6, and 10 h, the tissues (including heart, liver, spleen, lung, kidney, and tumor) were collected and imaged by Maestro 500FL *in vivo* optical imaging system. (Excitation light wavelength: 635 nm, filter wavelengths for acquisition: 665−900 nm).

1.13 *In vivo* antitumor efficiency

When the tumors of mice grew to 50–100 mm^3, the mice were randomly divided into 6 groups, which intravenously injected with NP-Por, NP-DN-Por with light irradiation, PBS, NP-DN, NP-

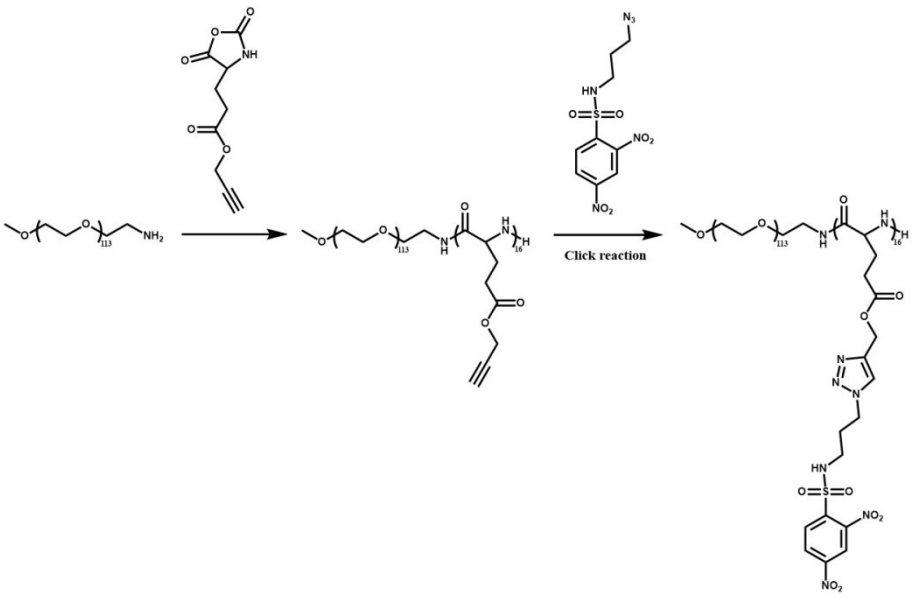
Por, or NP-DN-Por without light irradiation. The intravenously injections were performed on days 0, 4, 8, 12 and the doses of Por-NH_2 were about 8.0 mg kg^{-1} per mouse. 4 h after the injection, the NP-Por, NP-DN-Por groups were exposed to light irradiation (650 nm, 200 mW/cm^2) for 10 min, and the other groups did not expose to light irradiation. The mice weights and tumor volume were measured every two days, and the tumor volume (V; mm^3) was calculated as $V = 0.5 \times a \times b^2$, where a (mm) is the tumor length and b (mm) is the tumor width. The tumor inhibition rate (%) = ($V_{control}$ - V_{sample}) / $V_{control} \times 100\%$,and the $V_{control}$ and V_{sample} represented the tumor volumes in control and sample groups, respectively.

1.14 Histological analyses

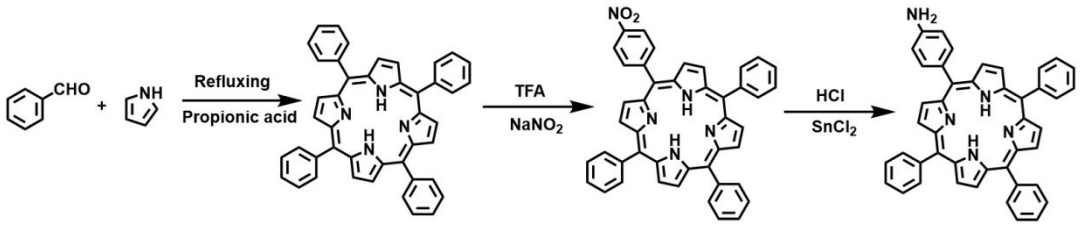
After the treatment is over, the mice were sacrificed. The heart, liver, spleen, lung, kidney and tumor of each group were excised and fixed in 4% paraformaldehyde for 24 h, embedded in paraffin and were sliced at 5 μm for haematoxylin and eosin (H&E) staining and *in situ* cell apoptosis analyses (TUNEL).

1.15 Statistical Analysis

All experiments were performed at least three times, and the data are shown as mean ± standard deviation (SD). All results were analyzed by one-way ANOVA. *p <0.05 is considered statistically significant. **p < 0.01 and ***p <0.001 are considered highly significant.



Scheme S1 Synthetic route of mPEG-PLG(DNs).



Scheme S2 Synthetic route of Por-NH_2.

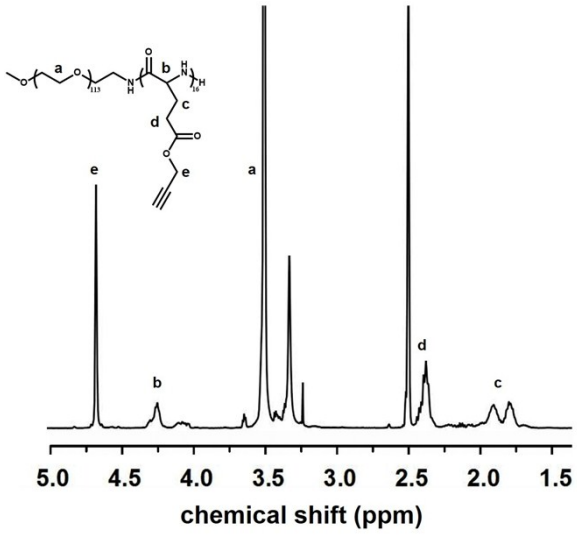


Fig. S1 Typical ^{1}H NMR spectrum of mPEG-PPLG in DMSO-d_6.

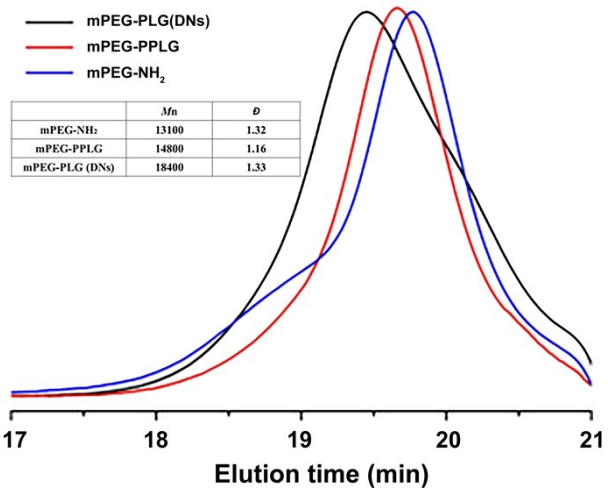


Fig. S2 GPC analyses of mPEG-PLG(DNs), mPEG-PPLG and mPEG-NH_2.

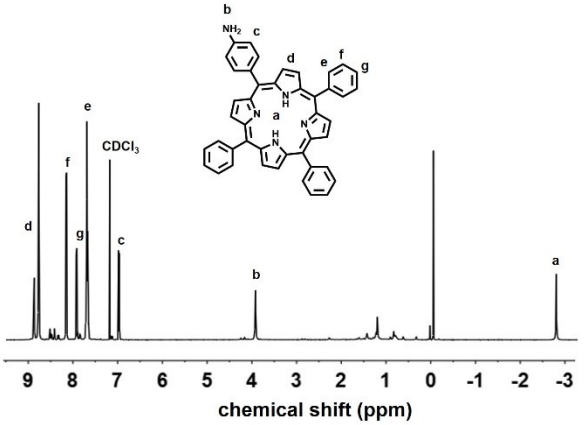


Fig. S3 Typical ^{1}H NMR spectrum of Por-NH_2 in $CDCl_3$.

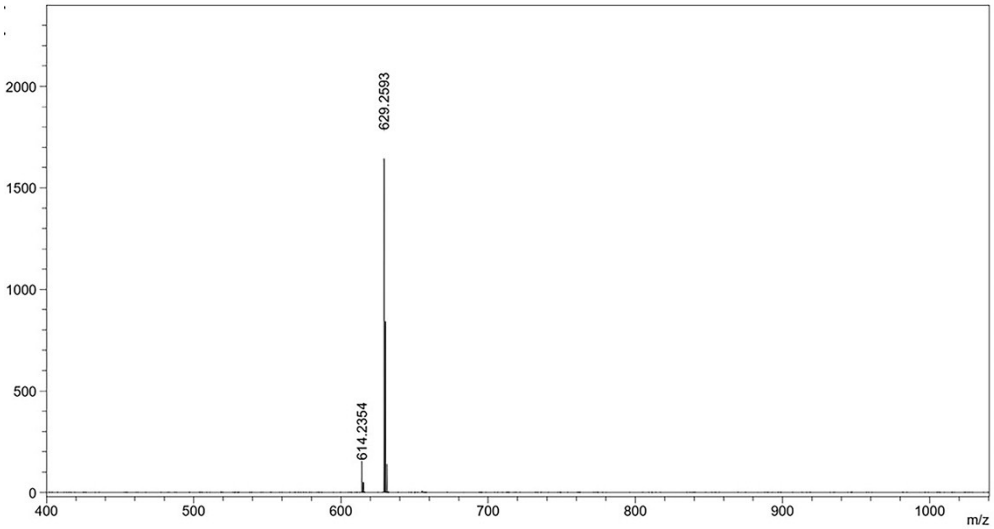


Fig. S4 High resolution electrospray ionization mass spectrometry (ESI-MS) of Por-NH_2

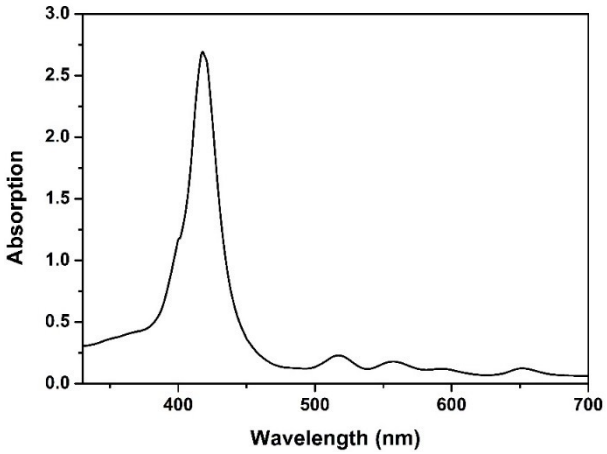


Fig. S5 The UV-Vis absorption spectrum of Por-NH_2.

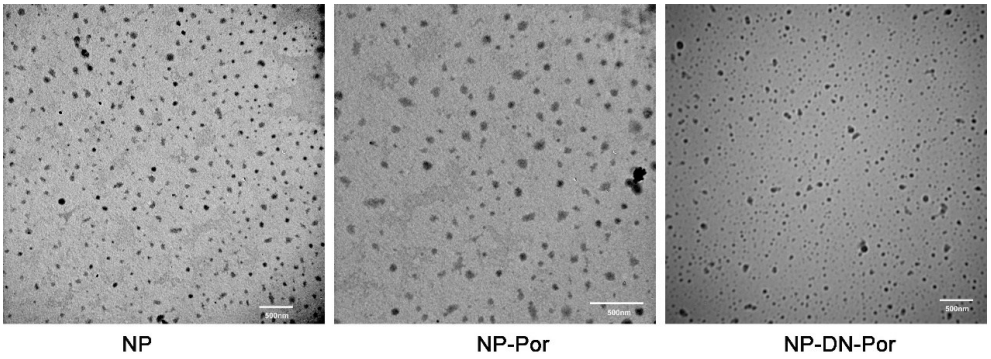


Fig. S6 The TEM images of NP, NP-Por and NP-DN-Por (Scale bar: 500 nm).

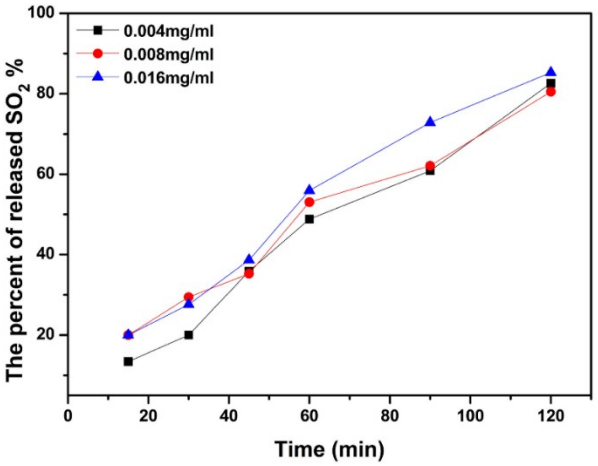


Fig. S7 Percent of released SO_2 in different concentrations of NP-DN solution.

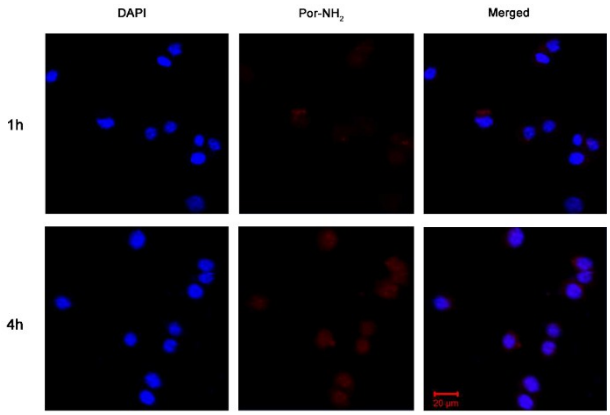


Fig. S8 The cellular internalization of NP-DN-Por in 4T1 cells at 1 h and 4 h by CLSM.

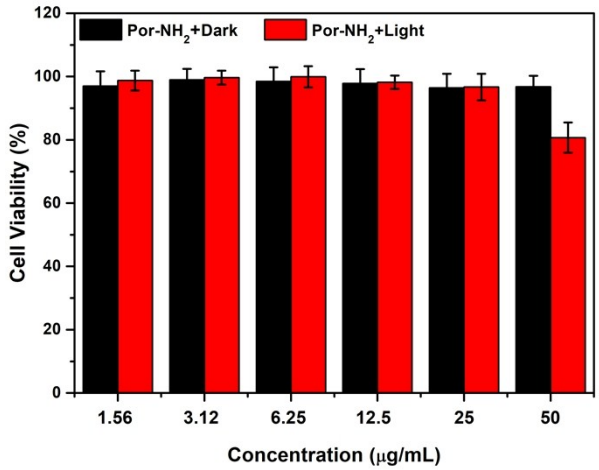


Fig. S9 Viability of 4T1 cells treated with Por-NH_2 in light or dark conditions for 72 h.

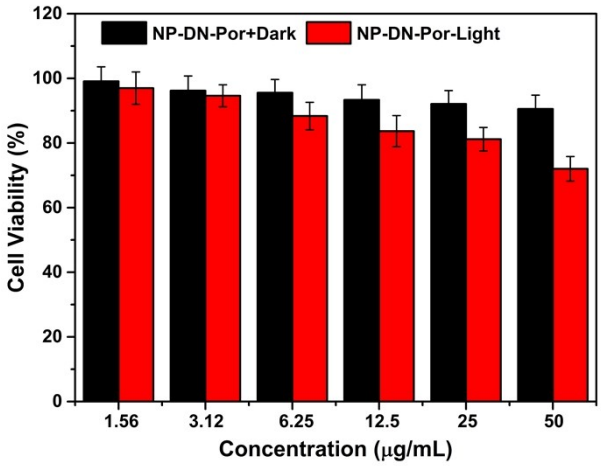


Fig. S10 Viability of L929 cells treated with NP-DN-Por in light or dark conditions for 72 h.

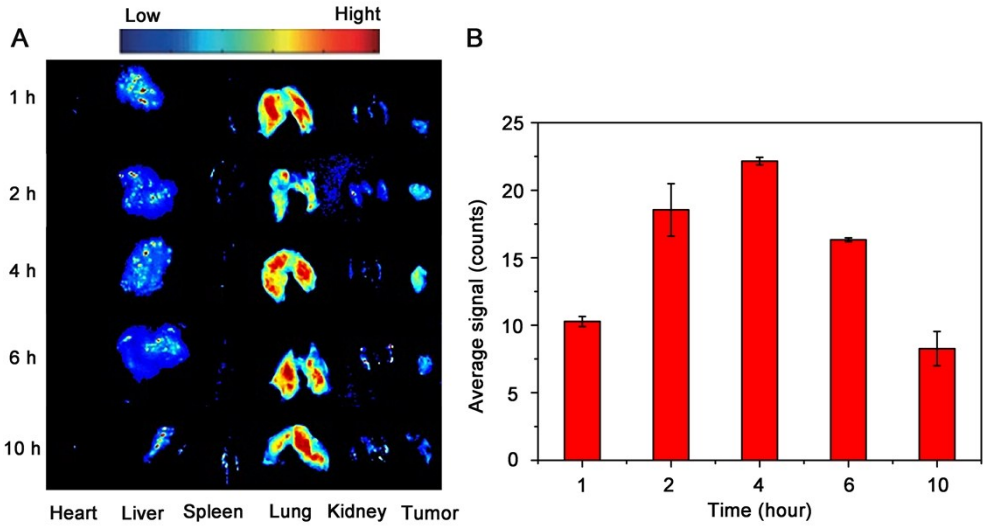


Fig. S11 (A) Fluorescence imaging of mouse tissues at 1, 2, 4, 6, and 10 h post-injection. (B) Average signals were counted from the tumors. This result suggests that the optimal time for illumination is at 2-4 h post-injection. Thus, in our work, the illumination was performed at 4 h post-injection.

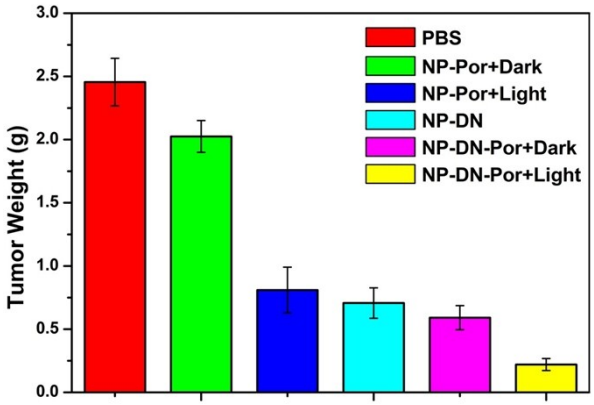


Fig. S12 Average tumor weight of mice after treatment. Data were presented as mean ± SD (n = 5).

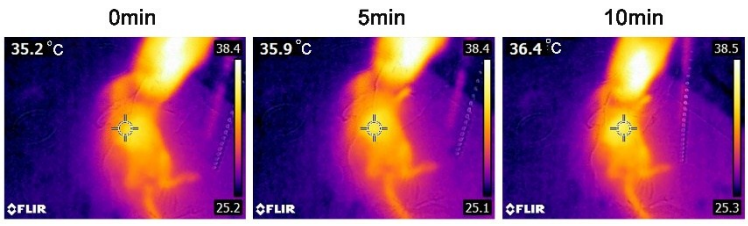


Fig. S13 Temperature detection of tumor sites in mice at 0, 5, and 10 min under light irradiation.

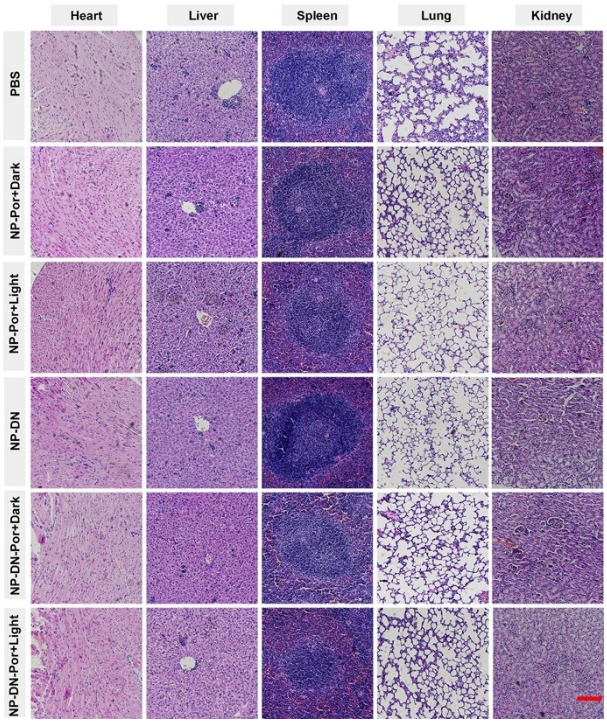


Fig. S14 H&E staining of the major organs in mice after treatment. Scale bar: 100 μm.

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