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Supporting Information

Enhanced photodynamic therapy based on an amphiphilic branched copolymer with pendent vinyl groups for simultaneous GSH depletion and Ce6 release

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Experimental section

Materials

Ethylene glycol dimethacrylate (EGDMA, 98%), Triethylamine (TEA, 99%), ascorbic acid (≥99.7%) and 2-butanone (≥99.5%) were purchased from Aladdin industrial corporation, China. Copper (II) chloride ($CuCl_2$, 99%), 2-bromoisobutyryl bromide (98%), sodium carbonate (Na_2CO_3, 99.8%), magnesium sulfate anhydrous ($MgSO_4$, 98%), hydrochloric acid (HCl, wt% = 36~38) were all purchased from Sinopharm Chemical Reagent Co., LTD., China. The solvents N,N-dimethylformamide (DMF, ≥99.5%), tetrahydrofuran (THF, ≥99%), diethyl ether (≥99.7%), dichloromethane (DCM, ≥99.5%), petroleum ether were purchased from Shanghai LingFeng Chemical Reagent Co., LTD. Poly(ethylene glycol) methyl ether (PEG_{45}-OH, 2000 g mol^{-1}, Sigma-Aldrich), reduced *L*-glutathione (GSH, ≥98%, Sigma-Aldrich) and GSH and oxidized glutathione disulfide (GSSG) assay Kit (Beyotime) were used as received. A dialysis membrane from Spectrum with MWCO = 6 to 8 kDa was used to purify the polymer and for self-assembly.

Characterization

The ^{1}H-NMR spectra were recorded at 400 MHz, using a German BRUKER AVANCE 400 spectrophotometer in deuterium chloroform ($CDCl_3$) and the internal standard is tetramethylsilane (TMS). The gel permeation chromatograph (GPC) was from Waters with the model of 1515. The standard sample is narrowly distributed polystyrene with a mobile phase of tetrahydrofuran (THF) at a flow rate of 1 mL/min. Dynamic light

scattering (DLS) measurements were carried out using a BECKMAN COULTER Delasa Nano C particle analyzer at room temperature. The UV-Vis spectras were recorded on UV-2550 UV-Vis spectrophotometer, which produced by SHIMADZU. The fluorescence spectras were performed using an FL-4500 fluorescence spectrophotometer, which produced by HITACHI. The transmission electron microscopy (TEM) test was carried out using a JEOL JEM-1400 transmission electron microscope at 100 kV voltage.

Prepare of PEG2K-Br macroinitiator

The procedure of preparation the PEG2K-Br macroinitiator was based on a reported article. PEG_{45}-OH (10.0 g, 5 mmol) and TEA (2.78 mL, 20 mmol) were added to a 250 mL three neck round bottom flask and dissolved with 100 mL DCM. Then, the mixture was bubbled with nitrogen for 10 minutes to remove residual moisture and air. 2-bromoisobutyryl bromide (2.48 mL, 20 mmol) was dissolved in 80 mL DCM. The solution was added dropwise to the flask from a constant pressure funnel. For the first 6 h, the mixture was stirred in an ice bath, for another 24 h, the mixture was stirred at room temperature. Reaction device was wrapped by silver paper to avoid light. After the reaction completed, the by-produced salt was removed by negative-pressure filtration, and the solution was evaporated to remove half of the solvent. And, washing with diluted hydrochloric acid (HCl, 0.1 M), sodium carbonate aqueous solution (Na_2CO_3, 0.1 M), and deionized water for 3 times. The organic solution was dried with anhydrous magnesium sulfate, then filtered, and precipitated into a 10-fold excess of ether for twice. The product was obtained by filtration under reduced pressure and was dried under vacuum at 40 ^oC. The purified macroinitiator was determined by ^1H NMR spectroscopy in $CDCl_3$ and the terminal hydroxyl group on the PEG_{45}-OH precursor was proved to be fully esterified: 1.97 ppm (6H), 3.40 ppm (3H), 4.20 ppm (2H).

Polymerization of amphiphilic block copolymers of PEG-*b*-PEGDMA

The PEG-*b*-PEGDMA polymer was synthesized by deactivation-enhanced atom transfer radical polymerization (DE-ATRP). EGDMA (0.79 g, 4 mmol), PEG2K-Br (0.4 g, 0.2 mmol), PMDETA (34.7 mg, 0.2 mmol) and $CuCl_2$ (26.9 mg, 0.2 mmol) were

dissolved in 10 mL 2-butanone in a three-neck round bottom flask. The mixture was bubbled with argon for thirty minutes to removal air. Then ascorbic acid (7.0 mg, 0.04 mmol) was added to the mixture under the protection of argon quickly. After that, bubbled with argon for another 10 minutes and the flask was sealed and the mixture was heated to 50 ^{o}C with oil bath. After a period of reaction, the flask was opened and air was introduced to stop the reaction. Subsequently, the reaction solution was added dropwise to 10 times excess of petroleum ether twice to precipitate polymer and remove unreacted monomer. After that, the obtained precipitation was dissolved in a small amount of THF and dialyzed against deionized water to remove residual macromolecular initiator PEG-Br and copper salt. At last, the pure dry polymer was obtained by lyophilizing.

Micelle fabrication

A component solvent of 1 mL of DMF and 4 mL of THF was prepared. Subsequently, 5 mg of the pure dry polymer was dissolved in 5 mL mixture solvent and stirred for 3 hours. Then 1 mL of the solution was added dropwise to 4 mL of deionized water and stirred for 8 hours to stabilize the assembled micelle. Finally, the solution was dialyzed against deionized water to remove organic solvent for 48 h with the change of water for every 8 hours.

Preparation of Ce6-loaded PEG-*b*-PEGDMA /Ce6 micelles

The PEG-*b*-PEGDMA polymer (5 mg) and Ce6 (1 mg) were dissolved in mixture solvent of 1 mL of DMF and 4 mL of THF and stirred for 3 hours. Subsequently, 1 mL of the solution was added dropwise to 4 mL of deionized water and stirred for 8 hours to stabilize the assembled micelle. The Ce6-loaded micelles were freeze-dried and analyzed through UV−vis spectrometry at 670 nm to calculate the drug content and loading efficiency.

Singlet oxygen ($^1O_2$) production of free Ce6 and Ce6-loaded micelles

As a singlet oxygen scavenger, 1, 3-diphenylisobenzofuran (DPBF) was used to determine $^1O_2$ production of Ce6 and nanoparticles. A solution containing a fixed concentration and DPBF was added into a quartz cuvette and irradiated at 660 nm for 25 s. The $^1O_2$ generation of Ce6 and Ce6-loaded micelles can be directly correlated with

the decrease of the DPBF absorbance in the UV/Vis spectrum, thus the absorbance of DPBF at 459 nm was measured every 5 s. PEG-*b*-PEGDMA nanoparticles at the same photosensitizer and GSH concentration was tested as a control.

In vitro release of Ce6

To study the release behavior *in vitro*, the Ce6-loaded polymer micelles were dispersed in a phosphate buffer (PBS, 10 mM, pH = 7.4) with 0, 2 and 10 mM GSH at 37 ^{o}C. 2.0 mL of the sample solution was taken out for a set of time and replaced with an equal volume of fresh PBS. The amount of release Ce6 in each sample was determined by a fluorescence spectrophotometer at the excitation wavelengths of 400 nm and the emission wavelength of 660 nm.

Cell culture

A549 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with high glucose containing 10% fetal bovine serum (FBS), 100 units per mL of penicillin, and 100 μg/mL of streptomycin at 37 ^{o}C in a 5% CO_2 humidified atmosphere.

Cellular uptake assays

Flow cytometry and confocal laser scanning microscopy (CLSM) were used to study the cellular endocytosis of free Ce6 and PEG-*b*-PEGDMA/Ce6 drug-loaded micelles.

For flow cytometry analysis, the A549 cells were seeded in 6-well plates at a cell density of 2×10^5 cells per well and the cells were incubated for 24 h. Then, the A549 cells were treated with free Ce6, PEG-*b*-PEGDMA/Ce6 micelles with a Ce6 concentration of 5 μg/mL in DMEM for 4 h and 24 h, respectively. After the specified time, the A549 cells were detached by trypsin and resuspended in PBS. Finally, the fluorescence intensity of suspended A549 cells was determined by a BD FACS Calibur flow cytometer.

For CLSM analysis, A549 cells were seeded in 6-well plates, and the cell density were 1×10^4 A549 cells per well. Then the cells were incubated for 24 h. Subsequently, the free Ce6 and PEG-*b*-PEGDMA/Ce6 drug-loading micelles were added with a Ce6 concentration of 5 μg/mL in DMEM, and the cells were cultured for 4 h and 24 h. After that, the medium removed and the cells washed with PBS for 3 times. Next, the cells were fixed with 4% paraformaldehyde for 15 minutes and washed with PBS for 3 times.

Then the cell nucleus were stained with Hoechst 33342 for 10 minutes and washed with PBS for 3 times. Confocal fluorescence imaging studies were performed with fluorescence microscope (Nikon AIR).

Assessment of GSH levels

The intracellular levels of GSH were measured using GSH and GSSG Assay Kit (Beyotime). According to the manufacturer's instructions, the standard curve of GSSG levels was first measured. Then, 0.25, 0.65 and 1.3 μM PEG-*b*-PEGDMA/Ce6 micelles-treated A549 cells were collected and homogenized. The total GSH levels were carefully measured by using glutathione reductase and 5′, 5′-dithio-bis (2-nitrobenzoic acid). The sulfhydryl group of GSH reacts with DTNB and produces a yellow-colored 5-thio-2-nitrobenzoic acid (TNB), which has an absorbance at 405 to 414 nm. The value (A412) of TNB could be detected by a microplate reader and the levels of reduced GSH were calculated by subtracting the GSSG levels from the total GSH (GSH = total GSH − 2 × GSSG).

Cell viability assay

MTT assay was used to evaluate the toxicity of PEG-*b*-PEGDMA/Ce6 drug-loaded micelles, pure PEG-*b*-PEGDMA micelles, and free Ce6 to A549 cancer cells. The experiments were divided into phototoxic and dark toxicity.

For the dark toxicity, A549 cells were cultured in 96-well plate containing 200 μL of culture medium at a cell density of 5×10^3 per well, then placed in a specialized incubator for 24 hours. Afterwards, the A549 cells were cultured with PEG-*b*-PEGDMA micelles, free Ce6 and Ce6-loaded micelles PEG-*b*-PEGDMA/Ce6 at different concentrations in DMEM at 37^{o}C in the dark. Continue to culture for 24 hours, the medium were replaced with fresh DMEM, and the fresh DMEM containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (concentration of 5 mg/mL). The A549 cells were further incubated for another 4 h. Then, 150 μL of DMSO solution was added instead of the MTT-containing solution and gently shaken to extract the formazan product. Finally, the spectrophotometer was used to detect the

absorbance of formazan in DMSO at a wavelength of 560 nm. The cell viability (%) was calculated by the following equation:

Cell viability (%) = $(OD_{test} - OD_{background}) / (OD_{control} - OD_{background}) \times 100$

Where OD_{test} is the absorbance of sample solutions, and $OD_{control}$ is the absorbance of the control group and the $OD_{background}$ is the absorbance of empty plates.

For the phototoxicity, A549 cells were seeded into a 96-well plate at a density of 5×10^3, and they were incubated for 24 hours. Then, the A549 cells were treated with PEG-*b*-PEGDMAs micelles, free Ce6 and PEG-*b*-PEGDMAs/Ce6 drug-loaded micelles at different concentrations in DMEM at 37 ^oC. The A549 cells were incubated for another 24 hours, the medium removed and the cells washed 3 times with PBS. Next, the fresh DMEM was added, and the A549 cells were irradiated with a laser (50 mW/cm^2) at a wavelength of 660 nm for 3 minutes. After irradiation, the A549 cells continuously incubated for 24 hours. Subsequently, the old culture medium was replaced by fresh DMEM with MTT solution (concentration 5 mg/mL). Finally, cell viability was determined using the MTT assay.

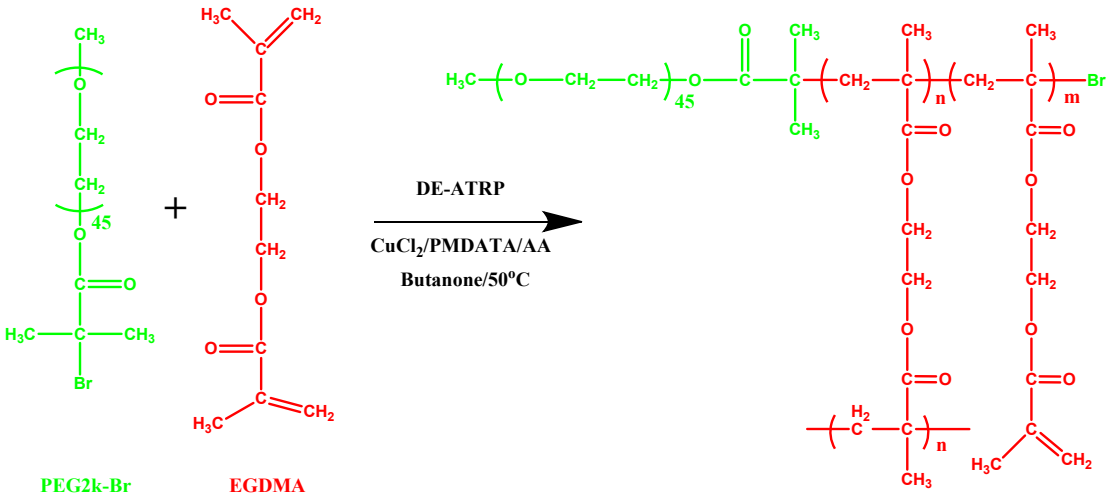
***In Vivo* Fluorescence Imaging.**

All animal procedures were performed in accordance with Chinese legislation on the Use and Care of Research Animals (Document No. 55, 2001), and institutional guidelines for the Care and Use of Laboratory Animals established by the East China University of Science and Technology Animal Studies Committee, and the experiments were approved by the committee. When the tumor volume reached to 50 mm^3, PEG-*b*-PEGDMA/Ce6 micelles (100 μL, 2.5 mg/kg) were injected into the tail vein of nude mice. Fluorescence imaging was performed at 2, 6, 12 and 24 h after injection using an *in vivo* imaging system. Mice were euthanized and their tumors and major organs (heart, liver, spleen, kidneys and lungs) were imaged by a fluorescence imaging system.

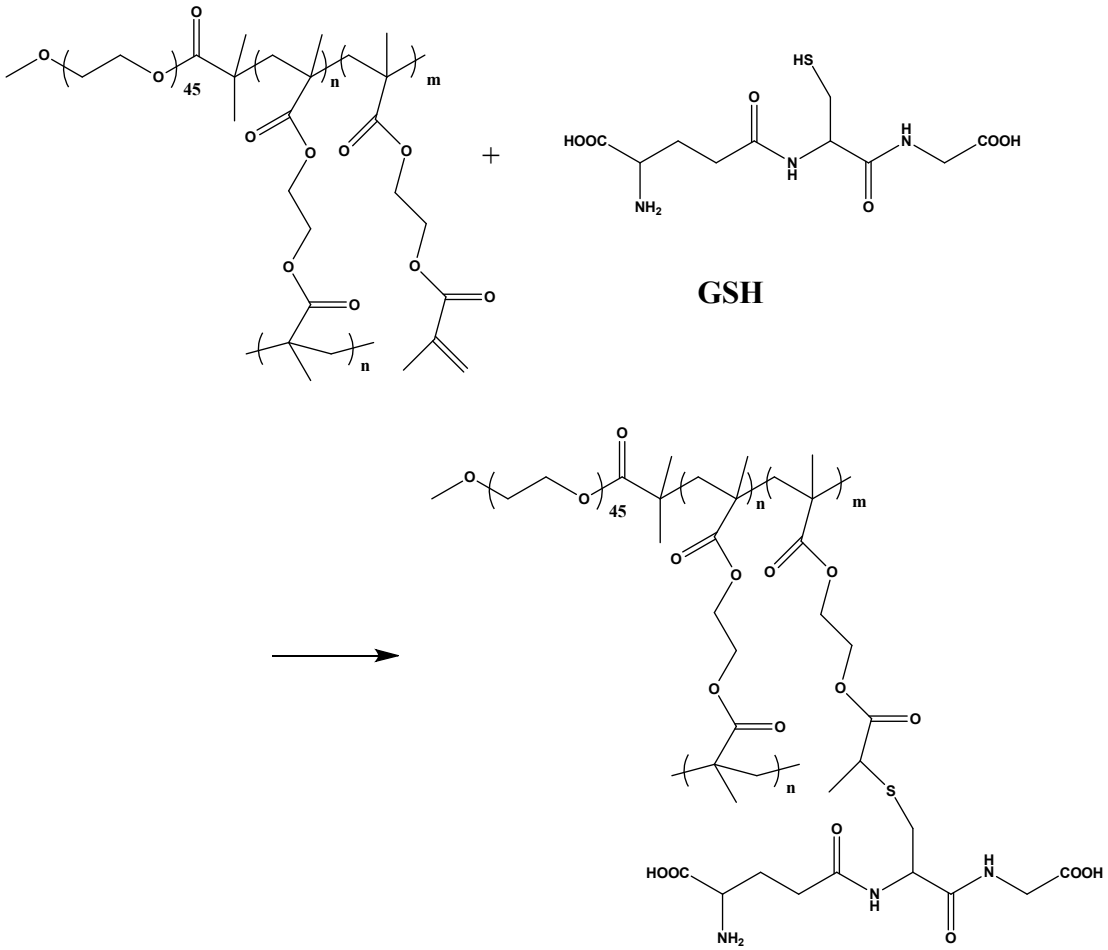
***In Vivo* Photodynamic Therapy**

I*n vivo* photodynamic therapy was performed on tumor-bearing mice. When the tumor size of the mouse reached 50~60 mm^3, the mice were randomly divided into 5 treatment groups (n = 4 per group): (i) PBS, (ii) PEG-*b*-PEGDMA, (iii) PEG-*b*-PEGDMA/Ce6

micelles (no laser), (iv) free Ce6 with laser (free Ce6 + L), (v) PEG-*b*-PEGDMA/Ce6 micelles with laser (PEG-*b*-PEGDMA/Ce6 + L). Each group of mice was then treated on day 1. After the injection, the mice of groups (d) and (e) were partially irradiated with 660 nm laser (50 mW/cm^2, 30 minutes). The tumor volume was measured every other day for two weeks, and the tumor volume (0.5 × L × W^2, where L is the tumor length and W is the tumor width) was calculated according to the above formula, and the relative tumor volume (day n tumor volume / day 0 tumor was calculated). After treatment on 14th day, the mice were euthanized by inhalation of CO_2. The tumors and main organs in the mic were excised, treated and stained for H&E imaging excision and fixed with 4% formaldehyde, then embedded in paraffin blocks, sectioned, stained with hematoxylin and eosin (H&E), mounted on glass slides.



Scheme S1 The reaction of the branched polymer of PEG-*b*-PEGDMA via an *in situ* DE-ATRP.



Scheme 2 The reaction of PEG-b-PEGDMA with GSH *via* Michael addition.

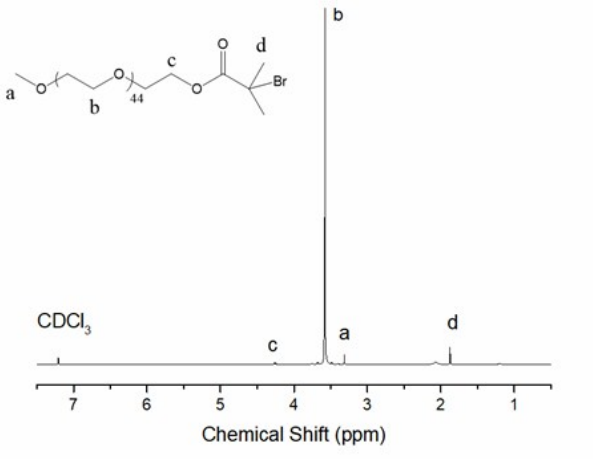


Fig. S1 ^{1}H-NMR spectrum of PEG-Br.

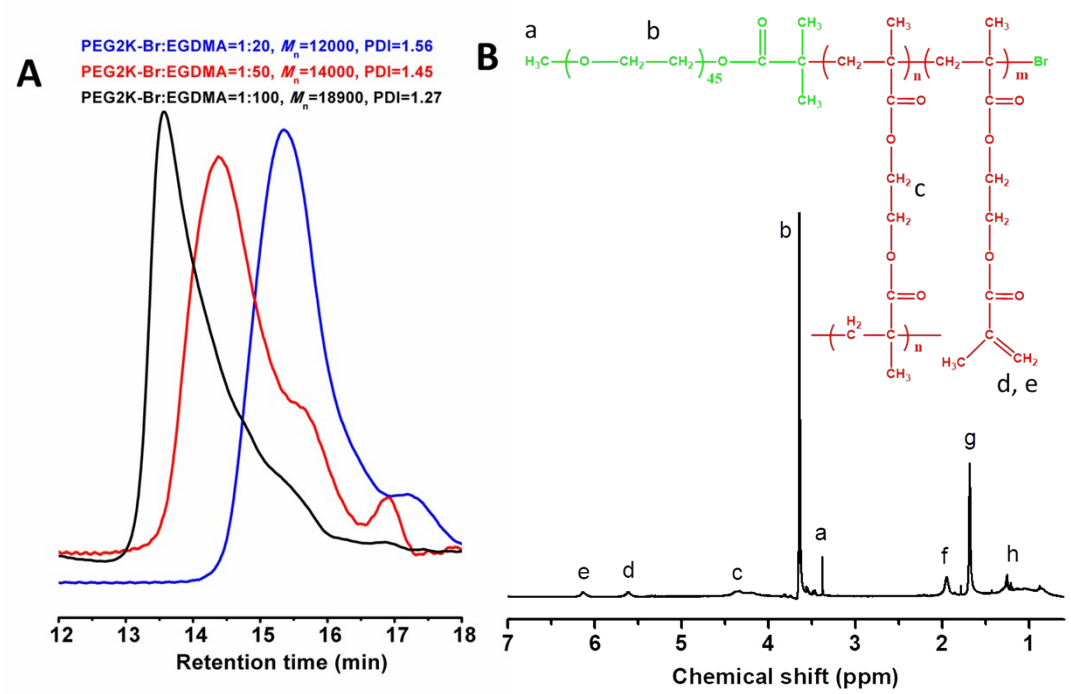


Fig. S2 (A) The GPC of PEG-*b*-PEGDMAs at different feed ratio; (B) the ^{1}H NMR spectrum of PEG-b-PEGDMA for the feed ratio of 1/20 of PEG-Br/EGDMA.

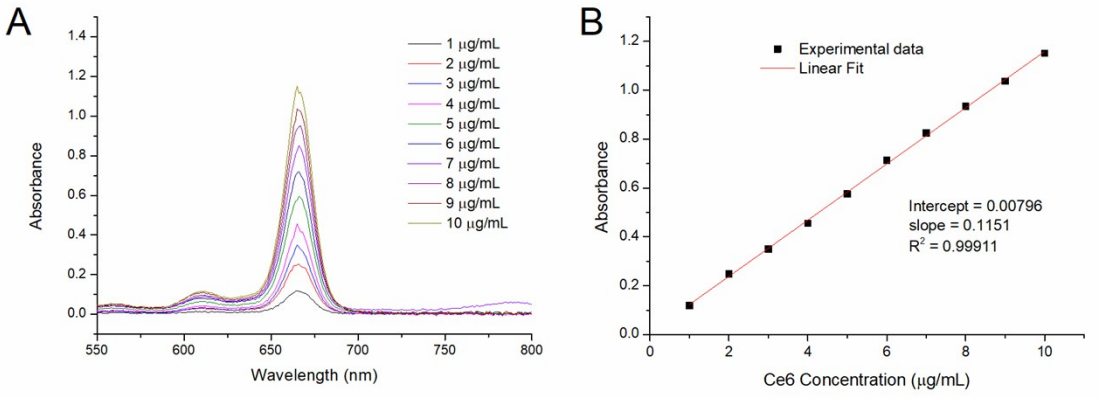


Fig. S3 UV-Vis absorption spectra of Ce6 at various concentrations (A) and the corresponding standard line (B).

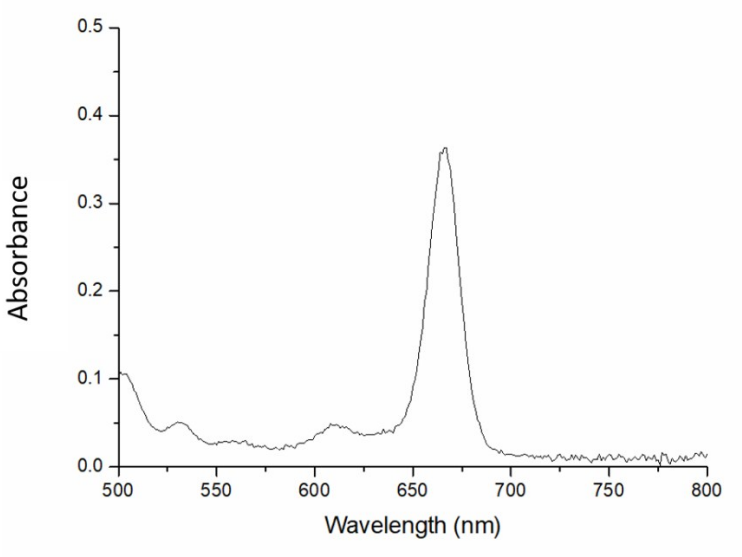


Fig. S4 UV-Vis absorption spectrum of PEG-*b*-PEGDMA/Ce6.

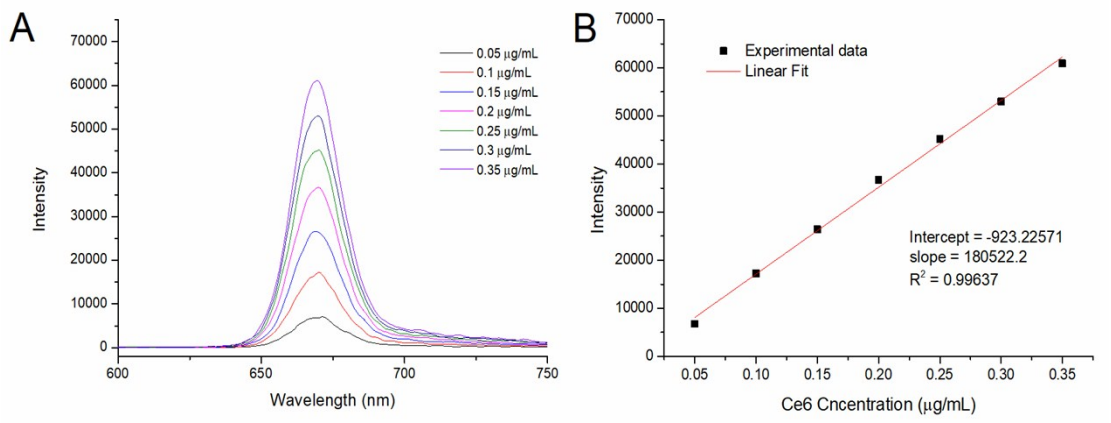


Fig. S5 Fluorescence emission spectra of Ce6 at various concentration (A) and the corresponding standard line (B).

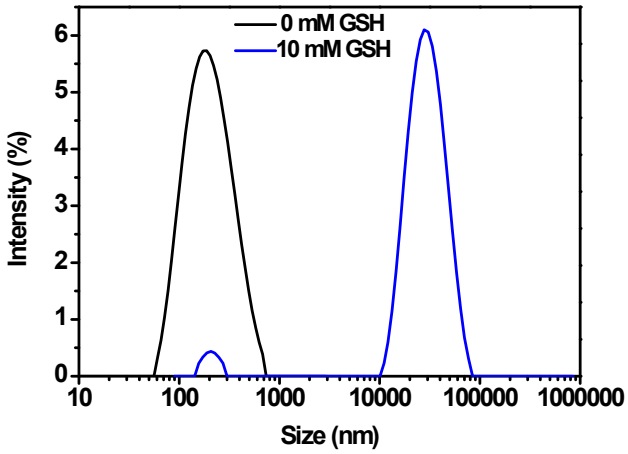


Fig. S6 The size change of the micelles at different GSH concentration for 48 h determined by DLS

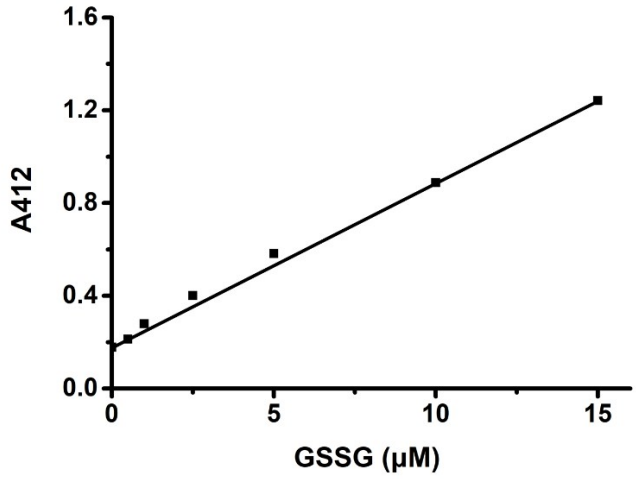


Fig. S7 The standard curve of GSSG levels.

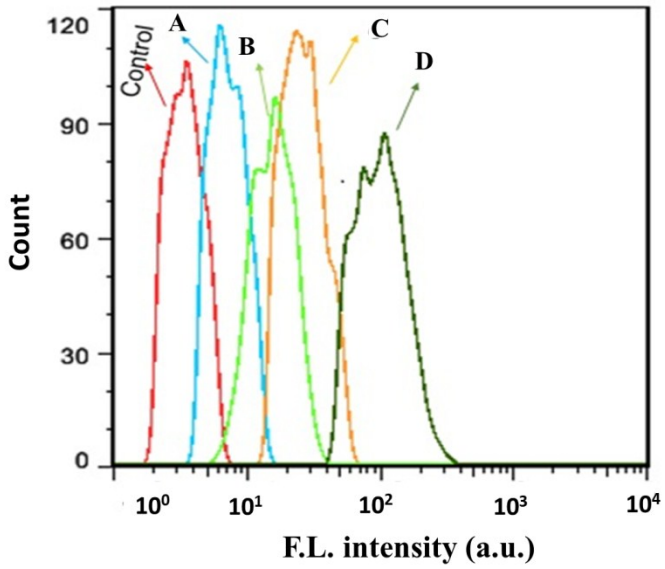


Fig. S8 The cellular uptake of A549 incubated with free Ce6 for 4 hours (A) and 24 hours (C), incubated with PEG-*b*-PEGDMA/Ce6 micelles for 4 hours (B) and 24 hours (D).

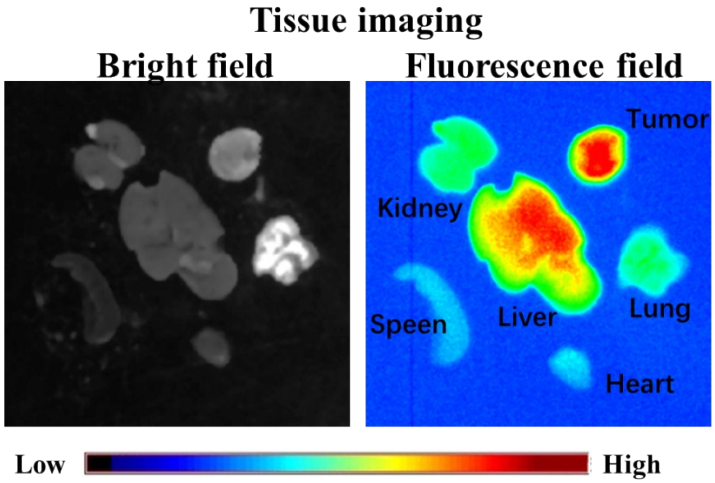


Fig. S9 Fluorescence images of major organs after treatment of PEG-*b*-PEGDMA/Ce6 micelles within 24 h.

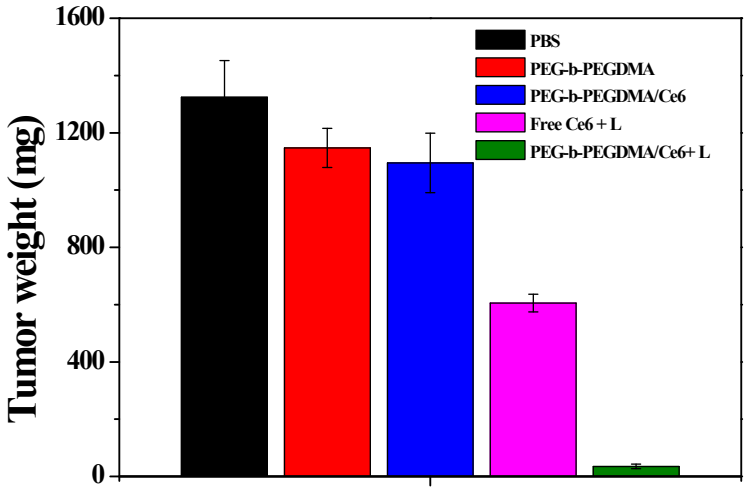


Fig. S10 Tumor weights measured after various treatments on the 14th day.

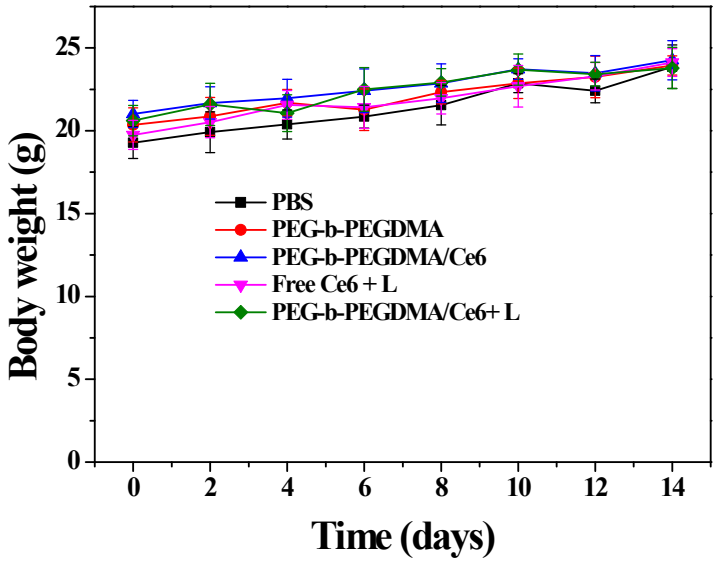


Fig. S11 Body weight changes of tumor-bearing nude mice VS time after various treatments.

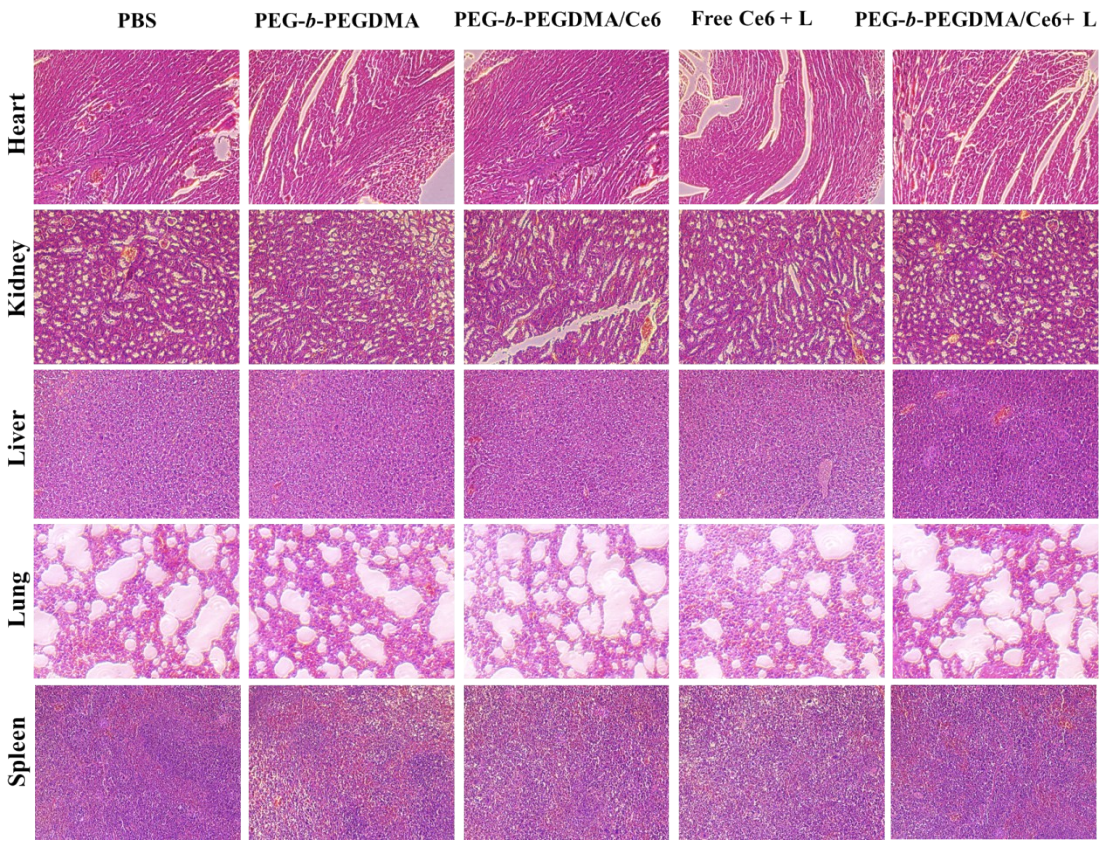


Fig. S12 H&E staining of organs after treating with different agents.