Support information

GSH-activated Porphyrin Sonosensitizer Prodrug for Fluorescence Image-guided Cancer Sonodynamic Therapy

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Materials and Methods

Materials

All complete media, fetal bovine serum and penicillin and streptomycin for cell culture were from Gibco brand. The petri dishes and consumables used for cell culture were purchased from Sangon Biotech (China). 5,10,15, 20-tetrakis (4-hydroxyphenyl) -21H, 23H-porphine was purchased from HEOWNS (CAS: 51094-17-8). Distearoyl-sn-glycero-3-phosphoethanolamine-n-(polyethylene)-5000 was purchased from Xian Ruixi Biotech Co. Ltd. (China). 1,3-Diphenylisobenzofuran (DPBF) was purchased from Saen Chemical Technology (Shanghai) Co., Ltd. (China). Calcein AM/PI kit was purchased from Beyotime Biotechnology. L-buthionine-sulfoximine was purchased from Yuanye Bio-Technology.

Preparation of drug and prodrug nanoparticles

Distearoyl-sn-glycero-3-phosphoethanolamine-n-(polyethylene)-5000 (DSPE-PEG_{5000}) and prodrug or 5,10,15, 20-tetrakis (4-hydroxyphenyl)-21H, 23H-porphine (THPP) were respectively weighed at a ratio of 9:1 and dissolved in sufficient amount of THF. Then, the mixture solution was dried by rotary evaporation and redissolved with ddH_2O with the assistance of ultrasonication to form prodrug- and THPP- loaded nanoparticles, respectively. The nanoparticles were named as prodrug nanoparticle and drug nanoparticles (NPs), respectively. As with the previous methods, we replaced DSPE-PEG_{5000} with FITC-DSPE-PEG_{5000} to prepare the prodrug nanoparticles, which we named FITC-prodrug NPs.

Characteristic of prodrug and drug nanoparticles

Tetrahydroxy porphyrin and prodrug were dissolved in DMSO to form solutions (1 μM), and full spectral scanning was performed by UV-Vis-NIR spectrometer (759S, Lengguang Technology, China). In addition, the fluorescence emission spectra at 600 - 800 nm was recorded by fluorescence spectrometer (Hitachi F-4700, Japan) with an excitation wavelength at 430 nm. The size and morphology of nanoparticles in water, PBS, DMEM and RPMI 1640 complete medium were characterized by DLS (Litesizer™ 500, Anton paar) and transmission electron microscope (TEM, H-7650), respectively. The nanoparticle size of prodrug NPs was measured for five consecutive days to determine the size stability.

To explore the stability of prodrug NPs, prodrug NPs were incubated with 10% Fetal bovine serum (FBS) and 90% FBS for 24 h and then analysis using LC-MS. FITC- prodrug NPs were

prepared with loaded prodrug to FITC-labeled DSPE-PEG_{5000}, and then incubated with 22rv1 tumor cell for 4 h and 12 h. The cells were imaged using a fluorescence microscope. Tumor cells were lysed to obtain cellular contents, which were incubated with prodrug NPs for 24 h, and then analysis the components using the LC-MS.

Cells cultivated and animal models

4T1 cells were cultured in DMEM complete media with 10% serum and 1% penicillin-streptomycin. Human Umbilical Vein Endothelial Cells (HUVEC) were cultured in Ham's F12 media supplement with 10% fetal bovine serum and 100 U mL^{-1} penicillin and streptomycin. They were all cultured in a cell incubator containing 5% CO_2 at 37 °C.

BAL B/C mice (4 - 8 weeks, 20 g, female, SPF) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. and kept in the animal center of Xi'an jiaotong university health science center. All animal experiments have been approved by the Biomedical Ethics Committee of Health Science Center, Xi'an Jiaotong University (No. 2021-1613). All procedures were carried out in accordance with the NIH Principles of Laboratory Animal Care and the Guidelines of the Xi'an Jiaotong University Laboratory Animal Care Committee. 4T1 cells were digested, washed with 1×PBS for 3 times and re-suspended with 1× PBS. The tumor-bearing animal model was established by subcutaneous injection of 100 μL cells in 1× PBS (1×10^6) at the right back of the BAL B/C mice. After 7 days, the tumor volume reached ~ 100 mm^3 (Volume = 0.5 × Length × $Width^2$) and were used for subsequent imaging and sonodynamic therapy.

ROS detection in vitro

DPBF was used to detect the $^1O_2$.THPP and prodrug were dissolved in DMSO to form solutions (5 μM), respectively. Then, DPBF was added into the solutions to ensure the DPBF has an absorbance value of ~ 1.0 at the wavelength of 410 nm, respectively. Then, the solution was transferred to a 6-well plate. With the assistance of ultrasonic coupling agent, the ultrasonic probe was ultrasonically irradiated from the bottom of the 6-well plate (1.7 W/cm^2, 30 KHZ, 50% Cycle duty), and the absorption value of DPBF at 410 nm was measured every two minutes to draw the attenuation curve of DPBF. A DMSO solution contained DPBF only was set as the control group, and the change of absorbance value was also detected after US irradiation. TMB, that can be oxidized by ·OH to form OXTMB, which is a blue substance and has absorbed peak at 370nm and 654 nm, was used to detect

the ·OH. THPP and prodrug (10 μM) were dissolved in the distilled water and mixed with TMB solution. The mixture solution was treated with US irradiation for 10 min (1.7 W/cm^2, 30 KHZ, 50% Cycle duty), and then the color and absorption value changes of the solution were detected. NBT served as the O^{2-} probe, can produce methyl hydrazone which has aborption peak at 595 nm in the presence of O^{2-}. THPP and prodrug (10 μM) were dissolved in PBS (pH = 7.4) and then mixed with NBT solution. The mixture solution was treated with US irradiation for 10 min (1.7 W/cm^2, 30 KHZ, 50% Cycle duty), and then the color and absorption value changes of the solution were detected. Prodrug NPs (5 μM) was pre-incubated with GSH for 24 h in mixed solution (DMSO/H_2O=7/3), and then added to the DPBF solution and further treated with the US irradiation for 10 min to detect the decreased absorbance value of DPBF.

Prodrug (8 μM) was added to 4T1 cells and HUVEC cells, respectively. After 24 hours' incubation, the cells were further added with DCFH-DA (10 μM as a final concentration) and incubated for another 20 min. Then, the cells were irradiated with US for 2 min (0.85 W/cm^2, 50% cycle duty, 30 KHZ), followed by another 30 minutes' culture. Finally, the cells were washed with 1 × PBS for 3 times carefully and stained with DAPI for imaging with a fluorescence microscope at GFP channel and DAPI channel, respectively. The parameters for imaging and data processing were consistent in all groups.

4T1 cells were pre-incubated with BSO for 24 h, and then the prodrug NPs (8 μM) was added to culture another 24 h. The cells were further added with DCFH-DA and treated with US for 5 min (0.85 W/cm^2, 50% cycle duty, 30 KHZ). Finally, the cells were stained with DAPI and imaged with the microscope.

Sonodynamic effect in Vitro

4T1 and HUVEC were seeded into 96-well plates (5×10^3 cells/well), respectively. After 24 h, prodrug NPs, prodrug and prodrug+GSH at different concentrations in fresh medium were added into 96-well plates and incubated for another 24 h. Then, the cells were irradiated with US for 5 min (30 KHZ, 0.85 W/cm^2, 50% cycle duty) and cultured for another 24 h. The cell survival rates were determined by standard CCK-8 essay (GLPBIO Technology).

Flow cytometry detection

4T1 cells, 22rv1 cells and HUVEC were seeded into the 6- wells plates and incubated with

prodrug (10 μM) for 12 h, and then collected for fluorescence intensity detection using a flow cytometry (Cyto FLEX, Beckman Coulter Life Sciences, the United States) at APC channel.

4T1 cells were seeded into 96-wells plates and incubated with GSH pre-incubated prodrug (15 μM) for 24 h, and then treated with US irradiation for 5 min. After another 24 h incubation, the cells were collected and washed with PBS for 3 times, and then stained with Annexin V-FITC-PI kit for apoptosis detection using a flow cytometry.

Live-dead cell staining

1 $\times 10^5$ 4T1 cells were seeded in 24-well plates and then cultured with prodrug and prodrug + GSH respectively for 24 h, where prodrug had a final concentration of 15 μM and GSH had final concentration of 1 mM. After US irradiation for 5 min (30 KHZ, 0.85 W/cm^2, 50% cycle duty), the cells were cultured for another 6 h to apoptosis. Then, Calcein AM/PI reagent was added and incubated for 30 min to stain the cells. Finally, the cells were imaged with a fluorescence microscope. GFP channel was used to detect green fluorescence in living cells, and Dsred channel was used to detect red fluorescence in dead cells.

Histological examination

For histological examination, the mice were sacrificed at 24 h after treatment. Then, the tumors of different groups were collected. The tumor tissues were fixed with 4% paraformaldehyde (Solarbio) for 24 h, embedded in paraffin, and sectioned for H&E and TUNEL staining. At the end of 14 days' treatment of SDT *in vivo*, all mice were sacrificed to collect the heart, liver, spleen, lung and kidney. After fixation with 4% paraformaldehyde for 24 h, the organs were embedded in paraffin, and sectioned for H&E staining. All the pathological sections were imaged with microscope.

Blood panel assays

BAL B/C mice (n = 15) were intravenously injected (200 μL) with prodrug NPs at a concentration of 0.67 mg/mL (counted by prodrug). After 1,7 and 14 days of injection, blood from mice was collected and and tested immediately the level of white blood cell(WBC), red blood cell (RBC), hemoglobin (HGB), hematokrit (HCT), mean corpuscular hemoglobin content (MCH), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV) and platelet (PLT).

Results

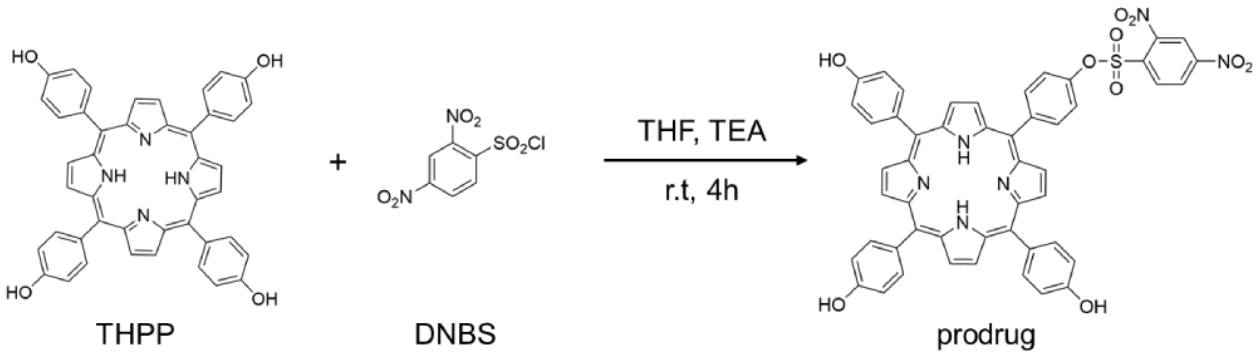

Figure S1. The scheme of prodrug synthesis.

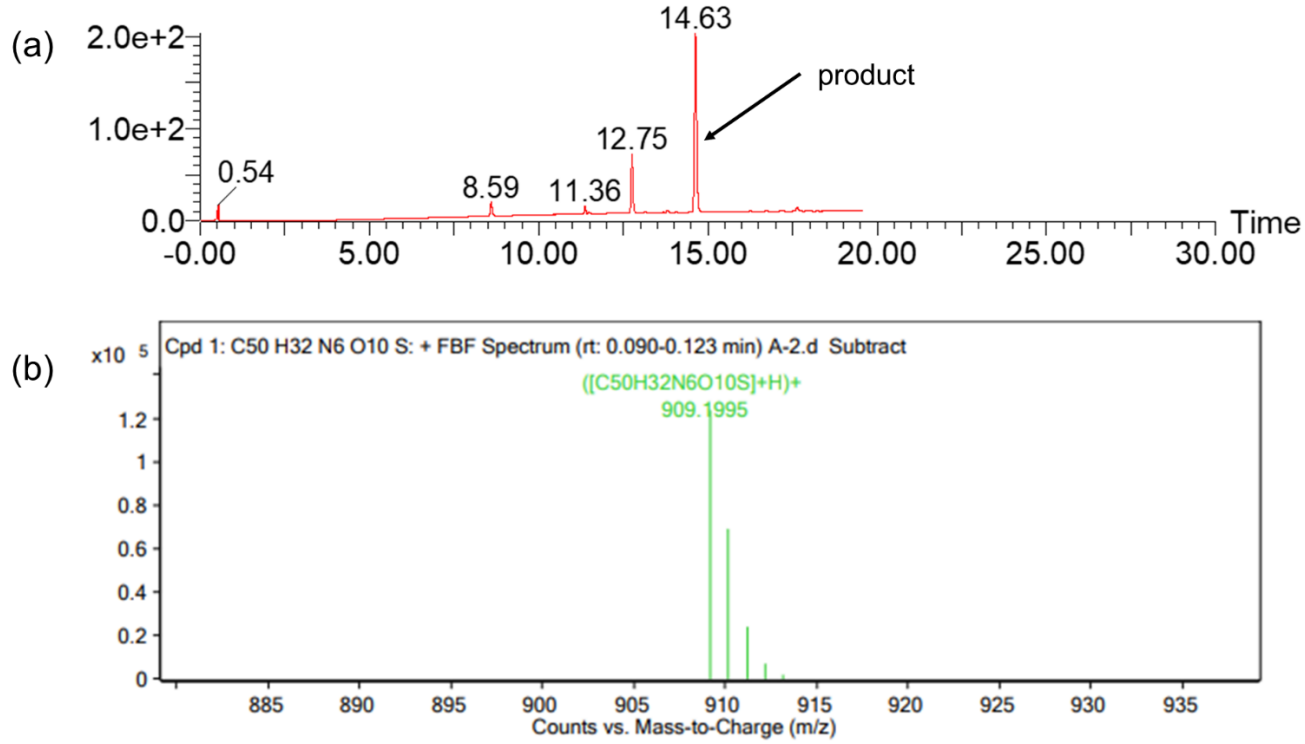

Figure S2. (a) HPLC profiles of purification. The peak at 14.63 min (arrow) was the target product.

(b) Extracted ion (EIC) mass chromatograms of prodrug obtained by HRMS.

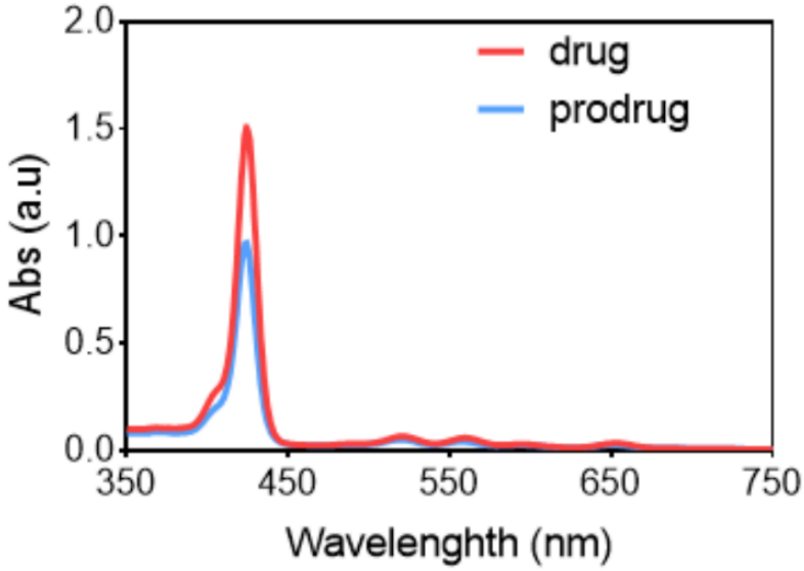

Figure S3. The absorption spectrum of drug and prodrug in mixture solution (DMSO/H_2O = 7/3), respectively.

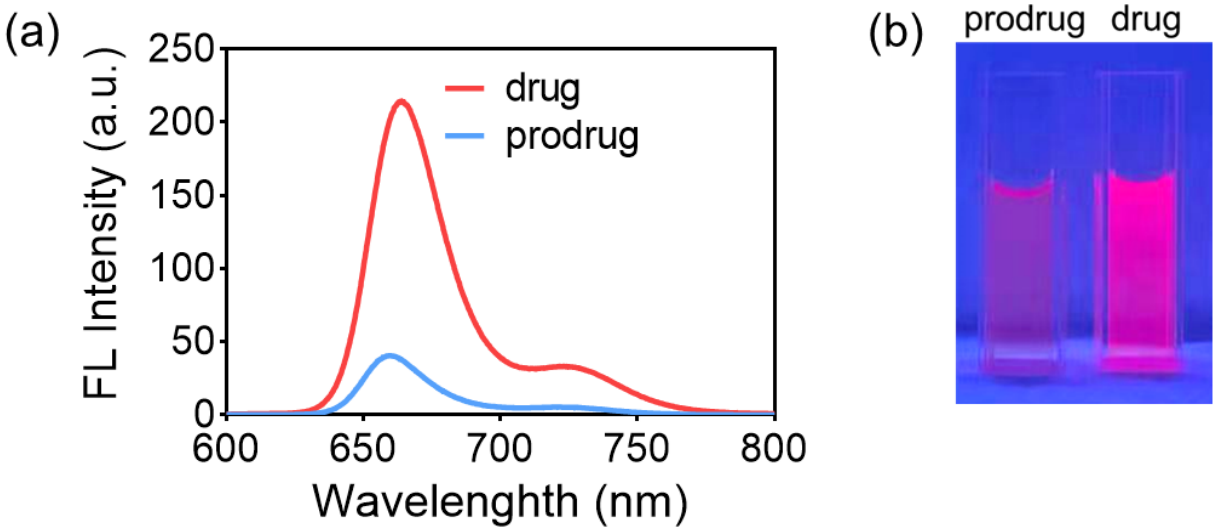

Figure S4. (a) The fluorescence emission spectrum of drug and prodrug of same concentration in solution (DMSO/water = 7/3). (b) The photo of drug and prodrug solution (30 μM) under 365 nm UV lamp.

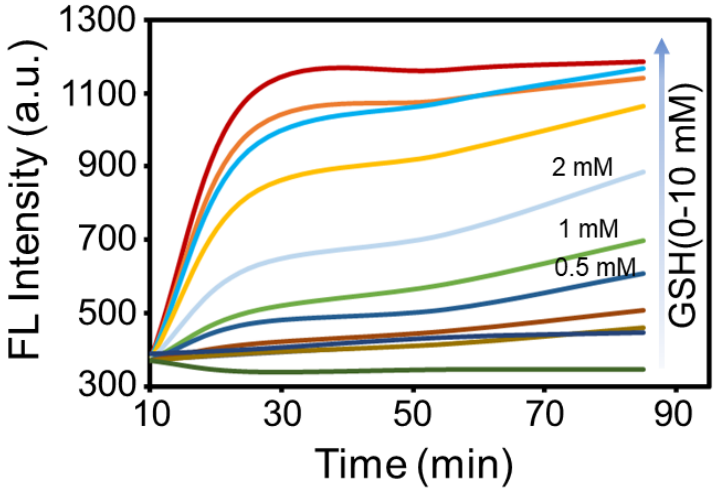

Figure S5. The fluorescence intensity of prodrug after incubation with different concentration GSH (0, 0.002, 0.005, 0.01, 0.25, 0.5, 1, 2, 4, 6, 8, 10 mM).

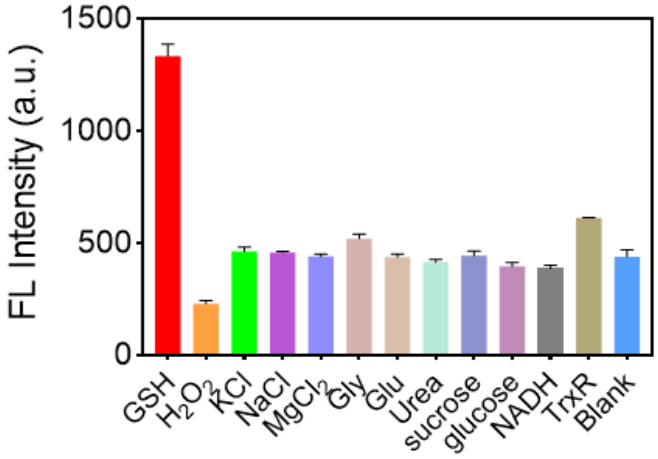

Figure S6. The quantified fluorescence values of prodrug after 1 hour's incubation with different chemicals.

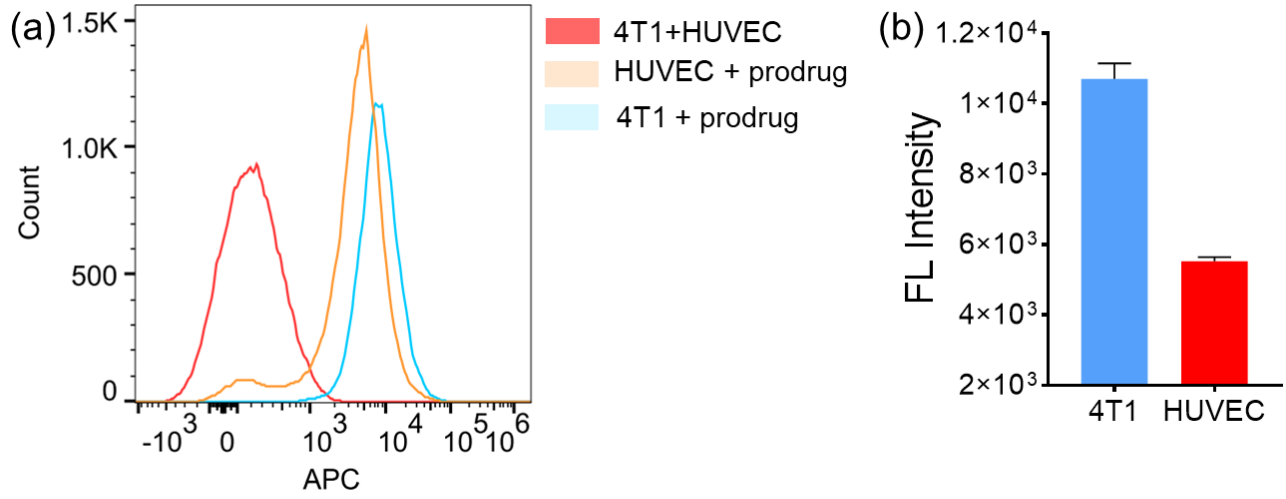

Figure S7. (a) Histograms of cells uptake were measured by flow cytometry. (b) Fluorescence intensity of prodrug in 4T1 cells and HUEVC, respectively.

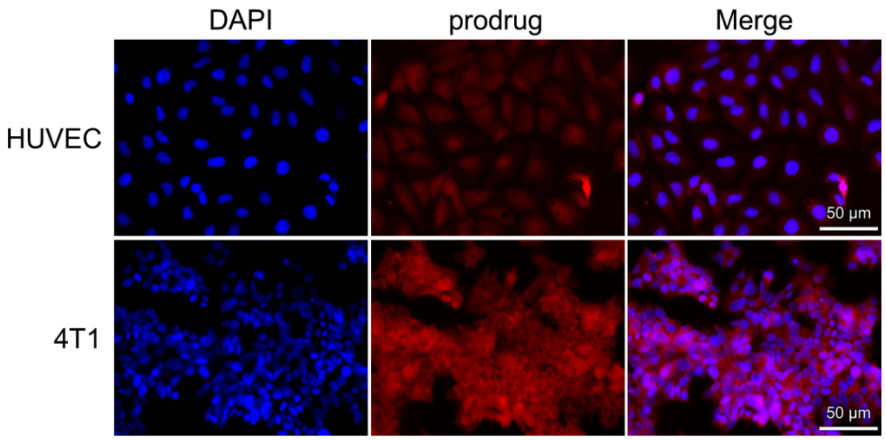

Figure S8. GSH activation of prodrug in 22rv1 and HUVEC cells.

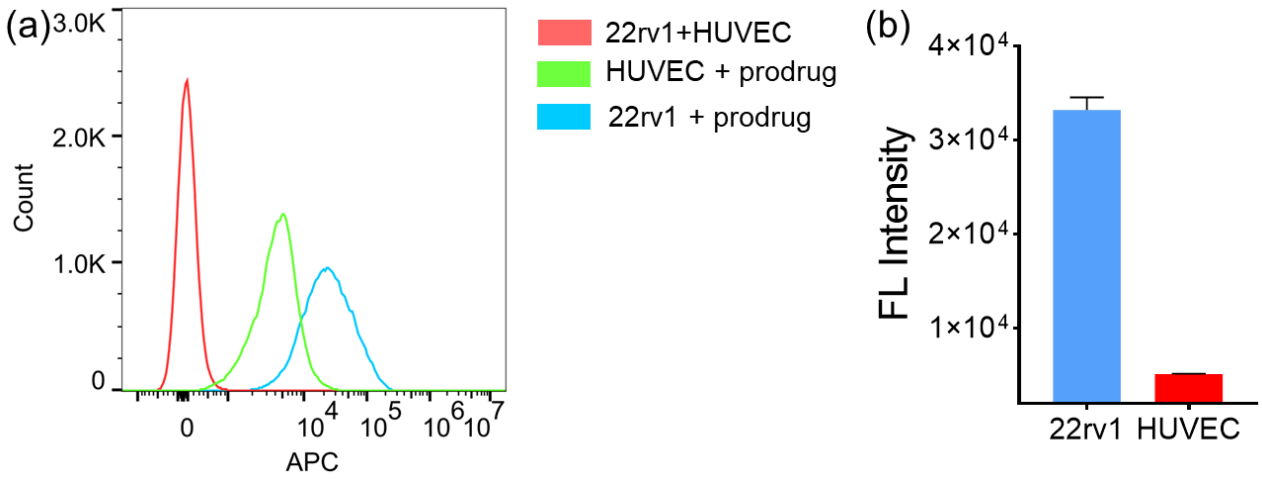

Figure S9. (a) Histograms of cells uptake were measured by flow cytometry. (b) Fluorescence intensities of prodrug in 22rv1 cells and HUEVC, respectively.

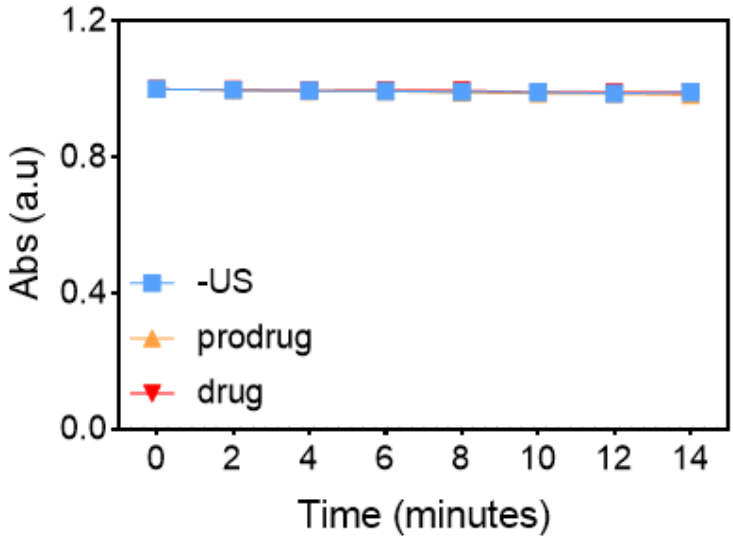

Figure S10. Absorption of DBPF in different DMSO solutions (DMSO only, DMSO + prodrug, DMSO + drug) across several timepoints (days) without ultrasound irradiation.

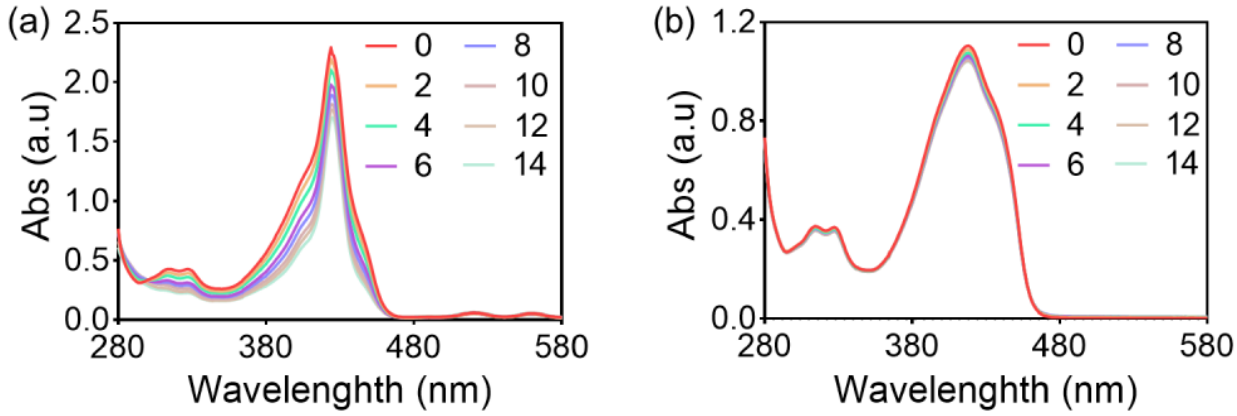

Figure S11. (a) The absorption spectra of DPBF with THPP (5 mM) in DMSO after US irradiation for different time. (b) The absorption spectra of DPBF in DMSO after US irradiation for different time.

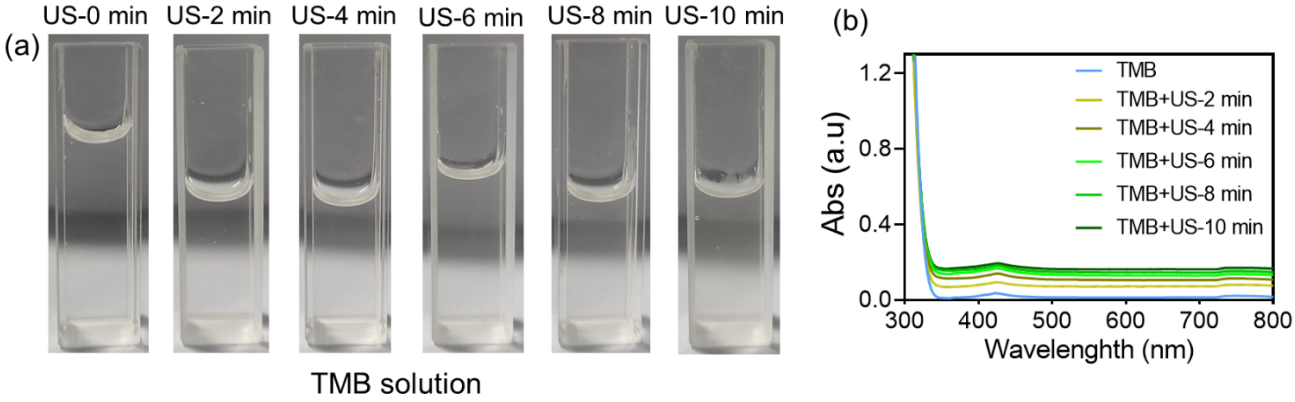

Figure S12. (a) The picture of TMB solution after different timepoints of US irradiation. (b) The absorption spectra of TMB solution after US irradiation for different time.

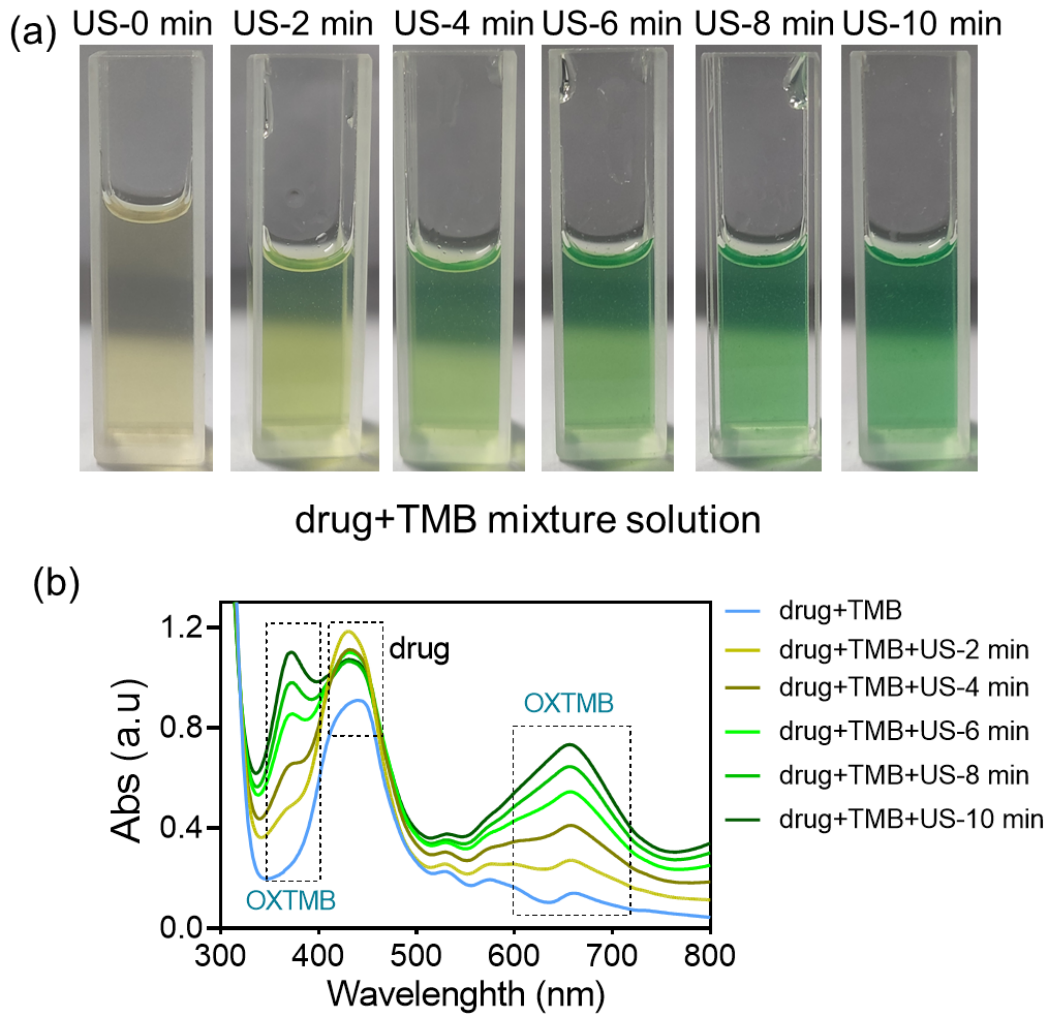

Figure S13. (a) The picture of drug+TMB solution after different timepoints of US irradiation. (b) The absorption spectra of drug+TMB solution after US irradiation for different time.

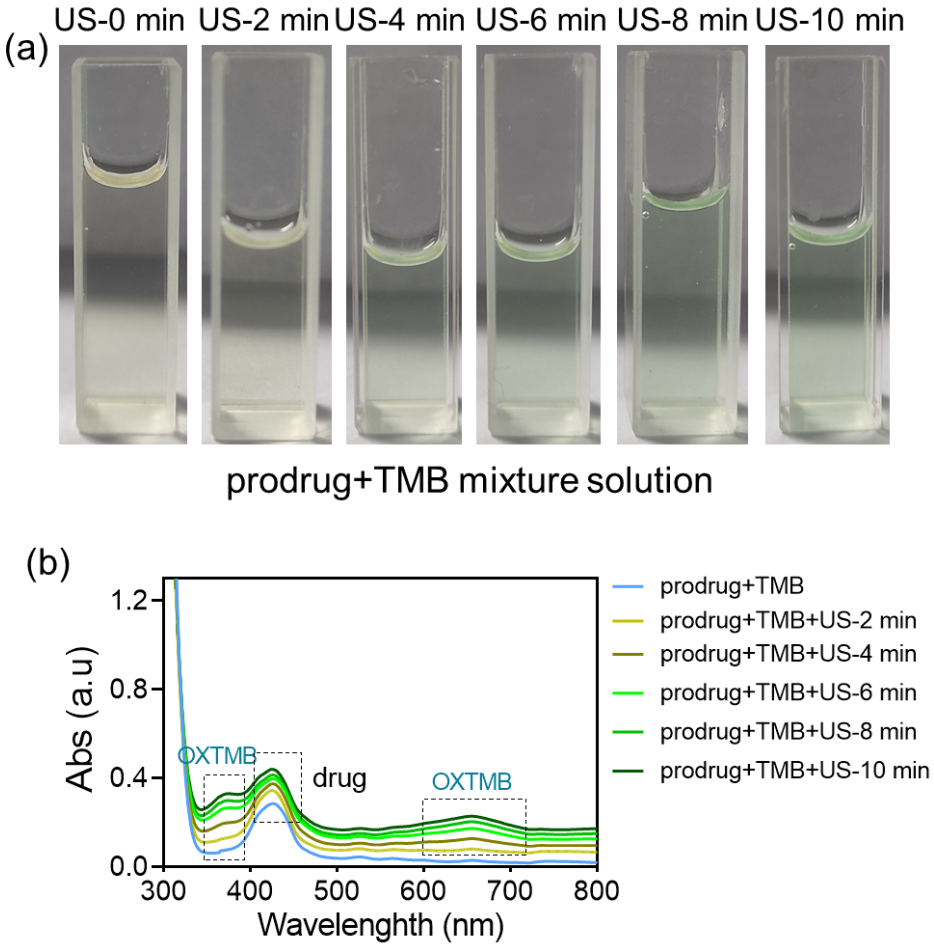

Figure S14. (a) The picture of prodrug+TMB solution after different timepoints of US irradiation. (b) The absorption spectra of prodrug+TMB solution after US irradiation for different time.

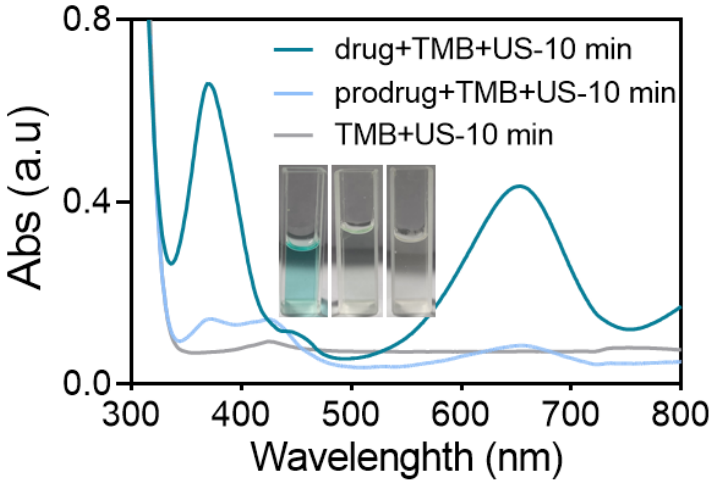

Figure S15. The centrifuged supernatant of drug+TMB solution, prodrug+TMB solution and TMB solution after US irradiation for 10 min.

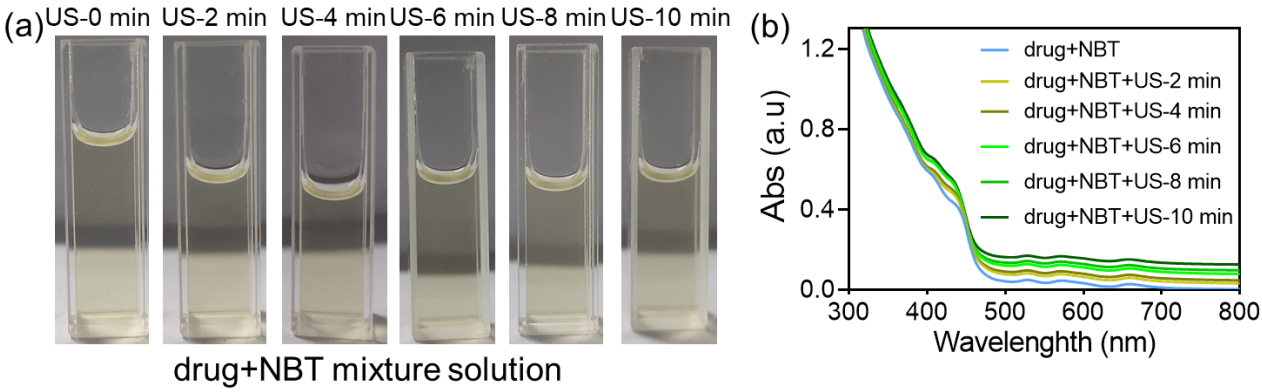

Figure S16. (a) The picture of drug+NBT solution after different timepoints of US irradiation. (b) The absorption spectra of drug+NBT solution after US irradiation for different time.

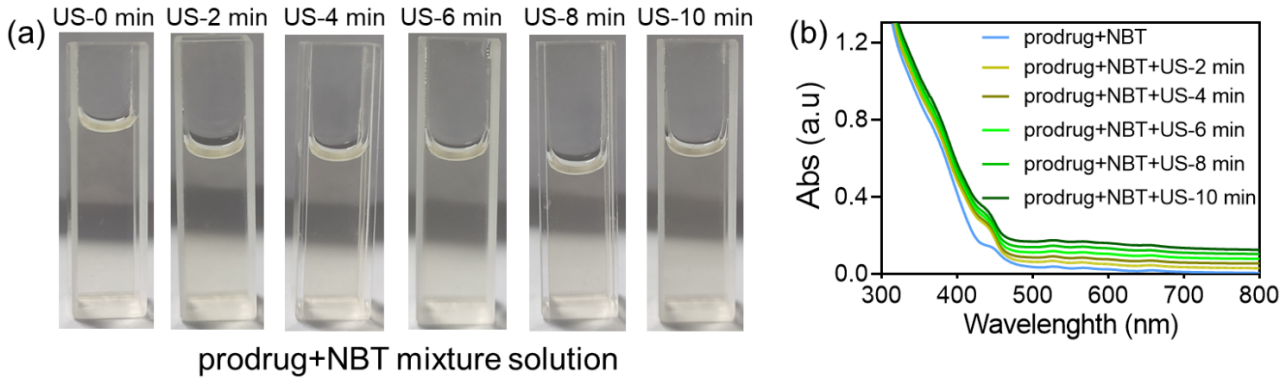

Figure S17. (a) The picture of prodrug+NBT solution after different timepoints of US irradiation. (b) The absorption spectra of prodrug+NBT solution after US irradiation for different time.

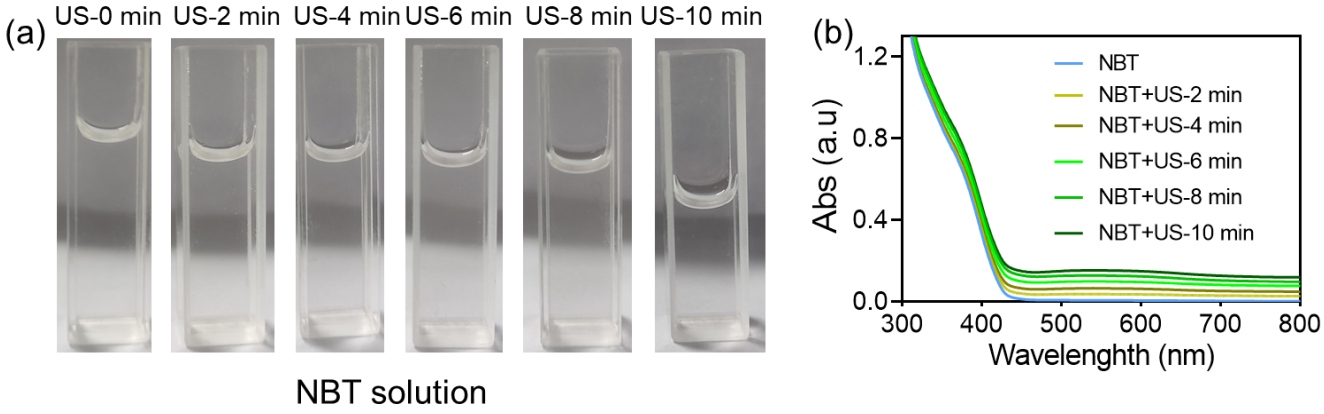

Figure S18. (a) The picture of NBT solution after different timepoints of US irradiation. (b) The absorption spectra of NBT solution after US irradiation for different time.

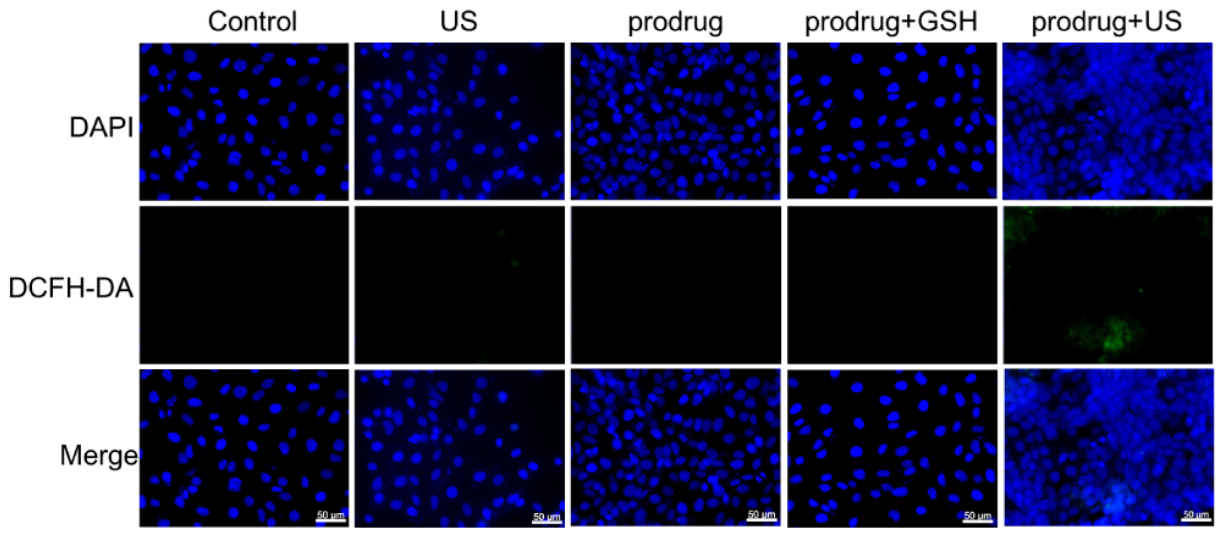

Figure S19. ROS detection by fluorescence image of DCFH-DA (10 μM) in HUVECs

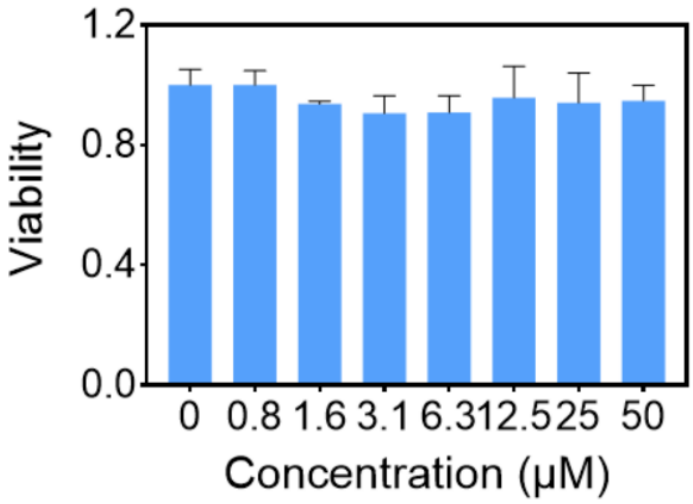

Figure S20. The 4T1 cytoviability of prodrug after 48 h incubation without US stimulus.

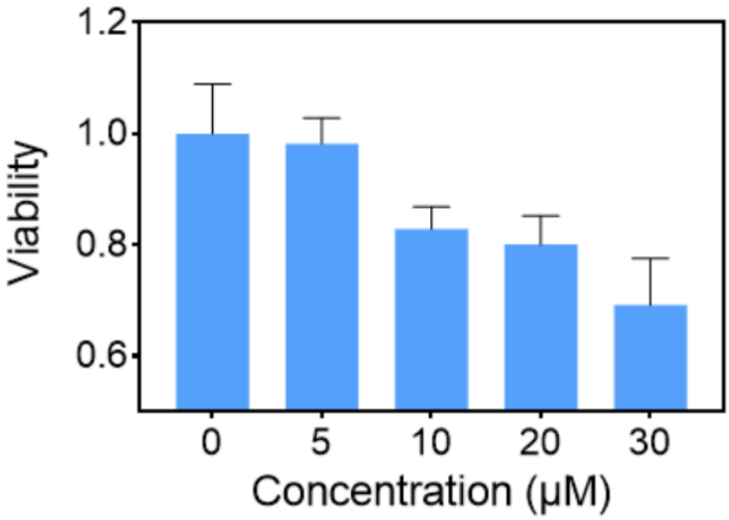

Figure S21. The viability of 4T1 cells after treatment with prodrug at different concentrations and US irradiation for 5 min (0.85 W/cm^2, 50% cycle duty, 30 KHZ).

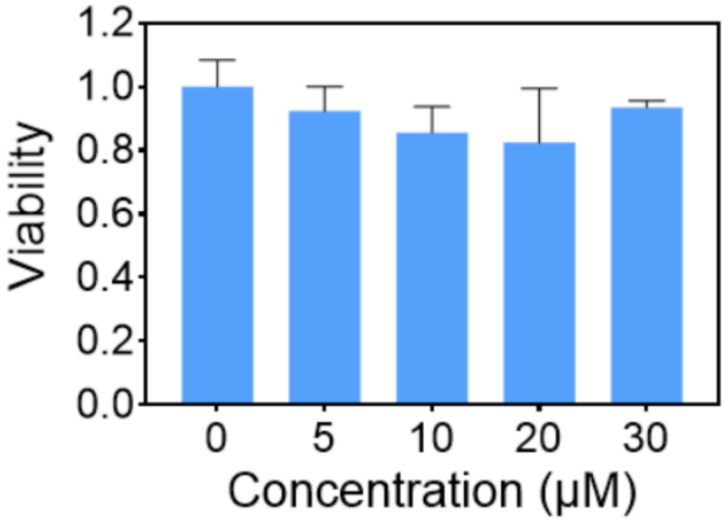

Figure S22. The viability of HUVEC after treatment with prodrug at different concentrations and US irradiation for 5 min (0.85 W/cm^2, 50% cycle duty, 30 KHZ).

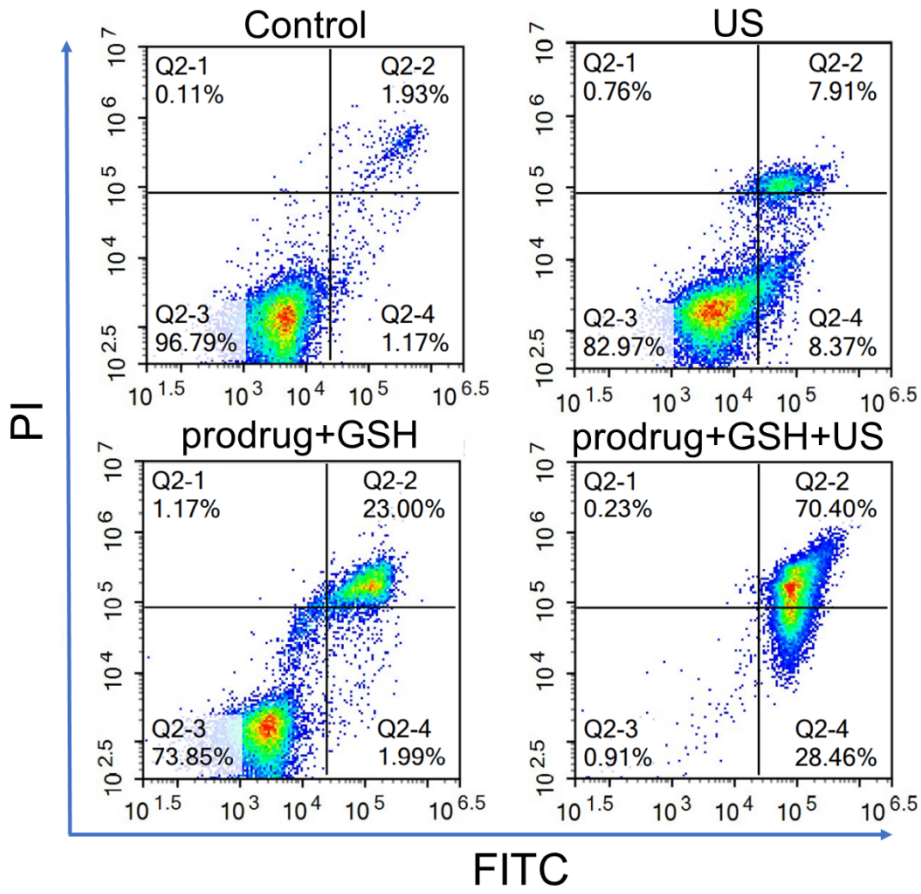

Figure S23. Apoptosis of 4T1 were detected using a flow cytometry after incubation with GSH pre-incubation prodrug (15μM) for 24 h and US radiation for 5 min.

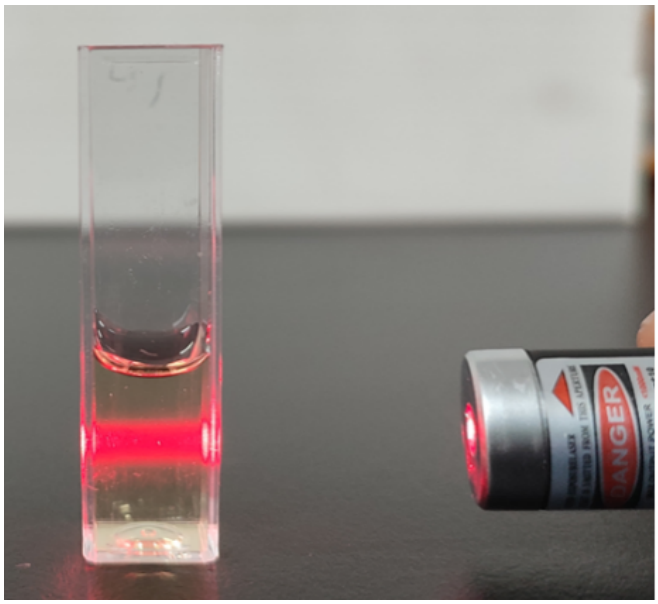

Figure S24. The Tyndall effect of prodrug NPs.

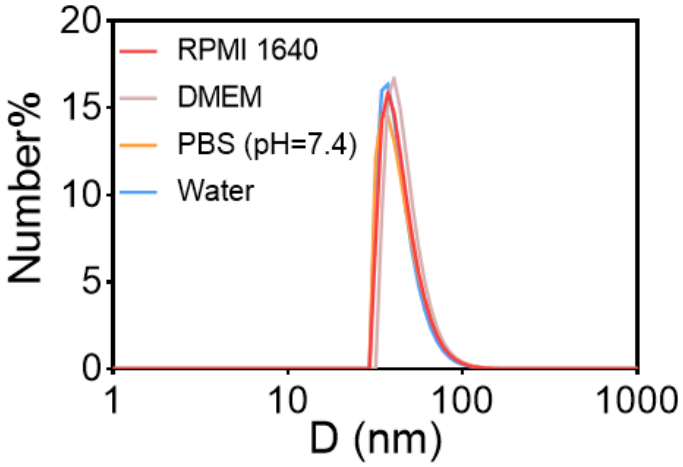

Figure S25. Size of prodrug NPs in water, PBS (pH = 7.4), DMEM, RPMI 1640 culture medium, as measured by DLS.

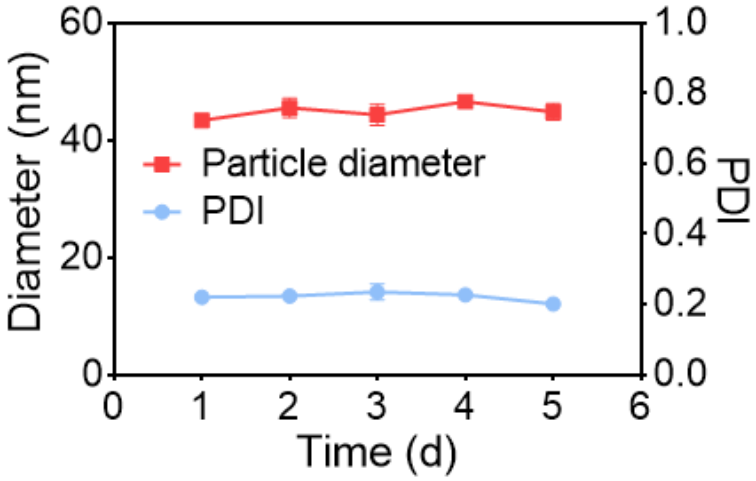

Figure S26. The particle size and PDI stability of prodrug NPs in five days.

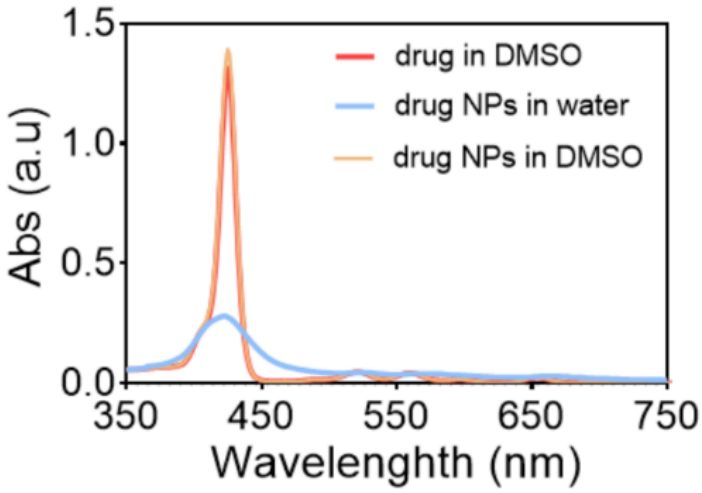

Figure S27. Absorption spectra of drug NPs and drug molecules in different solution, respectively.

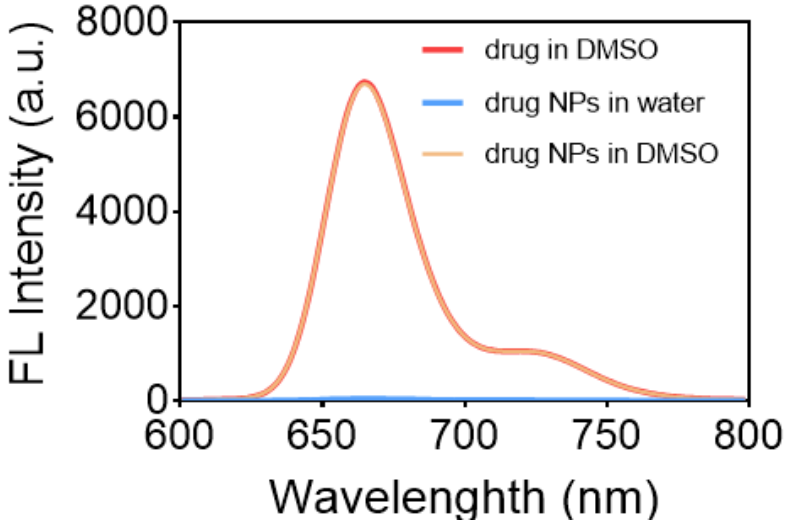

Figure S28. Fluorescence emission spectra of drug NPs and drug molecules in different solution, respectively.

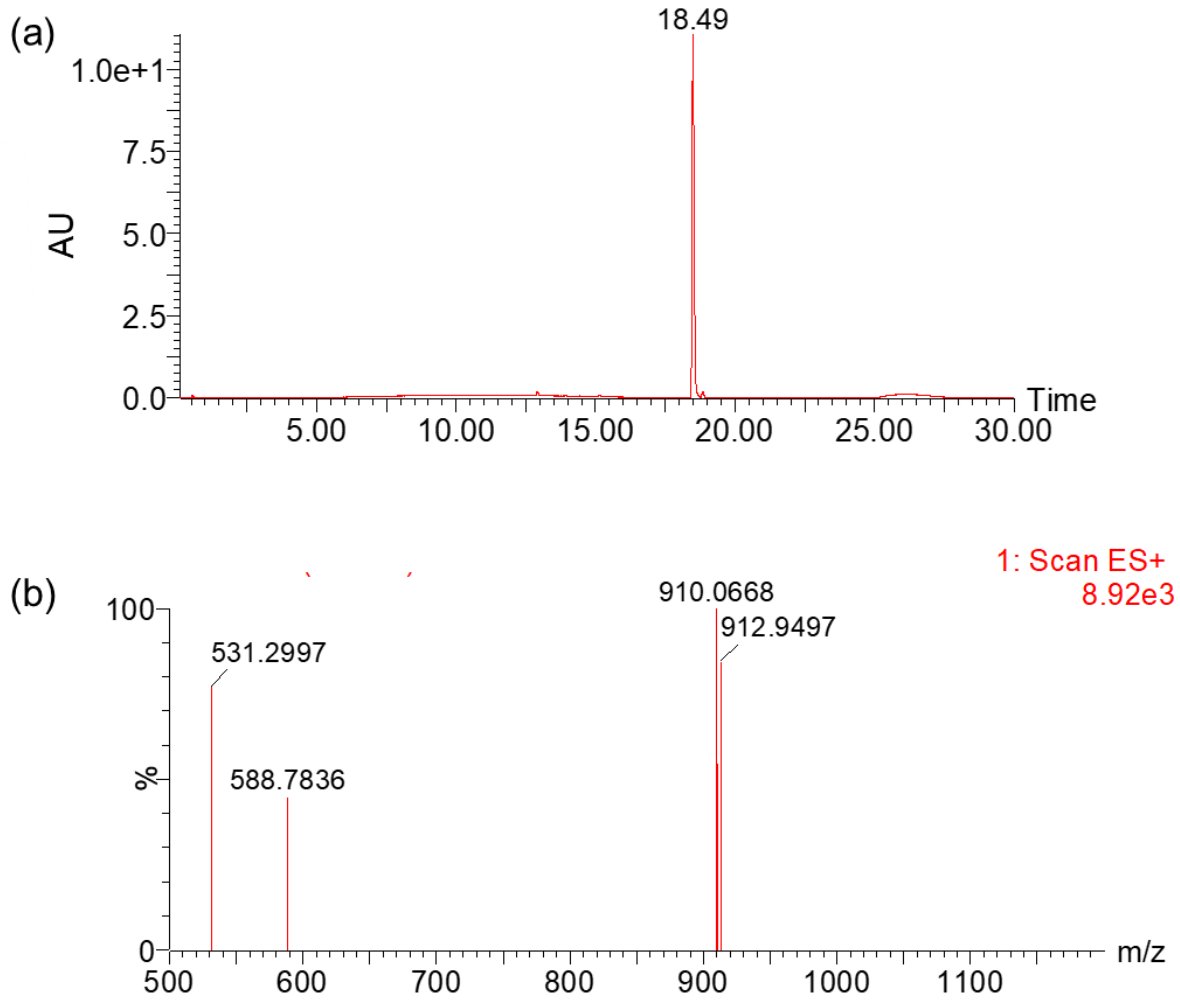

Figure S29. (a) HPLC profiles and (b) Mass spectrum of prodrug NPs after incubation with10% FBS for 24 hours, indicating sufficient stability of prodrug in 10% FBS. The peak at 18.49 min is the prodrug.

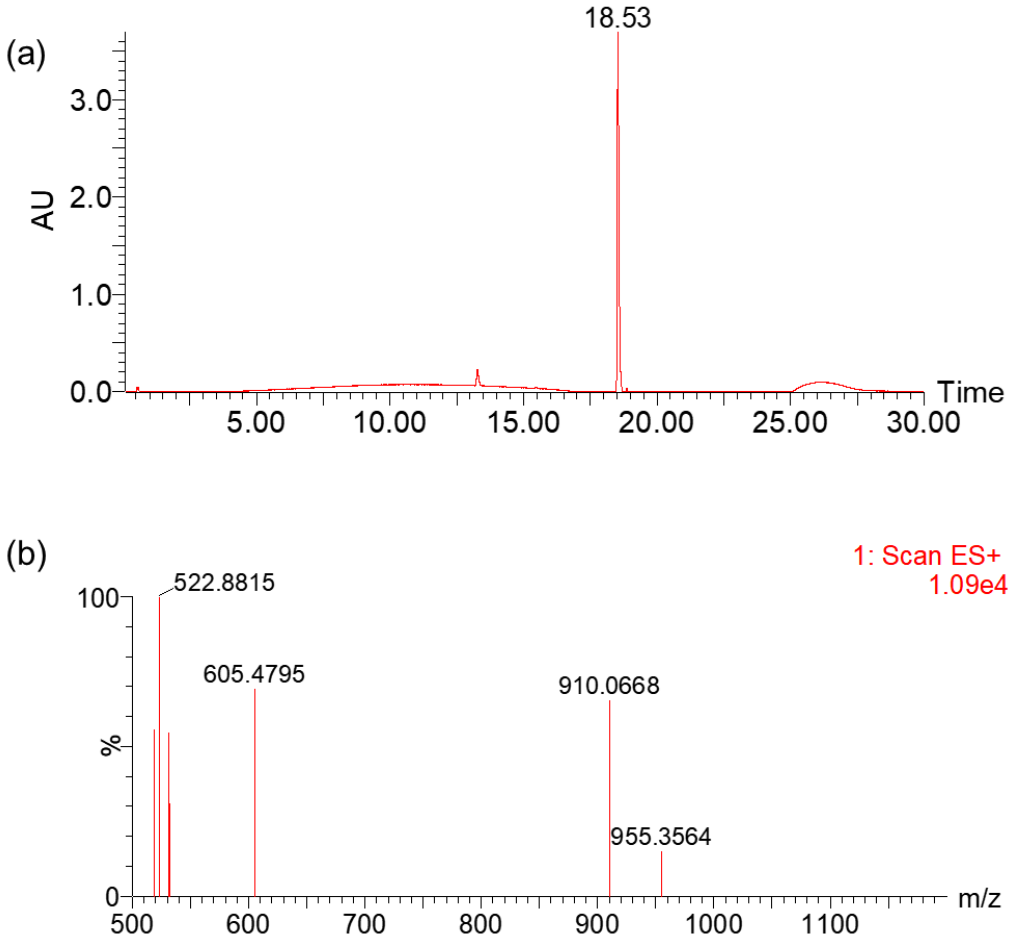

Figure S30. (a) HPLC profiles and (b) Mass spectrum of prodrug NPs after incubation with 90% FBS for 24 hours, indicating sufficient stability of prodrug in 90% FBS. The peak at 18.53 min is the prodrug.

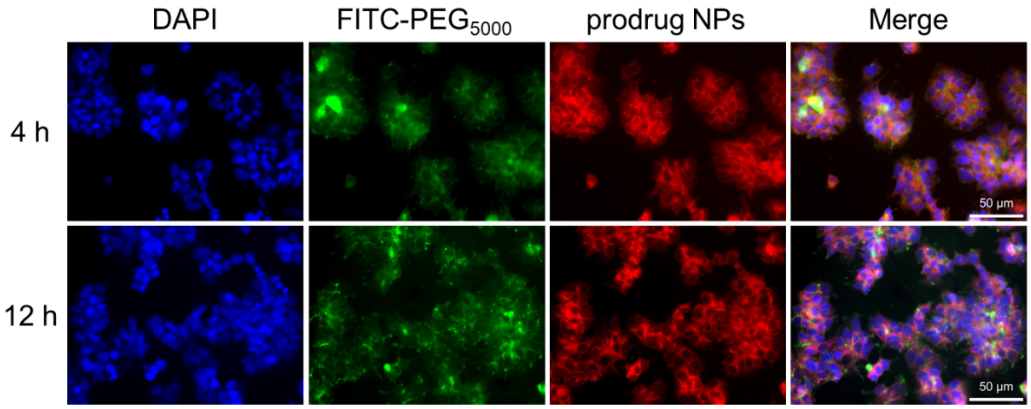

Figure S31. Fluorescence images of 22rv1 tumor cells after incubation with FITC-prodrug NPs for 4 h and 12 h.

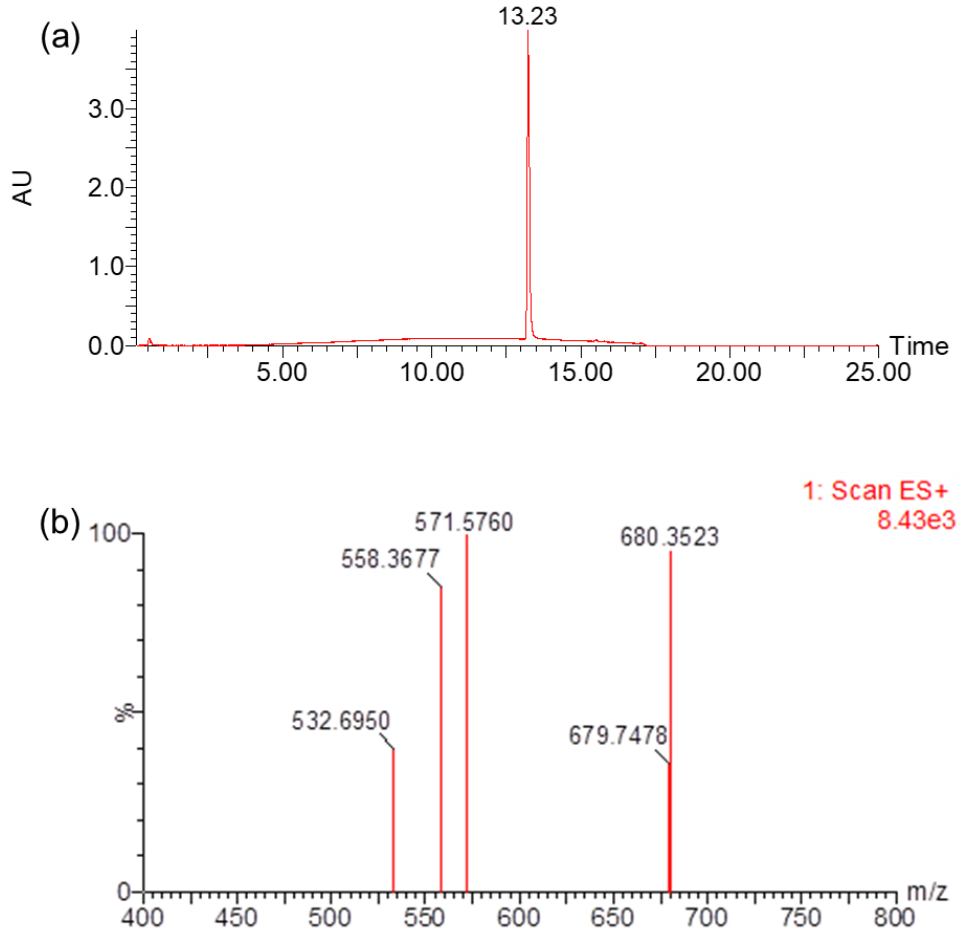

Figure S32. (a) HPLC profiles and (b) Mass spectrum of prodrug NPs after incubation with tumor cell lysate for 24 hours, indicating complete conversion of prodrug into drug. The peak at 13.23 min is the drug.

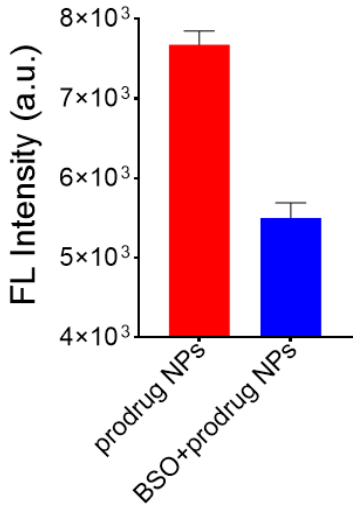

Figure S33. Quantification of fluorescence imaging of 4T1 cells after incubation with prodrug NPs and BSO + prodrug NPs, respectively.

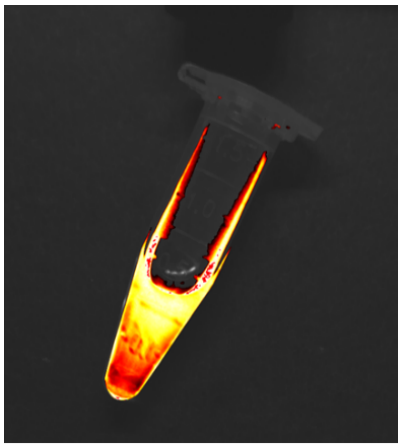

Figure S34. The fluorescence imaging of drug in DMSO in the blue excitation light and cy5.5 emitting channels.

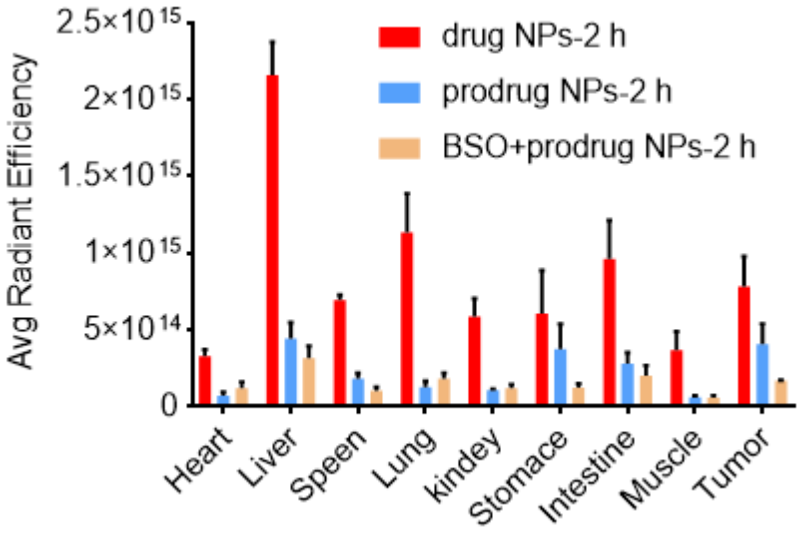

Figure S35. The fluorescence biodistribution at 2 h post injecting prodrug NPs.

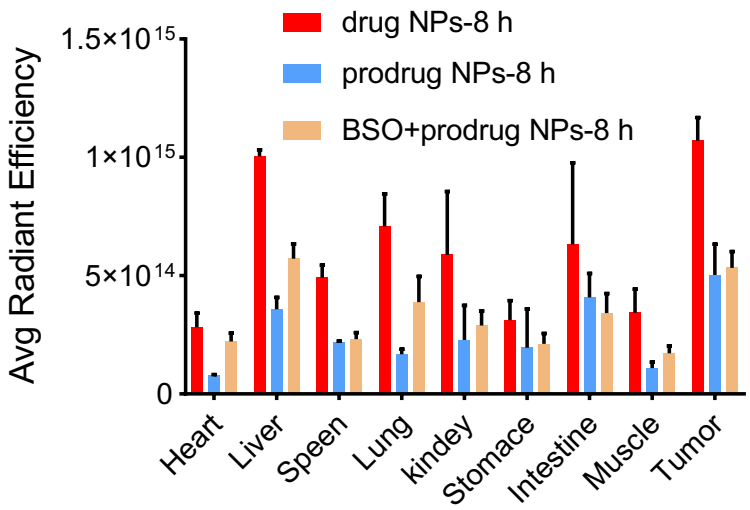

Figure S36. The fluorescence biodistribution at 8 h post injecting prodrug NPs.

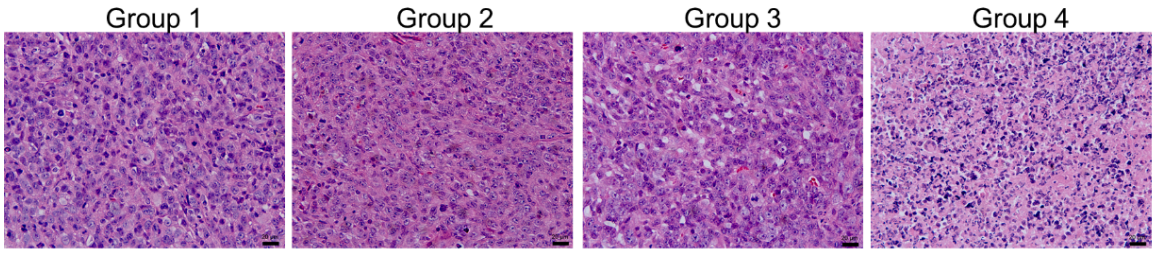

Figure S37. H&E staining of the tumor slices, scale bar = 20 μm.

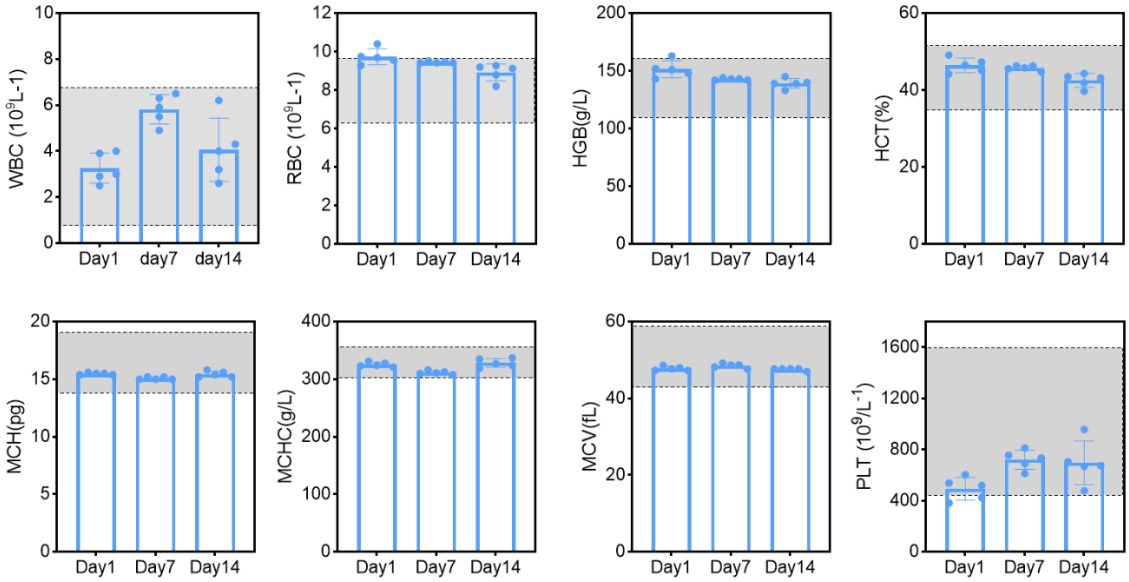

Figure S38. The levels of WBC, RBC, HGB, HCT, MCH, MCHC, MCV and PLT after intravenous injection of prodrug NPs for different times (1, 7 and 14 days).