

## Supporting Information for

## ORIGINAL ARTICLE

# GSH-sensitive polymeric prodrug: Synthesis and loading with photosensitizers as nanoscale chemo-photodynamic anti-cancer nanomedicine

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## 1. Experimental section

### 1.1. Materials and methods

2,2'-[Azobis(1-methylethylidene)] bis[4,5-dihydro-1H-imidazole] dihydrochloride (VA-044) and GSH were purchased from Sigma-Adrich (Shanghai, China). Chlorin e6 (Ce6, MW=596.68), doxorubicin (DOX) and phosphotungstic acid were obtained from Aladdin (Shanghai, China). Size and zeta potential were detected by Zetasizer (Malvern, Worcestershire, UK). Dulbecco's modified Eagle's medium (DMEM) was supplied by Nanjing KeyGEN Biotechnology Corp., Ltd. (Nanjing, China). Fetal bovine serum (FBS) was purchased from Hangzhou Sijiqing Biological Co., Ltd. (Hangzhou, China). Trypsin, Cell Counting Kit-8 for cell viability,

4',6-diamidino-2-phenylindole dihydrochloride (DAPI), syto9, 2',7'-dichlorofluorescein diacetate (DCFH-DA), methanol and acetone were produced by Sigma-Aldrich (Shanghai, China). 9,10-Anthracene-dipropionic acid disodium salt (ADPA) was purchased from Santa Cruz Biotechnology (California, USA). Regenerated cellulose dialysis membrane (Spectra/Por 6, MWCO = 1000 Da) was also supplied by Fisher (Massachusetts, USA). Monomer HPMA was prepared as previously reported<sup>1,2</sup>. The synthesis route for CTA-SS-CTA and the DOX-based monomer MA-SS-DOX was shown in Scheme S1.

<sup>1</sup>H NMR spectroscopy data were obtained *via* a 400 MHz Bruker Advanced Spectrometer, and the chemical shifts were reported in ppm on the  $\delta$  scale. The liquid chromatography-mass spectrometry (LC-MS) spectra were obtained on an Agilent 1200 series 6120 mass spectrometer with electrospray ionization with a Waters  $\times$  Bridge C18 column (50 mm  $\times$  4.6 mm  $\times$  3.5  $\mu$ m), flow rate: 2.0 mL/min, column temperature: 40  $^{\circ}$ C, and mobile phase: 1.0 min for a gradient from 95% [water + 10 mmol/L NH<sub>4</sub>HCO<sub>3</sub>] and 5% [CH<sub>3</sub>CN] to 0% [water + 10 mmol/L NH<sub>4</sub>HCO<sub>3</sub>] and 100% [CH<sub>3</sub>CN] in 3.0 min, then 0.70 min for a gradient from 0% [water + 10 mmol/L NH<sub>4</sub>HCO<sub>3</sub>] and 100% [CH<sub>3</sub>CN] to 95% [water + 10 mmol/L NH<sub>4</sub>HCO<sub>3</sub>] and 5% [CH<sub>3</sub>CN] in 0.05 min. MALDI high resolution mass spectra (HRMS) were recorded by ESI-TOF, and HRMS data were recorded on a Bruker Daltonics Bio TOF mass spectrometer.

Weight-averaged MW and polydispersity (PDI) of the polymers were measured *via* size-exclusion chromatography (SEC) on an ÄKTA/FPLC system (GE Healthcare) with a Superose 6 HR10/30 column. Sodium acetate buffer/methanol (7:3, pH 6.5) was used as the mobile phase with a flow rate of 0.4 mL/min. The polymers were purified by SEC via a Superose 6 HR10/30 column, while the mobile phase was

sodium acetate buffer/methanol (7: 3, pH 6.2), the flow rate was 2.5 mL/min, and the temperature was controlled at 4 °C. The copolymer polyDOX-*block*-(polyHPMA-SS-polyHPMA)-*block*-polyDOX was fractionated/purified by size exclusion chromatography using Superose 6 HR10/30 (MW range for hydrophilic neutral polymer 15–300 kDa/14 mL separation volume) column on an ÄKTA FPLC system (GE Healthcare) column with sodium acetate buffer containing 30% methanol (pH = 6.5) as the mobile phase.

### 1.2. Synthesis of functionalized chain transfer agent (CTA-SS-CTA)

Under nitrogen, 4-cyano-4-thiobenzoylsulfanyl-butyric acid (5.25 g, 18 mmol) and DMAP (2.21 g, 18 mmol) were dissolved in anhydrous DCM (200 mL). The solution was stirred for 5 min in ice bath, and EDCI (3.44 g, 18 mmol) was added and stirred for 30 min. 2-Hydroxyethyl disulfide (1.39 g, 9 mmol, in 10 mL DCM) was added and the solution was stirred in ice bath for 30 min and at room temperature for another 18 h. The solution was washed with 1 mol/L HCl and NaCl aq. (satd.). The solution was dried by anhydrous MgSO<sub>4</sub> and the solvent was removed by rotary evaporation. The crude product was isolated by column chromatography (silica gel) (EtOAc/Hexane = 1:4–1:3). The final compound was obtained as red oil in 78.5% yield (4.78 g, 7.06 mmol). <sup>1</sup>H NMR (400 MHz,  $\delta$  in ppm) (Fig. S1), <sup>13</sup>C NMR (100 MHz, *d*<sub>6</sub>-DMSO,  $\delta$  in ppm) (Fig. S2): LC-MS (ES<sup>+</sup>): *m/z* 677.1 [M+H]<sup>+</sup>, 699 [M+Na]<sup>+</sup> (Figs. S3 and 4). MALDI-HRMS: *m/z* 699.0583 [M+Na]<sup>+</sup> (Fig. S5).

### 1.3. Synthesis of functionalized monomer MA-SS-DOX

Under nitrogen, 2-hydroxyethyl disulfide (15.4 g, 100 mmol) was dissolved in 200 mL DCM) and 30 mmol DIPEA was added, and the solution was stirred at 0 °C for over 10 min. Methacryloyl chloride (10.4 g, 9.7 mL, 100 mmol, in 50 mL DCM) was added to the above solution by dropwise. The solution was stirred in ice bath for 30

min and at room temperature for another 2 h. The solution was removed by rotary evaporation. The crude product was isolated by column chromatography (silica gel) (EtOAc/Hexane = 1:6–1:3). The final compound (MA-SS-OH) was obtained as oil in 42.5% yield (9.44 g, 42.5 mmol). LC–MS (ES<sup>+</sup>): *m/z* 240.2 [M+NH<sub>4</sub>]<sup>+</sup> (Figs. S6 and 7).

#### *1.4. Preparation of drug loaded nanoparticles*

Blank nanoparticles [NPs(Ce6)] and Photosensitizer (Ce6) loaded nanoparticles [NPs(Ce6)] were prepared via a thin-film hydration method<sup>3</sup>. For the preparation of NPs(Ce6), Ce6 (1 mg) and polyDOX-*block*-(polyHPMA-SS-polyHPMA)-*block*-polyDOX (10 mg) were dissolved in methanol (3 mL) and chloroform (2 mL), respectively. The mixed solution was then evaporated at 40 °C under the vacuum to form a thin-film. 0.01 mol/L phosphate buffer saline (PBS, pH 7.4) was added dropwise to hydrate the thin-film. After stirring for 6 h at room temperature, the solution was filtered through a 0.22 μm membrane filter to remove unloaded hydrophobic drugs. The final product was stored at 4 °C for further use. NPs were prepared in the same manner except mixing with Ce6.

#### *1.5. Critical micelle concentration (CMC) determination*

The CMC value was determined by using pyrene as the fluorescence dye. The polymer concentration ranged from 1 × 10<sup>-4</sup> to 1 mg/mL with a pyrene concentration of 6 × 10<sup>-6</sup> mol/L. The emission spectra of pyrene (Ex = 334 nm) were determined by F-7000 fluorescence spectrophotometer (Hitachi, Japan).

#### *1.6. In vitro release and degradation*

For NPs(Ce6), DOX and Ce6 releasing profiles were tested by utilizing dialysis bag diffusion method. NPs(Ce6) solution was divided into four equal aliquots. Each

dialysis bag (MWCO = 3500 Da) was filled with 1 mL sample solution. The dialysis bags were immersed in 50 mL PBS solution with or without the presence of GSH (pH=7.4, pH=5.4, pH=7.4 + 3 mmol/L GSM and pH=5.4 + 3 mmol/L GSH) at 37 °C and shaking at 100 rpm, respectively. The solution was sampled at desired time intervals and the cumulative percentages were calculated by using the standard curves. Additionally, the samples were also used for SEC studies on the MWs.

#### *1.7. Particle size, zeta-potential and morphology characterization.*

The particle size and zeta potential of NPs and NPs(Ce6) were determined using Zeta-sizer (Malvern Instruments, Malvern, UK). The morphology of nanoparticles was observed under JEM1200EX (JEOL, Japan) transmission electron microscope (TEM). The micelles solutions were dropped onto the surface of copper grid with carbon film followed by removal of excess fluid with filter paper. They were negatively stained with uranyl acetate for 90 s and air-dried at room temperature before observation.

#### *1.8. In vivo safety evaluation.*

After administration of different formulations in tumor-bearing mice (15–20 g,  $n=6$ ), blood was withdrawn from the mice orbit to measure blood biochemical indicators including white blood cells (WBC), red blood cells (RBC), hemoglobin and mean RBC hemoglobin concentration (HGB and MCHC), hematocrit (HCT), average RBC hemoglobin (MCH) and mean platelet volume (MPV).

#### *1.9. Pharmacokinetic experiments:*

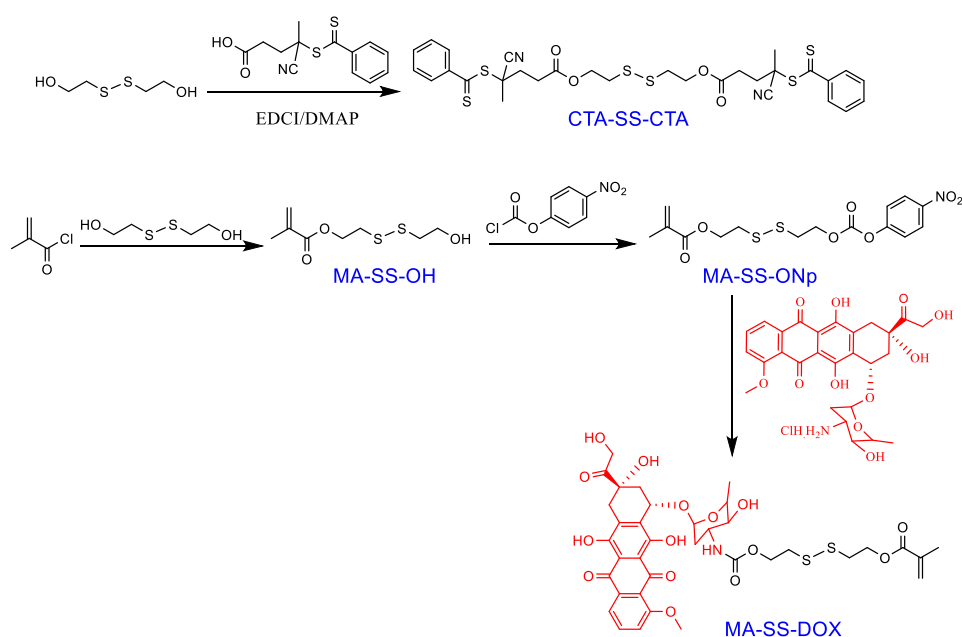
BALB/c mice, with an average body weight of about 20 g, half male and half female ( $n = 6$ ), were injected with DOX and NPs (Ce6) *via* tail vein at a dose of 4 mg/kg. Blood was taken from the orbit of the mice at 0.5, 2, 4, 8, 24, 48, 72 h and the content of DOX in plasma was determined by HPLC. The pharmacokinetic software

PKSolver 2.0 was used to calculate pharmacokinetic parameters.

### 1.10. Cell culture and animals

All cells were supplied by KeyGEN Ltd. The murine breast cancer cells (4T1) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (streptomycin, 100 U/mL, plus penicillin). Cells were cultured in an incubator at 37 °C with 5% CO<sub>2</sub>. 0.02% EDTA and 0.025% trypsin were applied to detach the cells.

The mice were purchased from the Experimental Animal Center, Chongqing University of Medicine in China. The experiment protocol was reviewed and performed in compliance with China's guidelines for care and use of laboratory animals, and approved by the experimental animal ethics committee of the College of Pharmaceutical Sciences, Southwest University (No. 001563). Some animal experiments were carried out at West China Hospital and the animal operation procedures were approved by the ethics committee of West China Hospital, Sichuan University (No. 2018148A).



**Scheme S1** Preparation of the chain transfer agent (CTA-SS-CTA) and the DOX-based monomer MA-SS-DOX.

## 2. Results

**Table S1** Characterization of the prodrug and NPs(Ce6).

|          | MW <sup>a</sup> | PDI <sup>b</sup> | DOX <sup>c</sup> | Ce6 <sup>d</sup> | EECe6 <sup>e</sup> | DLCE6 <sup>f</sup> | DLS <sup>g</sup> | TEM <sup>h</sup> | PDI <sup>i</sup> | $\zeta$ <sup>j</sup> |
|----------|-----------------|------------------|------------------|------------------|--------------------|--------------------|------------------|------------------|------------------|----------------------|
| Prodrug  | 93.5            | 1.18             | 8.9              | N/A              | N/A                | N/A                | 60.3±4.1         | 25.5 ±6.6        | 0.106            | -8.08±1.25           |
| NPs(Ce6) | N/A             | N/A              | 8.3%             | 7.1%             | 76.2±5.2           | 7.1±1.5            | 122.6±5.5        | 41.7 ±9.4        | 0.149            | -7.54±0.87           |

<sup>a</sup>Molecular weight (MW) with a unit of kDa, and <sup>b</sup> and <sup>i</sup> polydispersity index (PDI); <sup>c,d</sup>The contents of drug DOX and Ce6 content with weight percent (%); <sup>e</sup>Encapsulation efficiency of Ce6; <sup>f</sup>Drug loading capacity of Ce6; <sup>g</sup>Size from DLS as d.nm; <sup>h</sup>Size from TEM as d.nm; <sup>j</sup>Zeta potential ( $\zeta$ ) from DLS as mV.

**Table S2** Administration formulations for different groups

| Groups           | C <sub>DOX</sub> (mg/kg) | C <sub>Ce6</sub> (mg/kg) |
|------------------|--------------------------|--------------------------|
| NPs(Ce6) + Laser | 4.0                      | 5                        |
| NPs(Ce6)         | 4.0                      | 5                        |
| NPs + Laser      | 4.0                      | N/A                      |
| NPs              | 4.0                      | N/A                      |
| Ce6 + Laser      | N/A                      | 5                        |
| DOX              | 4.0                      | N/A                      |
| Laser            | N/A                      | N/A                      |
| Control          | N/A                      | N/A                      |

**Table S3** Degraded segments after incubation of the prepared prodrug in McIlvaine's buffer with 3 mM GSH at 37 °C.

| name      | 0 h  | 1 h  | 4 h  | 8 h  | 12 h |
|-----------|------|------|------|------|------|
| MWs (kDa) | 93.3 | 74.5 | 49.6 | 40.6 | 40.8 |
| PDI       | 1.16 | 1.44 | 1.29 | 1.20 | 1.21 |

**Table S4** Pharmacokinetic parameters of DOX and NPs (Ce6) in mice.

| Groups   | $t_{1/2}$ (h) | AUC ( $\mu\text{g/mL}\cdot\text{h}$ ) | CL (L/h)  | MRT (h)    | V <sub>ss</sub> (L) |
|----------|---------------|---------------------------------------|-----------|------------|---------------------|
| DOX      | 6.51±0.46     | 2.08±0.29                             | 0.04±0.02 | 4.57±0.82  | 0.17±0.03           |
| NPs(Ce6) | 21.93±3.31    | 10.14±2.36                            | 0.02±0.01 | 28.80±4.13 | 0.21±0.01           |

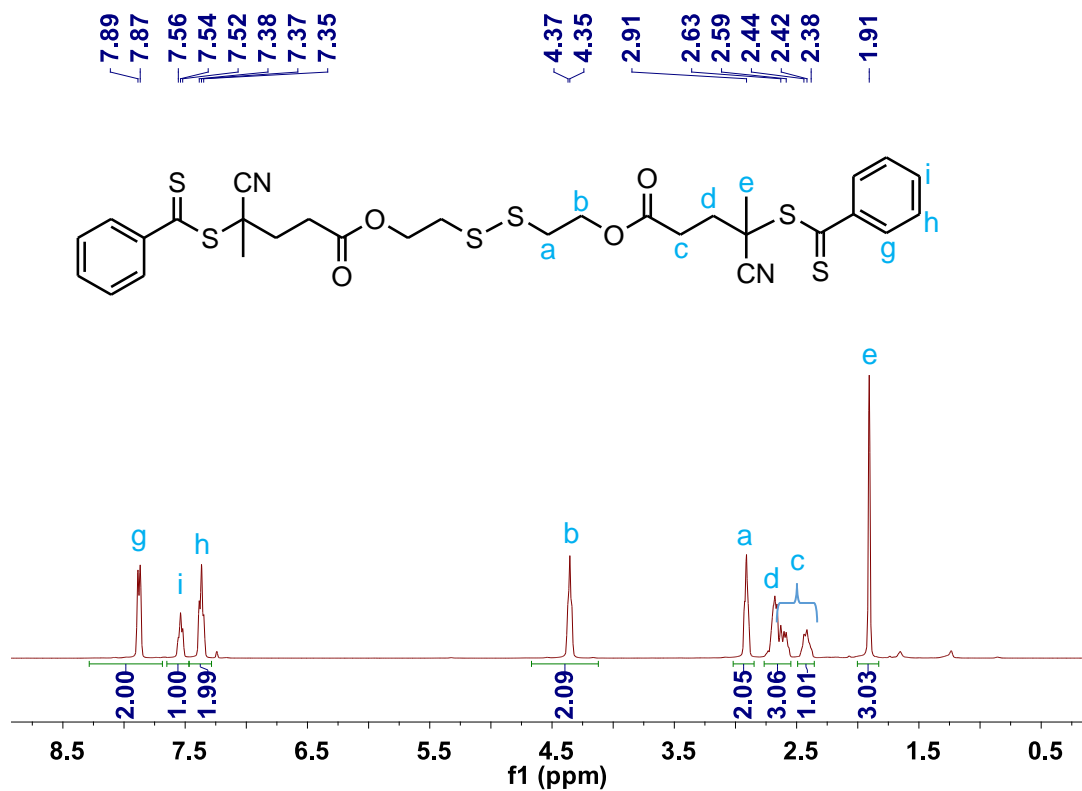


Figure S1  $^1\text{H}$  NMR spectrum of CTA-SS-CTA (recorded in CDCl<sub>3</sub>).

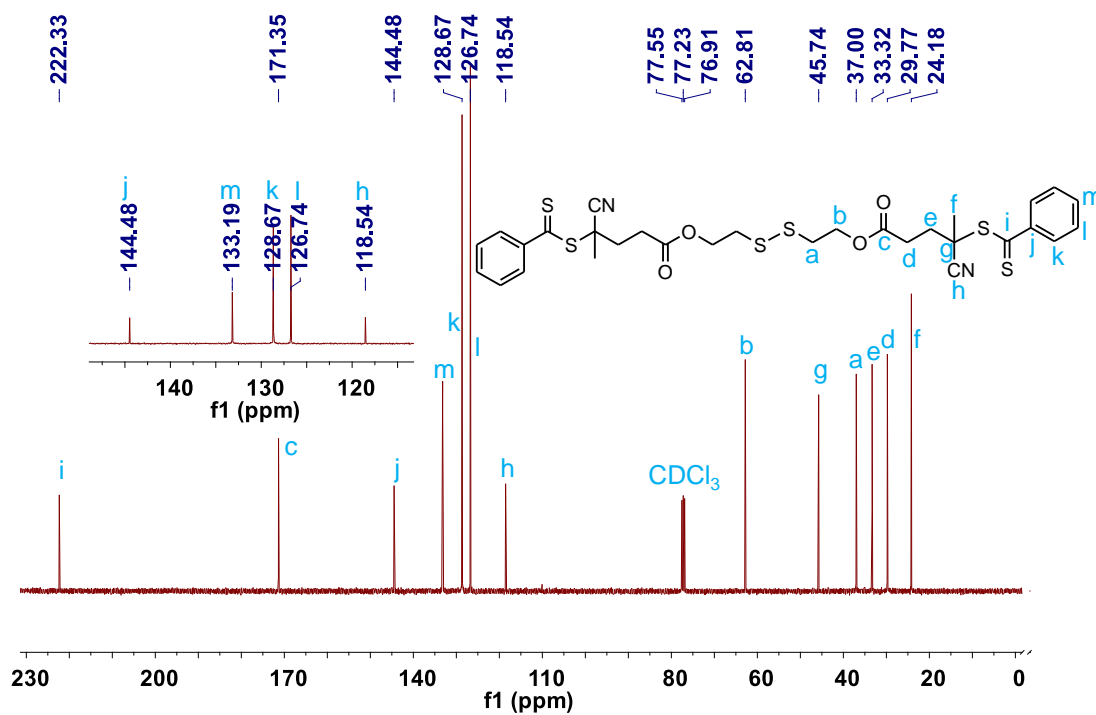
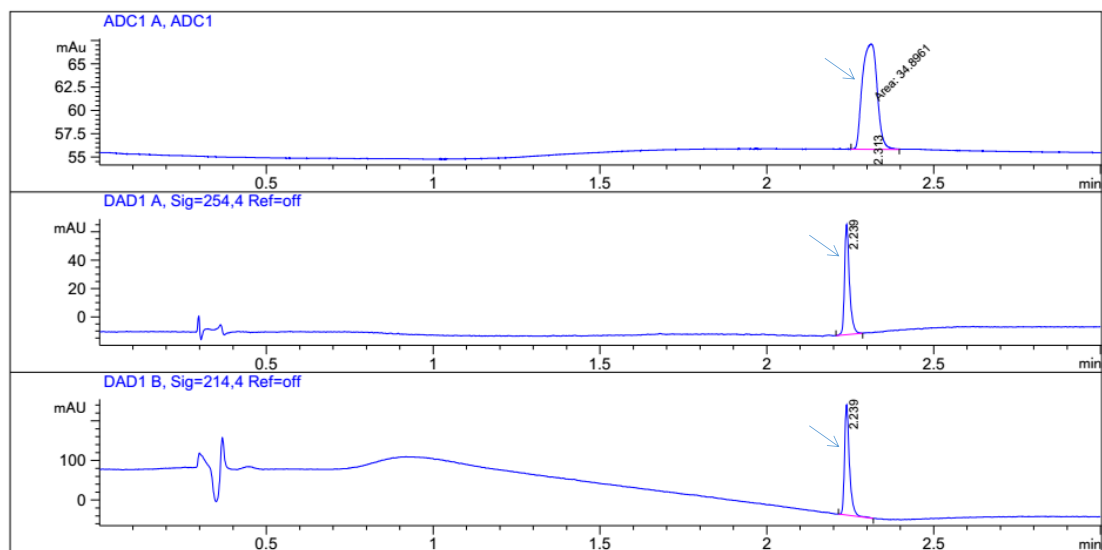
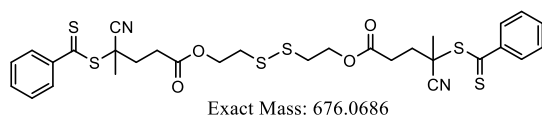
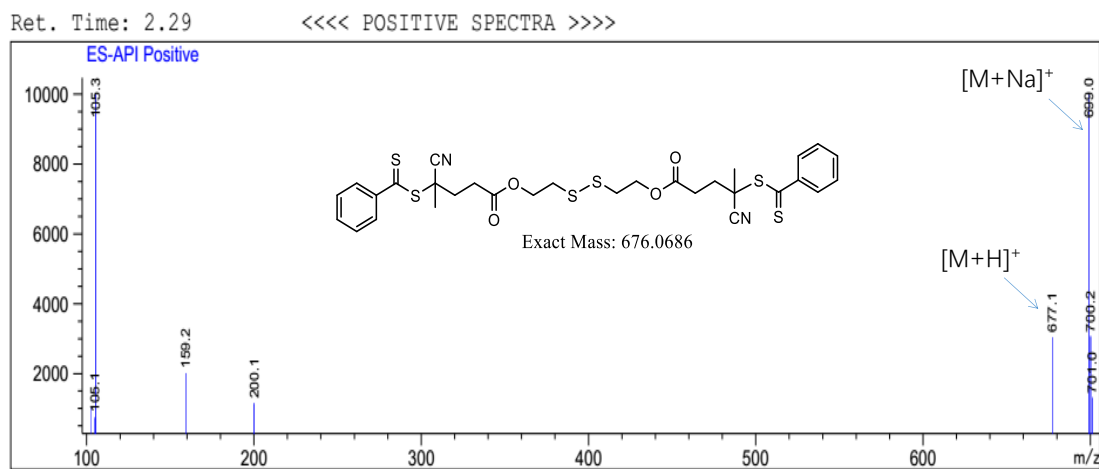


Figure S2  $^{13}\text{C}$  NMR spectrum of CTA-SS-CTA (recorded in CDCl<sub>3</sub>).

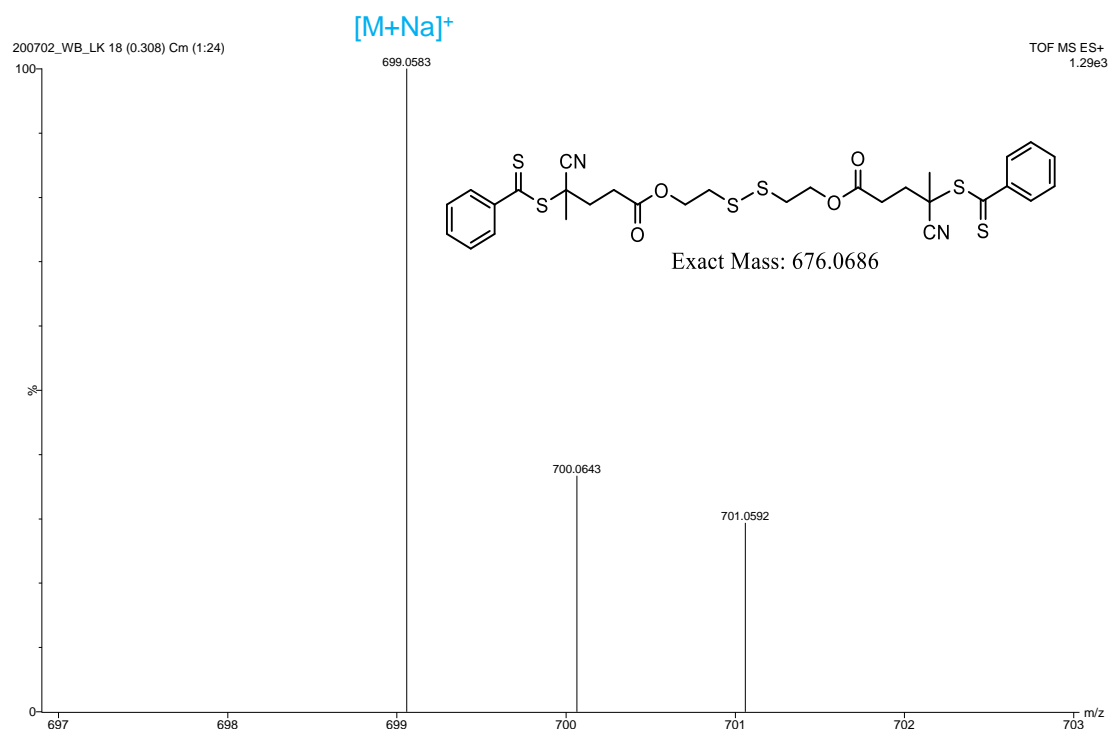




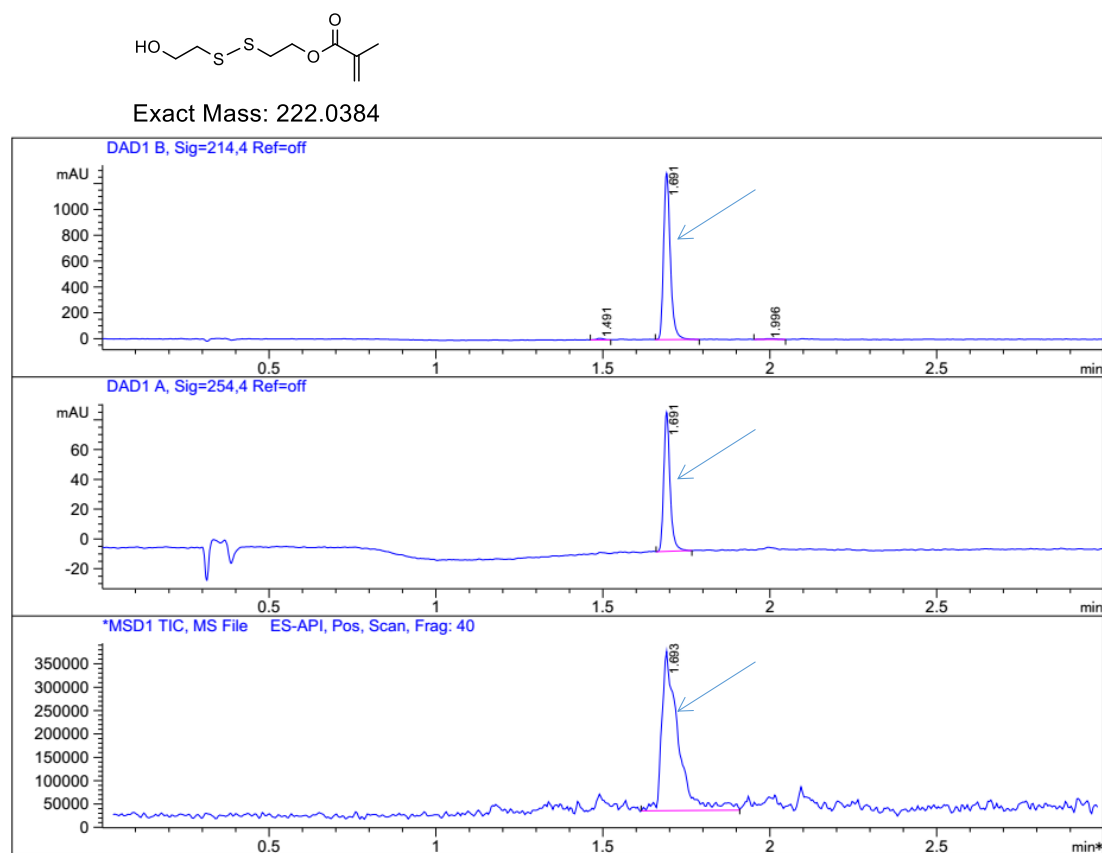
**Figure S3** LC-MS spectra of the CTA-SS-CTA. The labeled peaks are corresponded to the product peaks (recorded in the acid method).



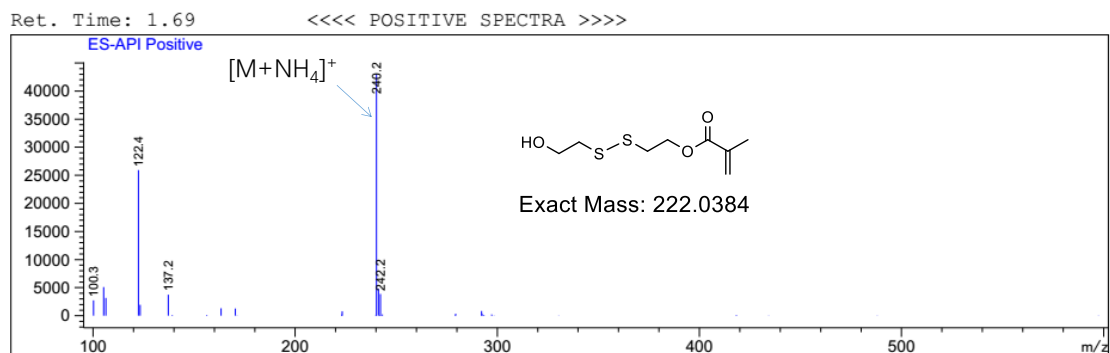
**Figure S4** LC-MS spectra of the CTA-SS-CTA. The product had ion peaks at 677.1  $m/z$  for  $[M+H]^+$  and 609.0  $m/z$  for  $[M+Na]^+$  (recorded in the acid method).



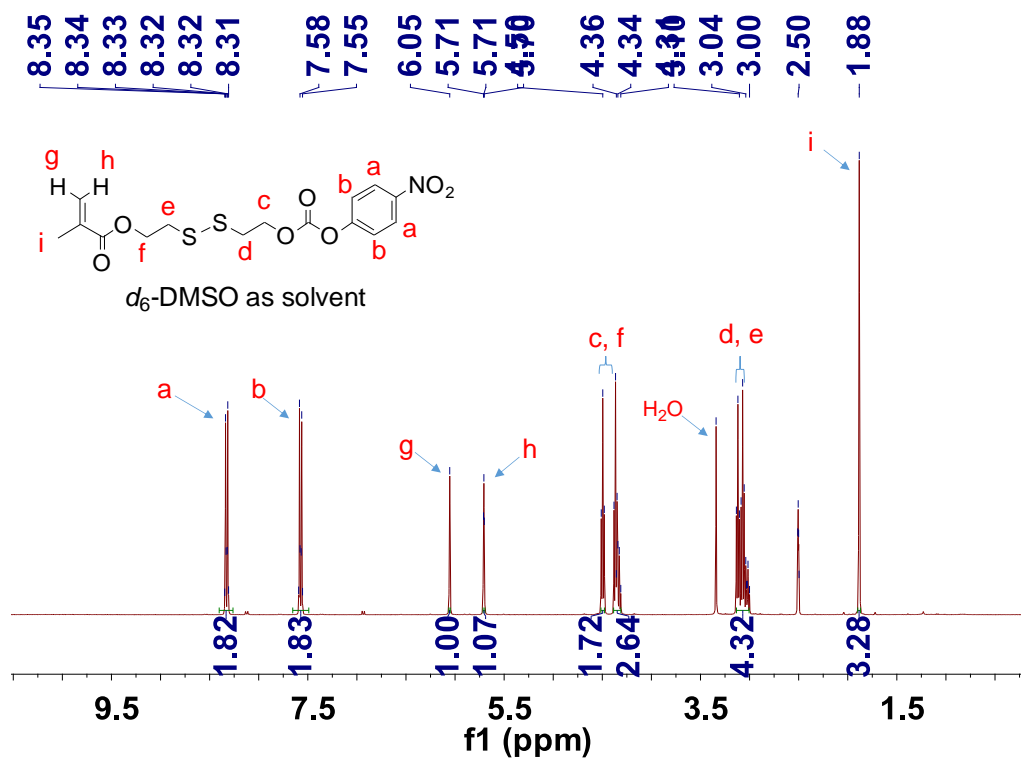
**Figure S5** MALDI-HR MS spectrum of CTA-SS-CTA, displaying ion peaks at 699.0583  $m/z$  for  $[M+Na]^+$ .



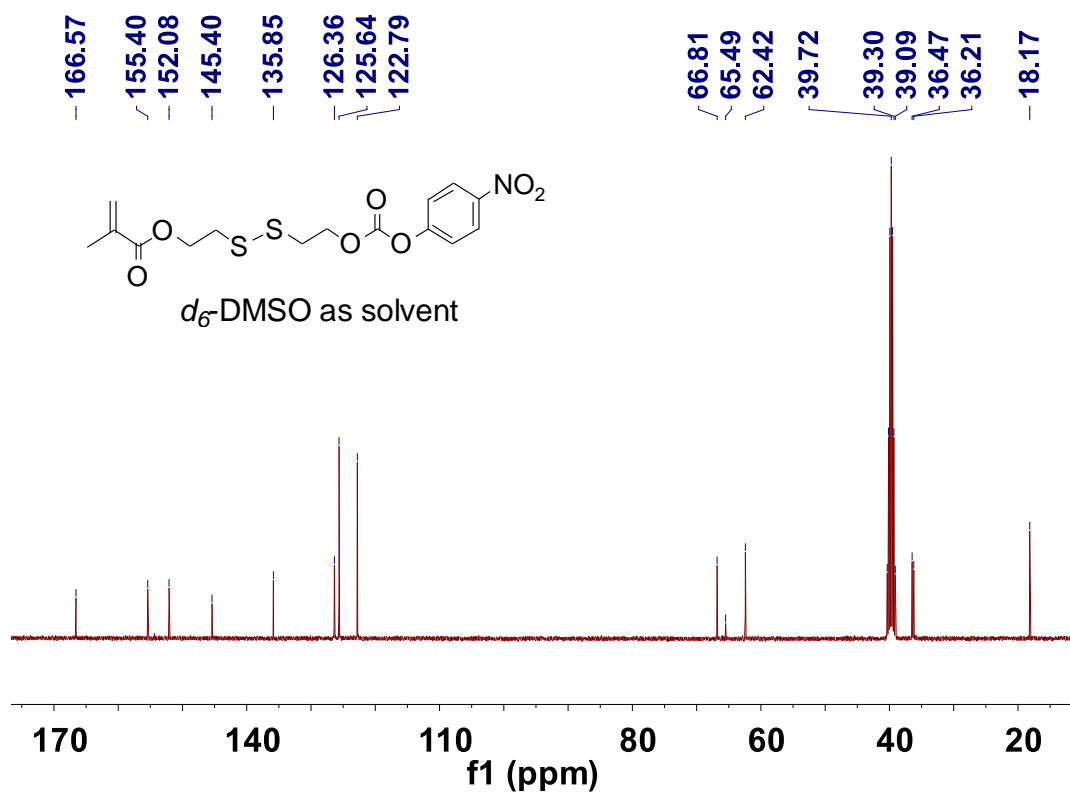
**Figure S6** LC-MS spectra of the MA-SS-OH. The labeled peaks corresponded to the product peaks (recorded in the acid method).



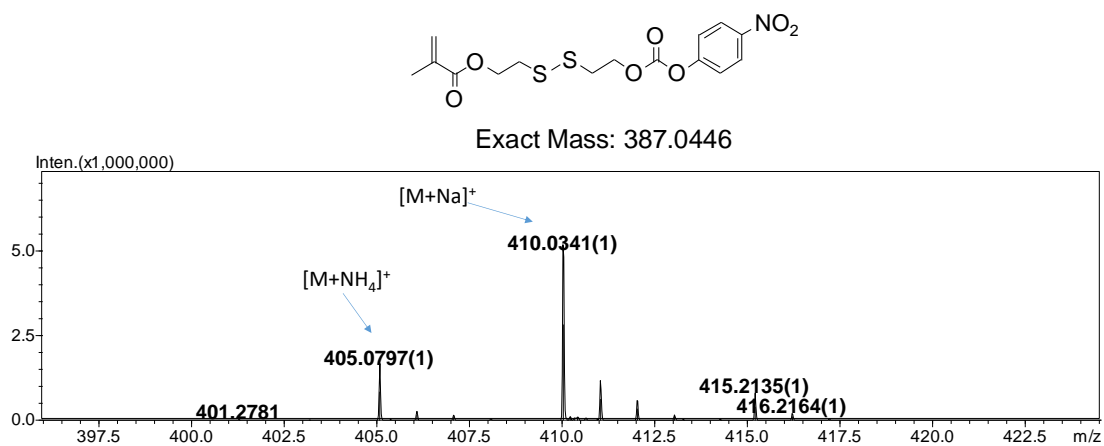
**Figure S7** LC-MS spectra of the MA-SS-OH. The product had an ion peak at 240.2  $m/z$  for  $[M+NH_4]^+$  (recorded in the acid method).



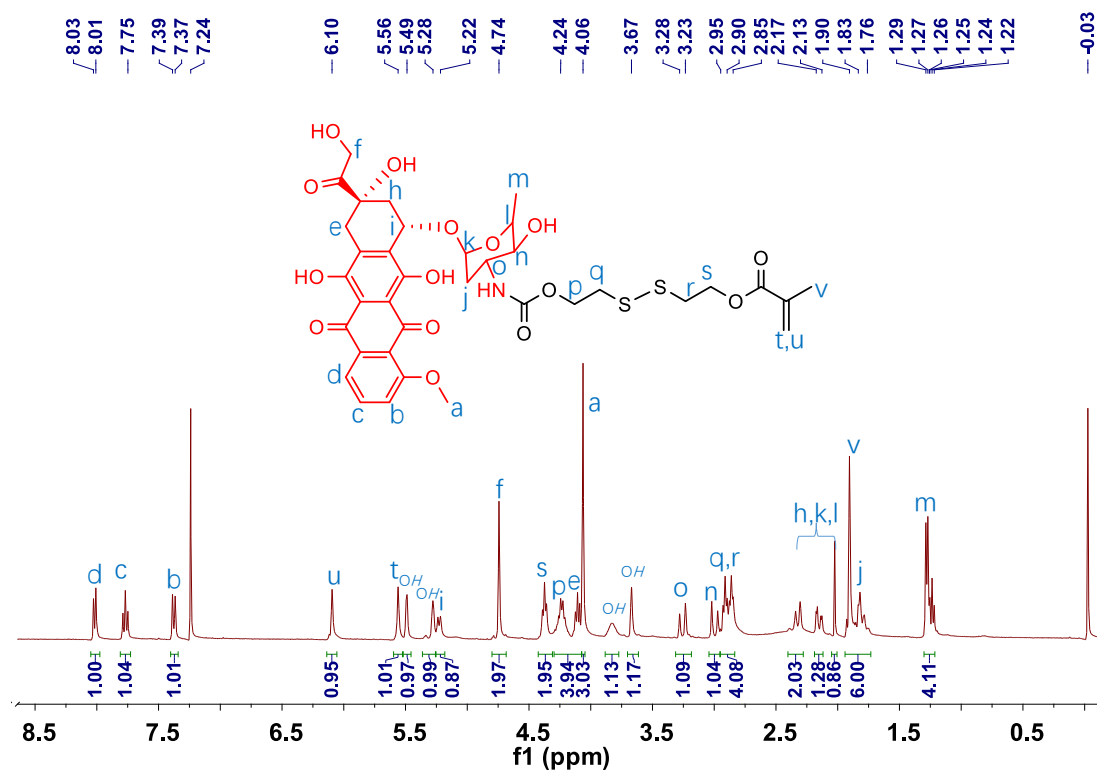
**Figure S8**  $^1H$  NMR spectrum of MA-SS-ONp (recorded in  $d_6$ -DMSO).



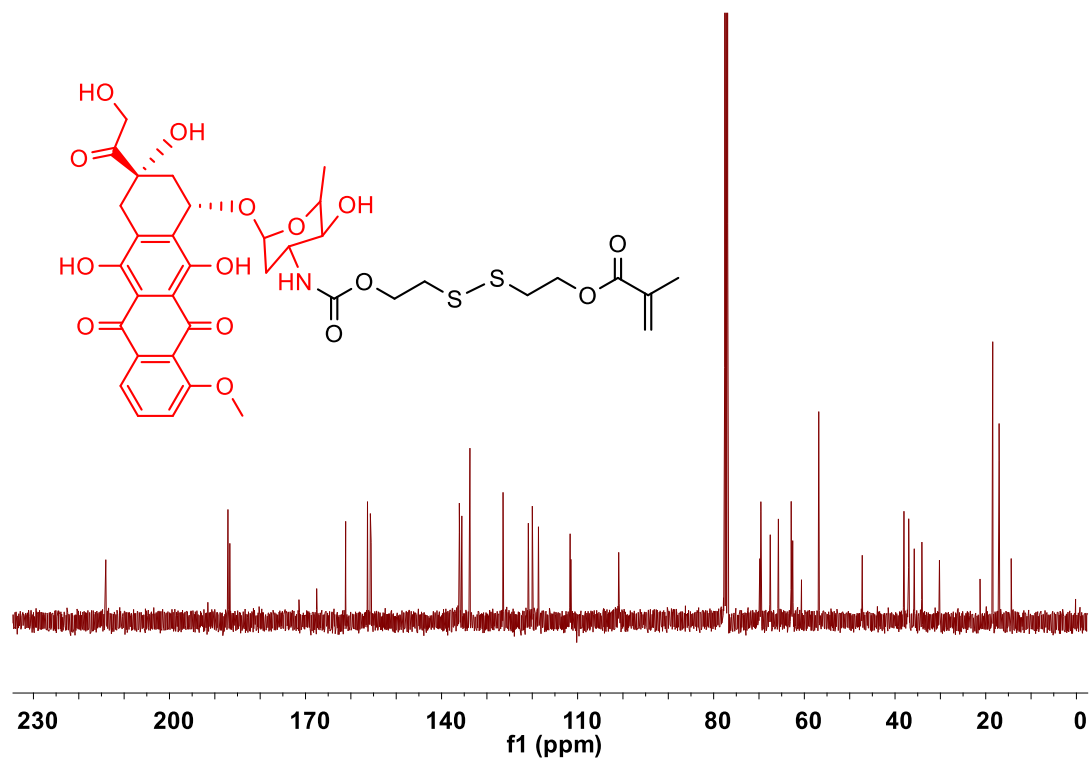
**Figure S9**  $^{13}\text{C}$  NMR spectrum of MA-SS-ONp (recorded in  $d_6$ -DMSO).



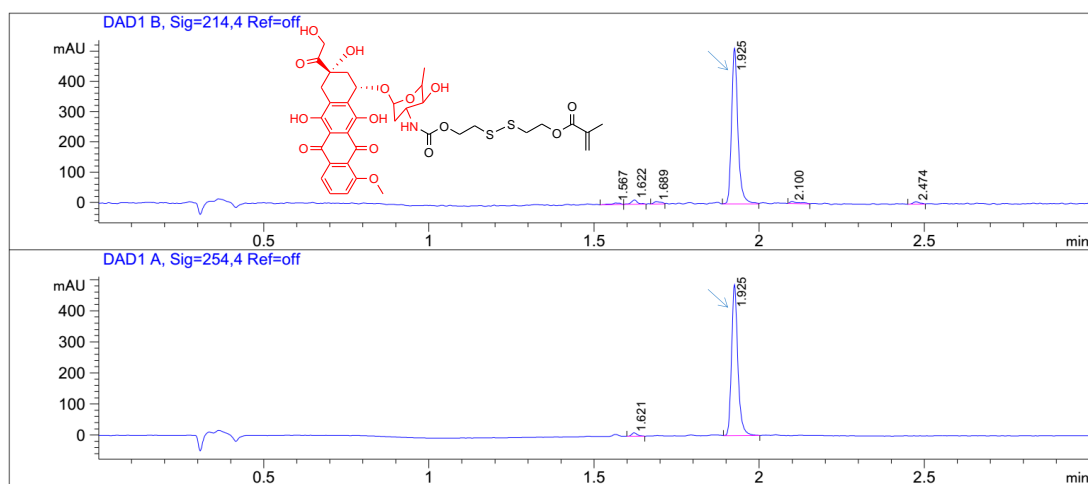
**Figure S10** MALDI-HR MS spectrum of MA-SS-ONp, displaying ion peaks at 405.0797  $m/z$  for  $[\text{M}+\text{NH}_4]^+$  and 410.0341  $m/z$  for  $[\text{M}+\text{Na}]^+$ .



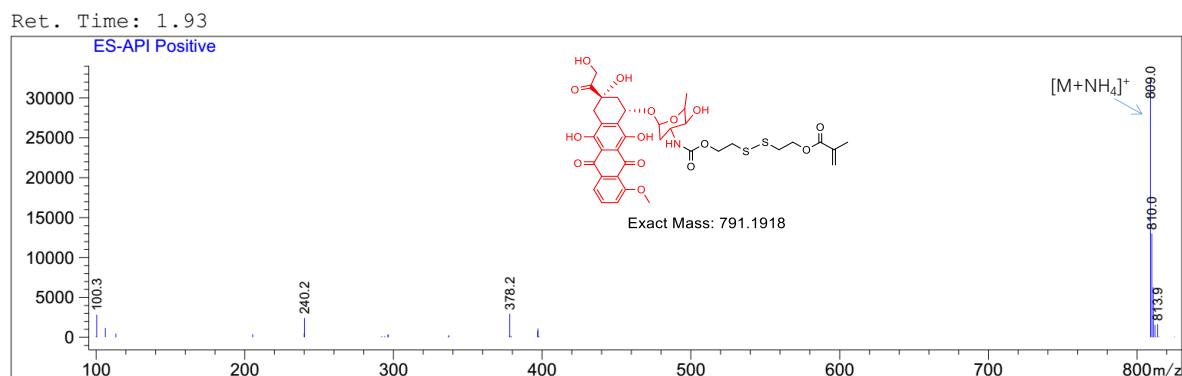
**Figure S11**  $^1\text{H}$  NMR spectrum of the monomer MA-SS-DOX (recorded in  $\text{CDCl}_3$ ).



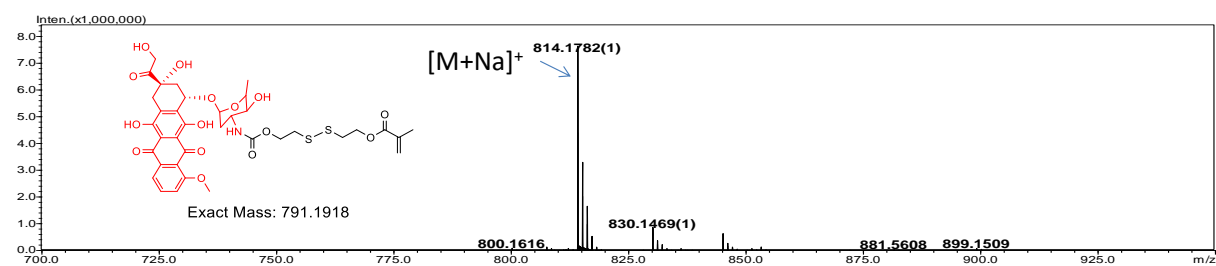
**Figure S12**  $^{13}\text{C}$  NMR spectrum of the monomer MA-SS-DOX (recorded in  $\text{CDCl}_3$ ).



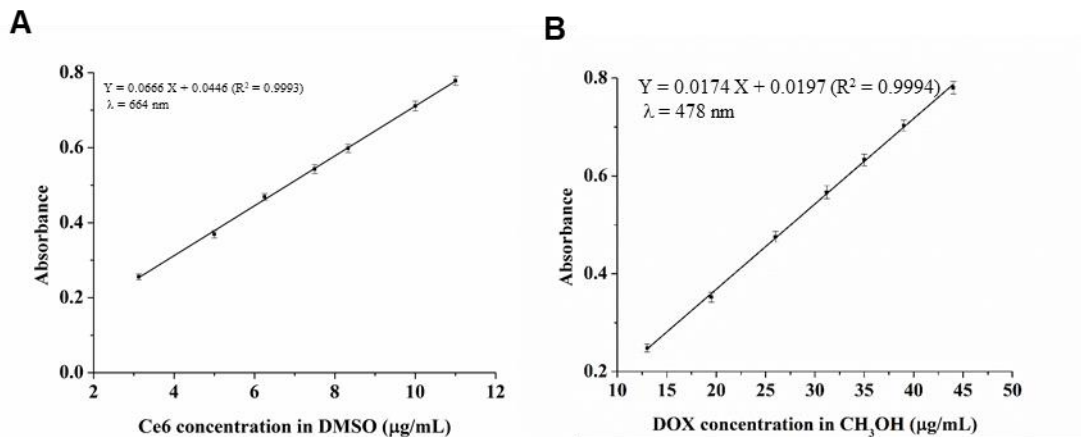
**Figure S13** LC-MS spectra of the MA-SS-DOX. The labeled peaks are corresponded to the product peaks (recorded in the acid method).



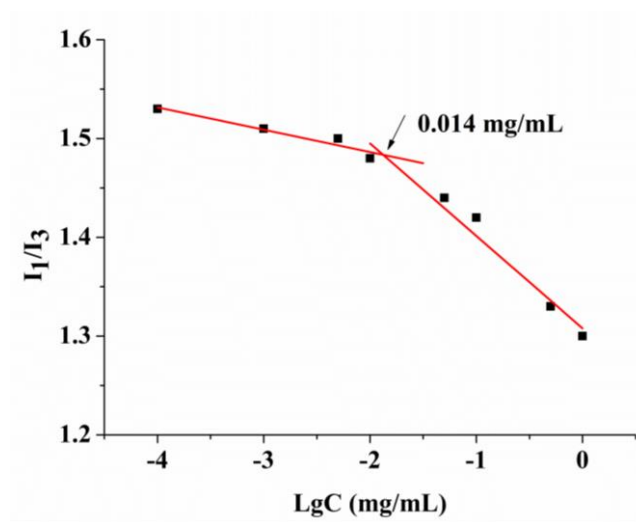
**Figure S14** LC-MS spectra of the MA-SS-DOX. The product had an ion peak at 809  $m/z$  for  $[M+NH_4]^+$  (recorded in the acid method).



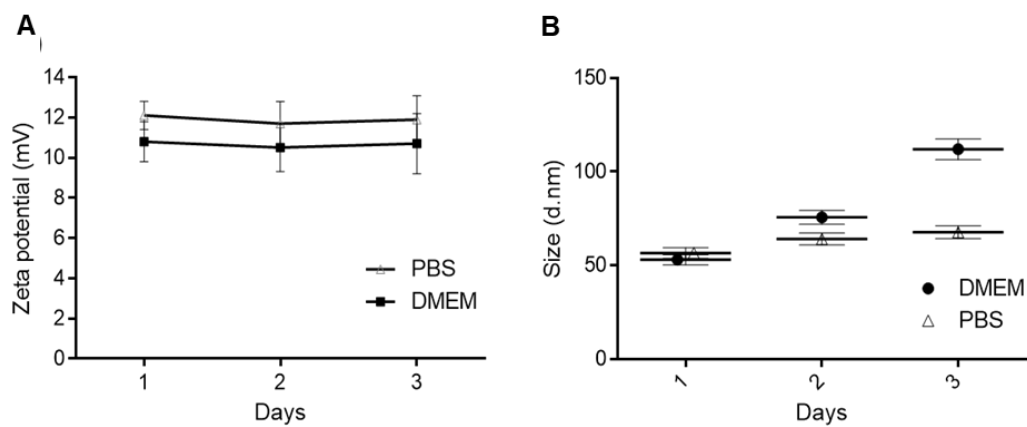
**Figure S15** MALDI-HRMS spectrum of MA-SS-DOX, displaying a single ion peak at 814.1782  $m/z$  for  $[M+Na]^+$ .



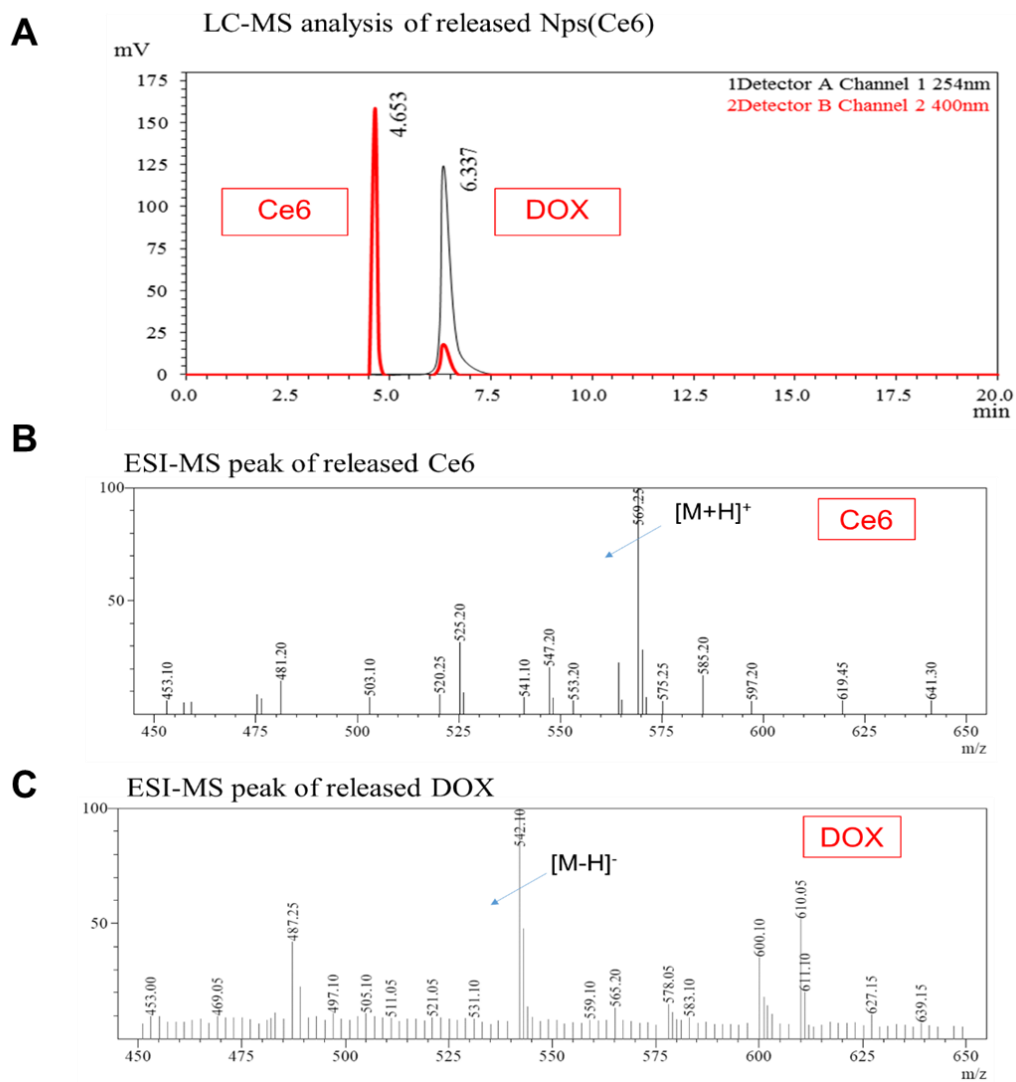
**Figure S16.** Standard curve of (a) Ce6:  $Y = 0.0666 X + 0.0446$  ( $R^2 = 0.9993$ ) and (b) DOX:  $Y = 0.0174 X + 0.0197$  ( $R^2 = 0.9994$ ).



**Figure S17** CMC of polyDOX-*block*-(polyHPMA-SS-polyHPMA)-*block*-polyDOX based nanoparticles.

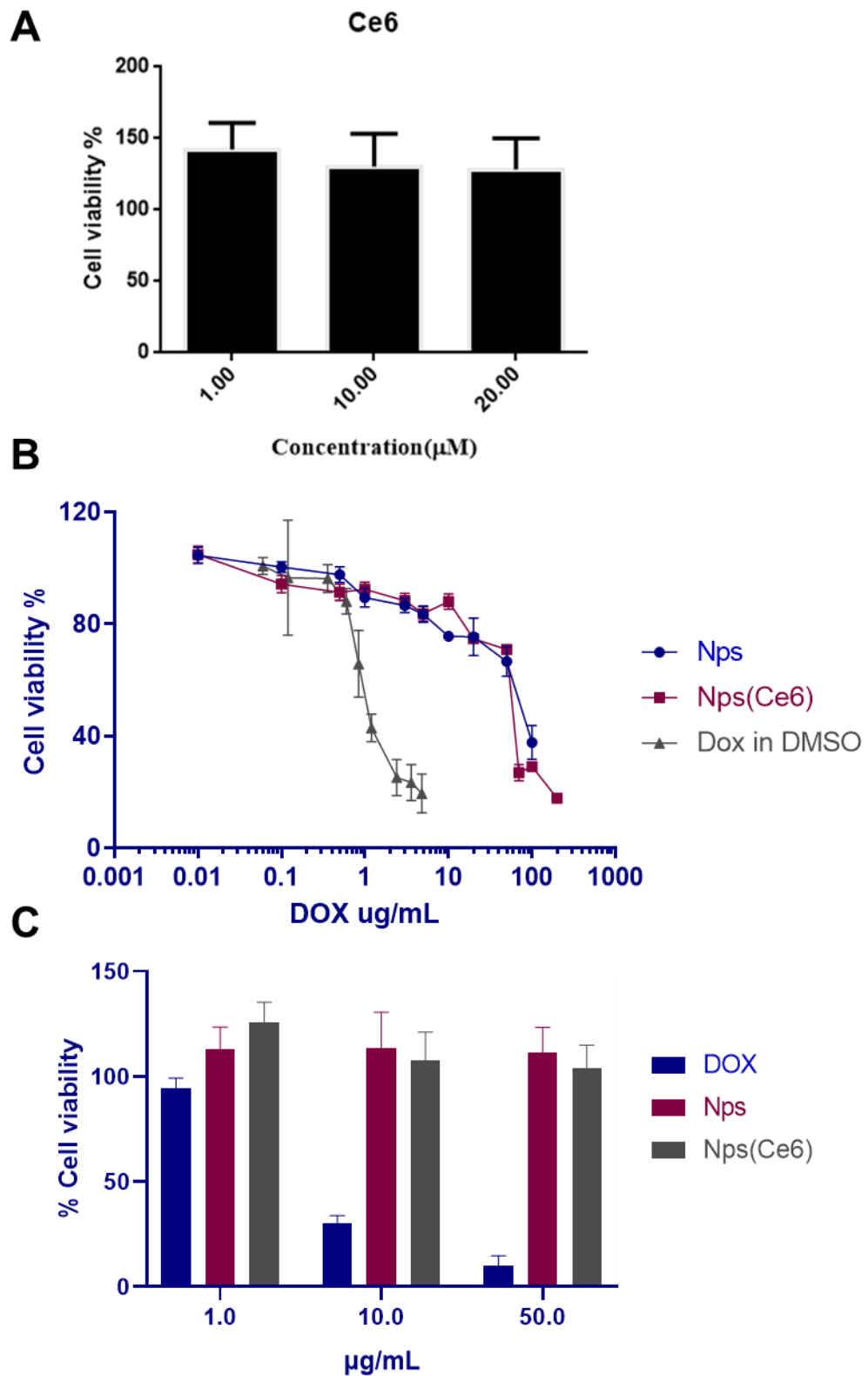


**Figure S18** Stability analysis of NPs(Ce6) by measuring zeta-potential and average sizes of NPs(Ce6) by DLS for 3 days.

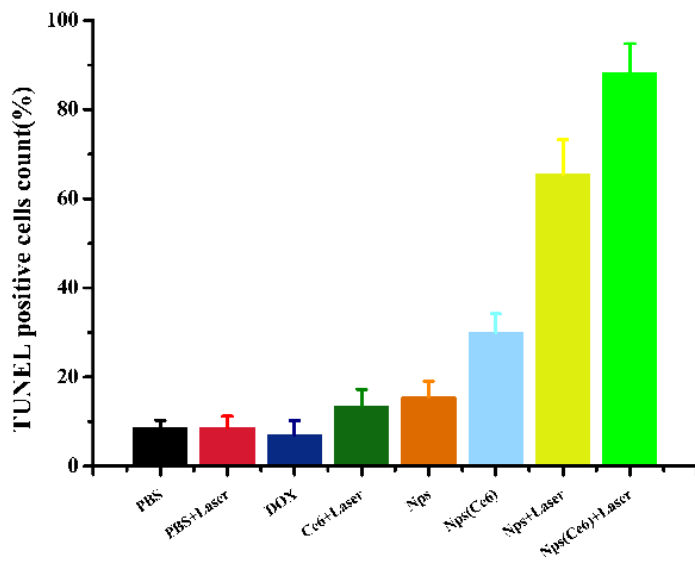


**Figure S19** LC-MS analysis of released DOX and Ce6 from NPs(Ce6) in pH 7.4 buffer solution containing 3 mmol/L GSH at 36 h (SHIMADZU, Shim-pack VP-ODS C18 column, 4.6 mm × 250 mm): (A) LC spectra, MS spectra of released (B) Ce6 and (C) DOX.

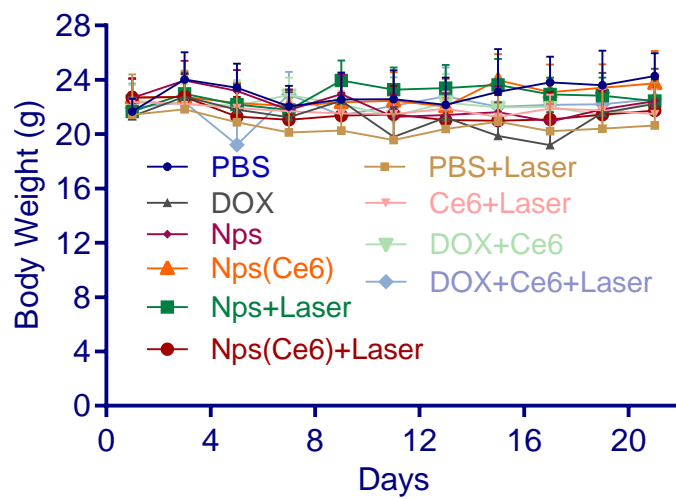




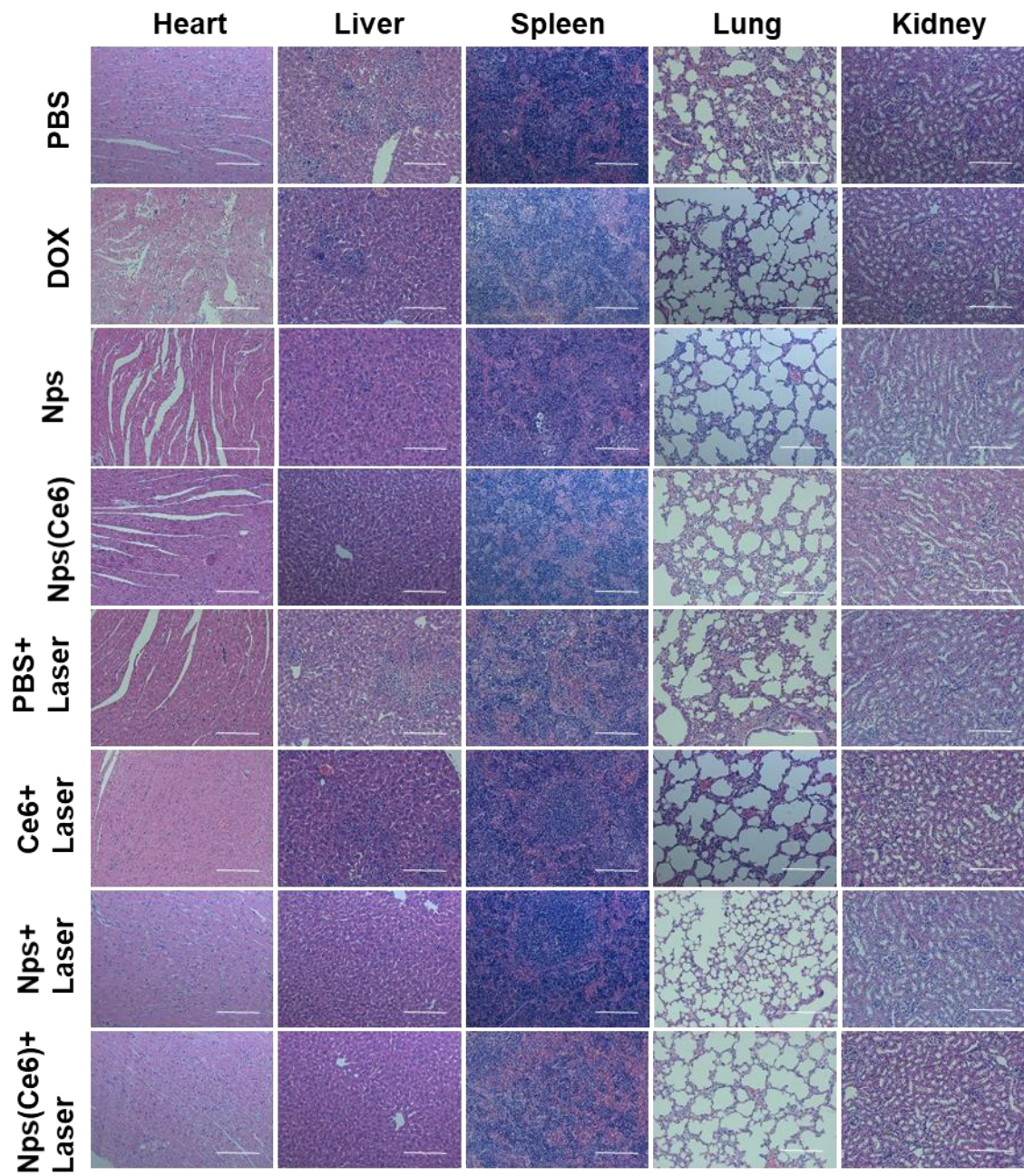
**Figure S20** Light-off cell viability with (A) Ce6 on 4T1 cells, (B) DOX, NPs, NPs(Ce6) on 4T1 cells and (C) different treatments on HELF cells,  $n=3$ , error bars:  $\pm$ SD.



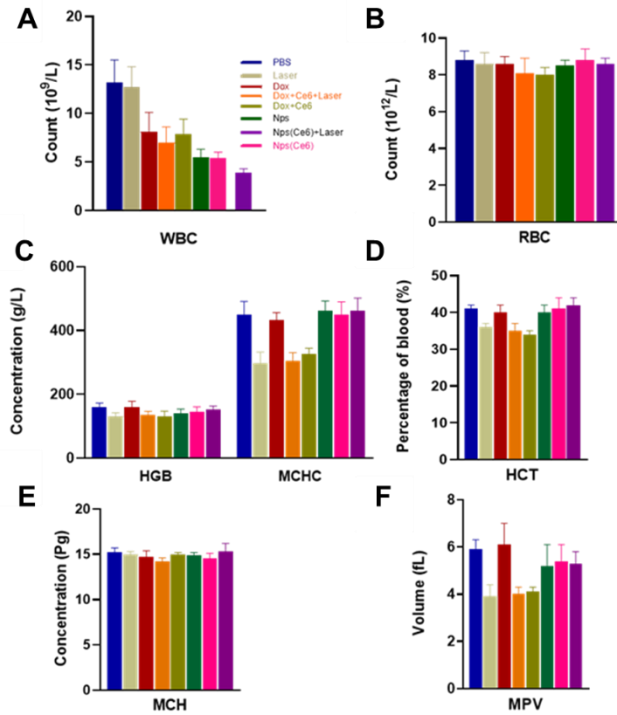
**Figure S21** Quantitative results of different groups of TUNEL.



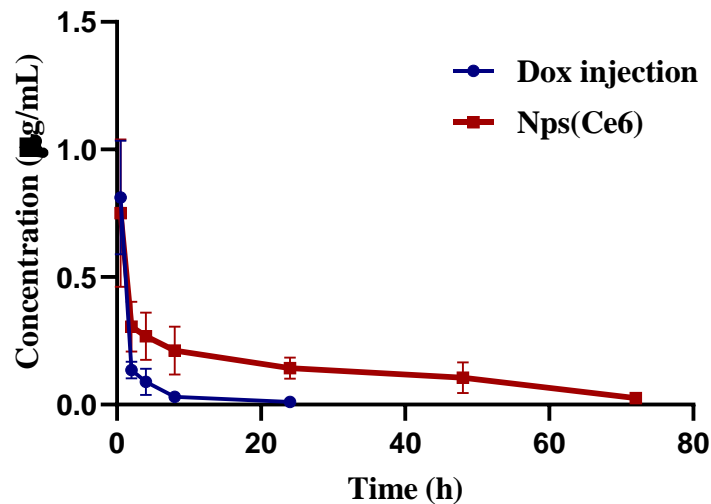
**Figure S22** Body weight changes of different treatment groups ( $n = 7$ ).



**Figure S23** Images of H&E stained heart, liver, spleen, lung, kidney tissues harvested from mice after treatment, scale bar: 100  $\mu$ m.



**Figure S24** Blood biochemical indicators of mice ( $n=6$ ) after injection with different formulations (A) WBC: white blood cells; (B) RBC: red blood cells; (C) HGB and MCHC: Hemoglobin and Mean RBC hemoglobin concentration; (D) HCT: Hematocrit; (E) MCH: Average RBC hemoglobin; (F) MPV: Mean platelet volume.



**Figure S25** *In vivo* pharmacokinetic data of NPs(Ce6) after i.v. injection to mice( $n=6$ ).

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