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Supporting Information

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Supporting experiments and materials

1 Materials

mPEG-NHS (molecular weight, 5,000 Da) was purchased from JENKEM Technology Co., Ltd (Beijing, China). Podophyllotoxin (PPT), 3,3'-dithiodipropionic acid (DTPA), dimethylmaleic anhydride (DMA), succinic anhydride (SA), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl), and N-hydroxysuccinimide (NHS) were purchased from Aladdin Industrial Corporation (Shanghai, China). Poly(allylamine hydrochloride) (PAH·HCl, molecular weight, 15,000 Da) and polyamidoamine dendrimers (PAMAM, generation 4) were obtained from Sigma-Aldrich (Shanghai, China).

2 Synthesis of DTPA-PPT

DTPA-PPT was synthesized through esterification between 3,3'-dithiodipropionic acid (DTPA) and PPT. Figure S1A illustrates the synthetic pathway of DTPA-PPT. Briefly, DTPA (1.4 mmol), EDC (1.5 mmol) and DMAP (1.5 mmol) were dissolved in 20.0 mL dichloromethane and stirred at 0°C for 30 min under dry nitrogen to activate the carboxyl group of DTPA. Subsequently, PPT solution (1.2 mmol PPT dissolved in 20.0 mL dichloromethane) was added dropwise to the mixture and further stirred at 37°C for 18 h under dry nitrogen. The reaction process was monitored via thin-layer chromatography. At the end of the reaction, the product was washed with 0.01 M diluted hydrochloric acid and distilled water three times, followed by drying under vacuum. DTPA-PPT was obtained through purification via silica gel column chromatography with ethyl acetate: petroleum ether (Rf = 0.36).

3 Synthesis of PAMAM-ss-PPT

PAMAM-ss-PPT was synthesized by conjugating DTPA-PPT to PAMAM through an amidation reaction, as shown in Fig. S1A. In brief, 0.1 mmol PAMAM, 6.6 mmol DTPA-PPT, 6.8 mmol 1-ethyl-3-(3-

dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl), and 6.8 mmol N-hydroxysuccinimide (NHS) were dissolved in 100.0 mL *N, N*-dimethylformamide (DMF) and stirred at 37°C for 48 h under dry nitrogen. The mixture was purified via dialysis (molecular weight cutoff (MWCO) 3,500 Da) in DMF for 24 h to remove unreacted DTPA-PPT and dialyzed against distilled water for 48 h to remove DMF. PAMA-ss-PPT was obtained after lyophilization and stored in methanol at 4°C until further use.

4 Synthesis of PEG-PAH-DMA and PEG-PAH-SA

To synthesize PEG-PAH-DMA, PAH·HCl was initially deprotonated. Briefly, 1.0 g PAH·HCl was dissolved in 10 mL aqueous NaOH (1.0 mol/L) and dialyzed (MWCO, 3,500 Da) against distilled water for 24 h at room temperature. After lyophilization, deprotonated PAH was obtained and stored at -20°C until further use.

PEG-PAH-DMA and PEG-PAH-SA were prepared in two steps, as shown in Fig. S1B. PEG-PAH was initially synthesized. In brief, PAH (50.0 mg), mPEG-NHS (500.0 mg), and triethylamine (TEA, 30.0 μL) were dissolved in 20.0 mL dry DMSO and stirred for 24 h at room temperature. At the end of the reaction, the mixture was dialyzed (MWCO, 12,000 Da) against distilled water for 48 h. PEG-PAH was obtained after lyophilization.

Subsequently, PEG-PAH-DMA was prepared by conjugating DMA to PAH. Typically, 50.0 mg PEG-PAH, 23.0 mg DMA and 20.0 μL TEA were dissolved in 10.0 mL dry DMSO and further reacted for 24 h under room temperature. The mixture was dialyzed (MWCO, 3,500 Da) against water (pH 8-9 adjusted with NaOH) for 48 h and lyophilized to obtain PEG-PAH-DMA. The control polymer, PEG-PAH-SA, was synthesized using the same method.

6 Synthesis of DMA-NPs and SA-NPs

DMA-NPs were formed using PEG-PAH-DMA and PAMAM-ss-PPT. Briefly, 10.0 mg PEG-PAH-DMA was dissolved in 1.0 mL methanol by vertexing. Next, 1.0 mL PAMAM-ss-PPT methanol solution containing 1.0 mg PAMAM-ss-PPT was mixed with PEG-PAH-DMA. The mixture was added to 10.0 mL PBS (pH 7.4) drop-

by-drop with violent stirring. After stirring for 1 h, methanol was removed by rotary evaporation at 30°C. DMA-NPs were obtained by filtration using a 420.0 nm filter. To obtain the optimal sizes and drug loading properties, a series of DMA-NPs with different weight ratios of PEG-PAH-DMA/PAMAM-ss-PPT were prepared using the same method. The control group with no size- and charge-changing ability was additionally prepared by complexing PEG-PAH-SA with PAMAM-ss-PPT.

7 Product characterization

^{1}H NMR spectra of products were obtained with the Bruker AV 300 NMR system (Bruker Biospin, USA) using tetramethyl silane (TMS) as the internal standard. Mass spectroscopy was performed using an AB SCIEX Triple Quad instrument (AB SCIEX, 5500, American). UV spectra were obtained using a UV-visible spectrophotometer (Shimadzu, UV-2450, Japan). Size distribution and zeta potential were determined via dynamic light scattering (DLS, Zetasizer Nano Zs90, Malvern, England). The morphology of nanoparticles was assessed using transmission electronic microscopy (TEM, Tecnai G2 20 WTWIN, Philips). PPT content in blood and organs was measured by high-performance liquid chromatography (HPLC) on a Shimadzu HPLC system (LC-20A, Japan) with the detector set at 292 nm using acetonitrile and water (80: 20, v/v) as the mobile phase.

8 pH stimuli-responsive size and charge changes of DMA-NPs and SA-NPs

DMA-NP and SA-NP PBS (1.0 mL, pH 7.4) solutions containing 5.0 mg NPs were dissolved in 10.0 mL PBS (pH 6.5 or 7.4) and incubated at 37°C with slight shaking. The sizes and zeta potentials of both NP types were determined at the designated time intervals. After 2 h incubation, the morphologies of both NP types were examined via TEM.

9 *In vitro* drug release

In vitro PPT release behavior was studied in PBS (pH 7.4 with 10% FBS) containing 0, 20 μM or 10 mM

GSH. In brief, 1.0 mL freshly prepared DMA-NP solution (containing 2.0 mg PPT) was transferred into a dialysis bag (MWCO: 3,500 Da) and immersed in 99 mL release medium. The release assay was performed in a thermotank at 37°C under gentle shaking at 100 rpm. At a predetermined time-point, 1.0 mL release solution was removed and an equal amount of fresh release medium added. The total amount of released PPT was detected via UV-Vis spectrometry at 292 nm.

10 Stability assays

PBS with or without 10% FBS (pH 7.4) was used to simulate storage and blood circulation conditions to investigate the stability of DMA-NPs and SA-NPs. Typically, 1.0 mL DMA-NP or SA-NP solution (3.0 mg/mL) was mixed with PBS with or without 10% FBS. Subsequently, mixtures were incubated at 37°C under gentle shaking at 100 rpm. Size changes in DMA-NPs and SA-NPs were measured using DLS at the designated time intervals.

11 Cell lines

Human non-small cell lung cancer cell lines (A549 and drug-resistant A549/PTX) and human breast cancer cell lines (MCF-7 and drug-resistant MCF-7/ADR) were supplied by KeyGEN BioTECH (Nanjing, China). A549 cells were cultured in F12K medium containing 10% (v/v) fetal bovine serum, 100.0 IU/mL penicillin and 100.0 μg/mL streptomycin at 37°C in a humidified 5% CO_2 atmosphere. A549/PTX, MCF-7, and MCF-7/ADR cells were cultured in RPMI1640 containing 10% (v/v) fetal bovine serum, 100.0 IU/mL penicillin and 100.0 μg/mL streptomycin at 37°C in a humidified 5% CO_2 atmosphere. Drug resistance of A549/PTX cells was maintained by the addition of Taxol (20.0 ng/mL) and that of MCF-7/ADR cells by the addition of doxorubicin (250.0 ng/mL) to the medium.

12 *In vitro* cellular uptake

Cellular uptake of DMA-NPs at different pH conditions was measured via confocal laser scanning

microscopy (CLSM, ZEISS LSM 780, Germany) and flow cytometry (FCM, FACScan; Becton Dickinson, USA). For CLSM analysis, A549/PTX cells were seeded in six-plates at a density of 1.0×10^4 cells/well and cultured at 37°C for 24 h. Next, cells were treated with FITC-labeled DMA-NPs and SA-NPs for 2 h at a final FITC concentration of 500.0 ng/mL in 2.0 mL complete RPMI 1640 at pH 7.4 or 6.5 At the end of the treatment period, cells were collected, washed, fixed with 4% formaldehyde for 10 min at room temperature, stained with DAPI, and subsequently examined using CLSM.

For FCM analysis, 549/PTX cells at a density of 1.0×10^4 cells/well were seeded in six-plates and cultured at 37°C for 24 h. Next, cells were treated with FITC-labeled DMA-NPs and SA-NPs for 2 h at a final FITC concentration of 500 ng/mL in 2 mL complete RPMI 1640 at pH 7.4 or 6.5 At the end of the treatment period, cells were collected, washed, and assessed via FCM.

13 Tumor spheroid penetration study

A 3D tumor spheroid model (3DTSM) was established using A549/PTX cells. Typically, cells were seeded in low-attachment 96-well plates at a density of ~2.0×10^4 cells per well and incubated at 37°C for 7 days. The 3DTSM was treated with FITC-labeled DMA-NPs or SA-NPs (2.0 μg/mL FITC) for 4 h at pH 6.5, respectively. Cells were washed three times with PBS and placed onto glass slides for CLSM analysis.

14 *In vitro* cytotoxicity

The cytotoxicities of PPT, DMA-NPs and SA-NPs against A549 and A549/PTX cells were analyzed via the MTT assay. Cells were seeded in 96-well plates at a density of 5.0×10^3 cells per well and cultured for 24 h, followed by treatment with PPT, DMA-NPs, and SA-NPs at pH 7.4 or 6.5 To determine the influence of intracellular reduction, cells were preincubated with GSH (10.0 mM) for 2 h before the addition of DMA-NPs and subjected to the MTT assay after incubation for 48 h. Absorbance values were detected at 490 nm on a Bio-Rad 680 microplate reader. Cell viability was calculated as follows:

Cell viability (%) = $\frac{Asample}{Acontrol} \times 100\%$

whereby "*Asample*"and "*Acontrol*" represent absorbance of sample and control wells, respectively.

15 Hemolysis assays

The hemocompatibility of different concentrations of DMA-NPs and SA-NPs was analyzed with the hemolysis assay. Typically, fresh mouse blood was diluted with PBS. Red blood cells (RBC) were collected via centrifugation and further diluted with PBS. The suspension obtained (2%) was used for evaluation of hemolysis. Different concentrations of DMA-NPs and SA-NPs (equivalent to 0.016, 0.03, 0.06, 0.13, 0.25, 0.5, and 1 mg/mL PPT) were added to the RBC suspension and incubated for 2 h at 37°C, followed by centrifugation of the mixtures at 1200 rpm for 10 min. Following collection of the supernatant, the amount of hemoglobin released was recorded on a microplate reader at 540 nm. Water was used as positive control and PBS as a negative control. The hemolysis ratio (HR) of RBCs was calculated according to the following formula:

Hemolysis (%) = (As – An) / (Ap-An) × 100, where "As" is the absorbance of sample, "An" the absorbance of negative control and "Ap" the absorbance of positive control. All hemolysis experiments were conducted in triplicate.

16 Animal and tumor xenograft models

BALB/c nude mice (male, 18.0–20.0 g, 5–6 weeks of age) and Sprague-Dawley (SD) rats (male, 200.0–220.0 g, 5–6 weeks) were purchased from the Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). All animals received care in compliance with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals and the procedures were approved by the affiliated Huaian NO.1 People's Hospital of Nanjing Medical University. The A549/PTX tumor-bearing nude mouse model was established by

injecting 5.0×10^6 cells in 100.0 μL PBS into the right flanks of BALB/c nude mice.

17 Pharmaceutical studies

SD rats were administered free PPT, DMA-NPs or SA-NPs (equivalent to 15 mg/kg PPT) via tail vein injection. At predetermined time intervals (0, 1, 2, 4, 8, 12, 24 and 48 h), 500.0 μL blood was collected from the orbital venous plexus and plasma obtained via centrifugation (1200 rpm, 5 min, 4 °C). Subsequently, 1.0 mL chloroform was added to extract PPT. After centrifugation at 15,000 rpm for 5 min, the supernatant was collected and dried under a nitrogen stream. Finally, the residues obtained were re-dissolved in methanol for HPLC analysis. Analysis of coupled PPT in DMA-NPs and SA-NPs required treatment of NPs with excessive GSH to liberate PPT.

2.18 *In vivo* biodistribution

A549/PTX tumor-bearing mice were treated with PPT, DMA-NPs or SA-NPs (equivalent to 15 mg/kg PPT) at a tumor volume of 50 mm^3. After injection for 6 h or 12 h, mice were anesthetized and applied heart perfusion by saline. Major organs and tumor tissues were harvested, rinsed in PBS, and homogenized in PBS. Subsequently, 1.0 mL chloroform was added to 200.0 μL samples to extract PPT. After centrifugation at 15,000 ppm for 5 min, the supernatant fraction was collected and dried under nitrogen steam. Finally, the residues obtained were re-dissolved in methanol for HPLC analysis. Analysis of coupled PPT in DMA-NPs and SA-NPs required treatment of NPs with excessive GSH to liberate PPT.

2.17 *In vivo* tumor penetration

A549/PTX tumor-bearing mice received intratumoral injections (at a fixed needle insertion depth) or tail vein injection of FITC-labeled DMA-NPs or SA-NPs and were euthanized after 12 h. Tumors were harvested, washed in PBS, and fixed with 4% paraformaldehyde. Next, tumors were sliced at different depths from the top to middle at a thickness of 10 μm. After staining with DAPI, tumor slices were imaged using

CLSM. The fluorescence intensity of FITC and DAPI in the tumor section were analyzed by software image J.

2.18 Determination of maximum tolerated dose

A549/PTX tumor-bearing mice were divided into 15 groups (n = 10.0) and injected intravenously with free PPT (5, 10, 15, 20, 30 mg/kg), DMA-NPs (20, 50, 100, 150, 200, 250, 300 mg/kg) or SA-NPs (20, 50, 100, 150, 200, 250, 300 mg/kg at PPT equivalent doses). Changes in body weight and survival of mice were monitored daily for 14 days. MTD was identified as the maximum dose of drug that did not induce animal death or > 20% body weight loss or other significant changes in general appearance throughout the experimental period.

2.19 *In vivo* antitumor studies

A549/PTX tumor-bearing mice with tumor volumes of about 50–80 mm^3 were randomly divided into six groups (n = 6). PBS, PPT (15.0 mg/kg), DMA-NPs (15.0 mg/kg, equivalent to the PPT dose), SA-NPs (15.0 mg/kg, equivalent to the PPT dose), DMA-NPs (160.0 mg/kg, equivalent to the PPT dose) and SA-NPs (160.0 mg/kg, equivalent to the PPT dose) were intravenously injected into mice via the tail vein once on day 0. Tumor volume and body were measured using calipers every three days. The tumor volume was calculated using the following formula: $V = L \times W^2/2$, where "*L*" represents the length and "*W*" the width of each tumor. At the end of the treatment course, mice were sacrificed, and tumors excised and weighed to calculate the tumor inhibition rate (TIR).

2.20 Statistical analysis

All data are presented as mean ± standard error of mean. Student's *t*-test was used to analyze differences between two groups. Differences with p-values < 0.05 were considered statistically significant.

Supporting Figures

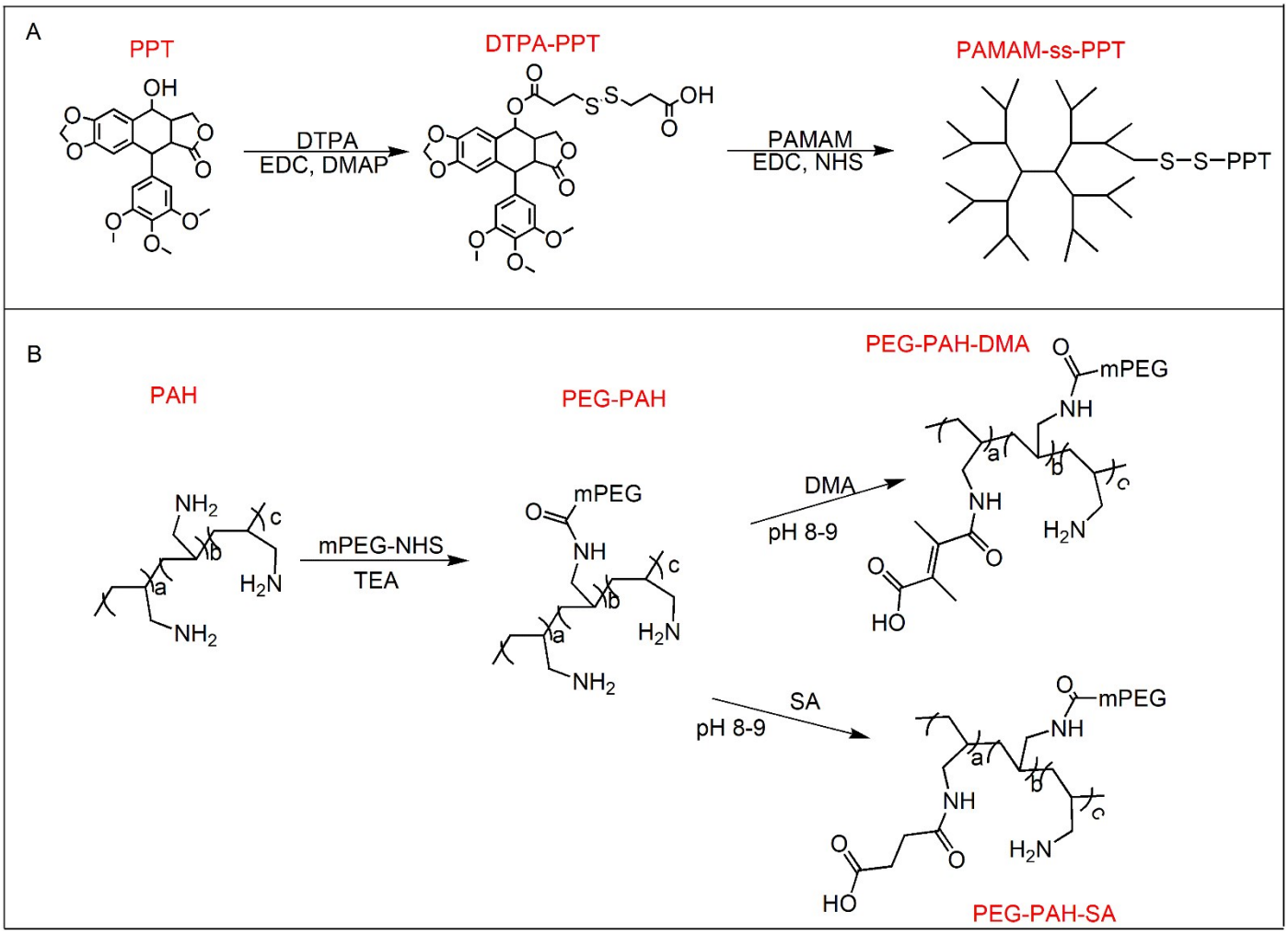

Fig. S1 Synthesis route of PAMAM-ss-PPT (A) and PEG-PAH-DMA/PEG-PAH-SA (B).

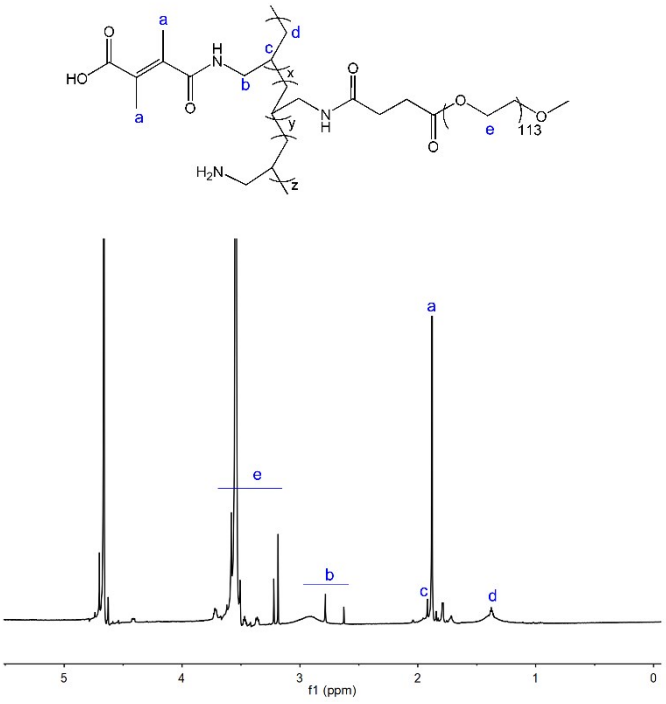

Fig. S2 ^{1}H NMR spectra of PEG-PAH-DMA in D_2O.

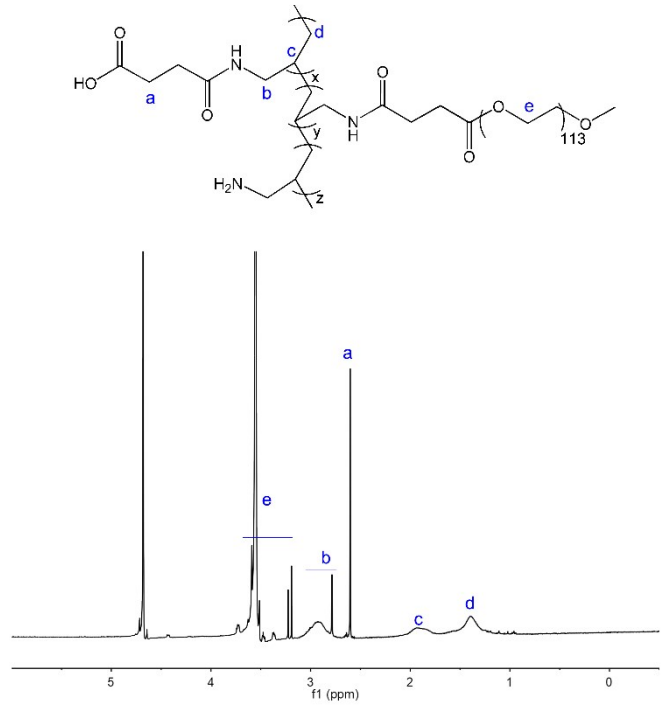

Fig. S3 ^{1}H NMR spectra of PEG-PAH-SA in D_2O.

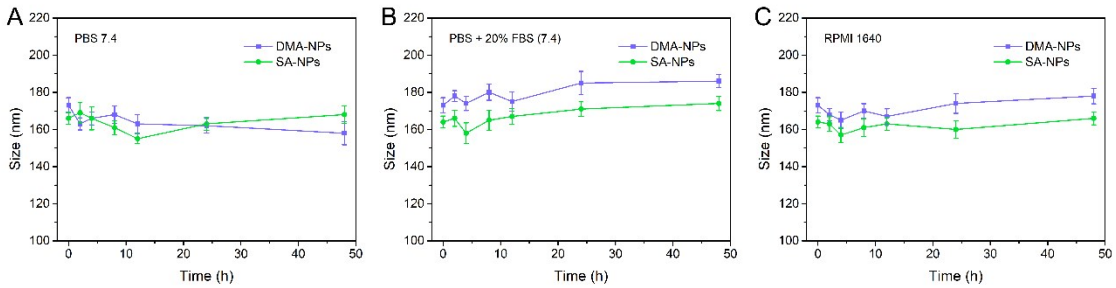

Fig. S4 Size changes in DMA-NPs and SA-NPs incubated in PBS (A), PBS +20% FBS (B) or RPMI 1640 (C) for different time-periods. Data are presented as means ± SD, *n* = 3.

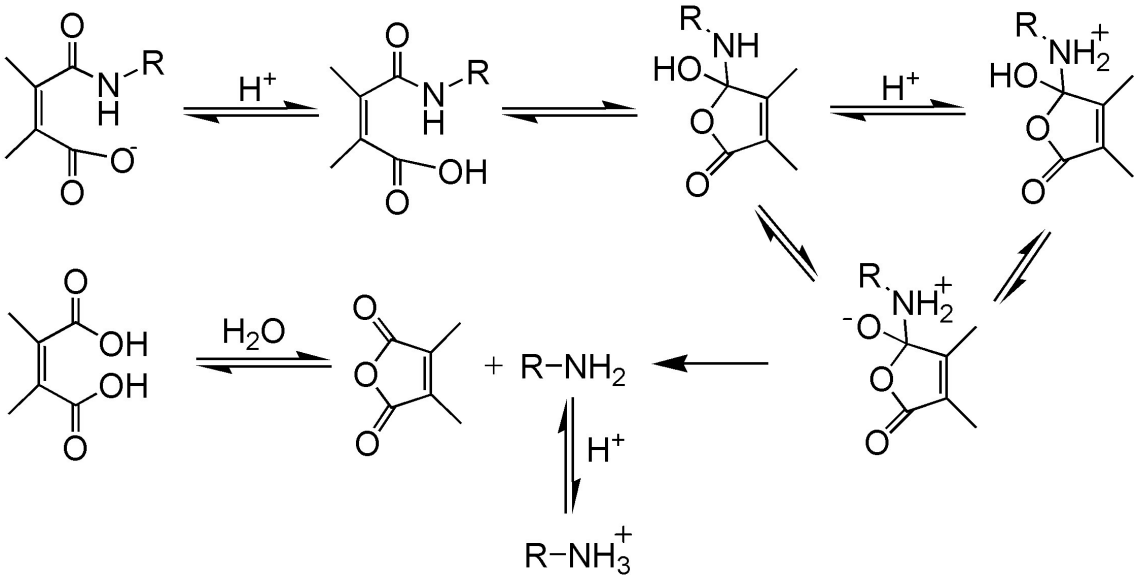

Fig. S5 Mechanism underlying hydrolysis of dimethylmaleic amide (DMA) at pH 6.5

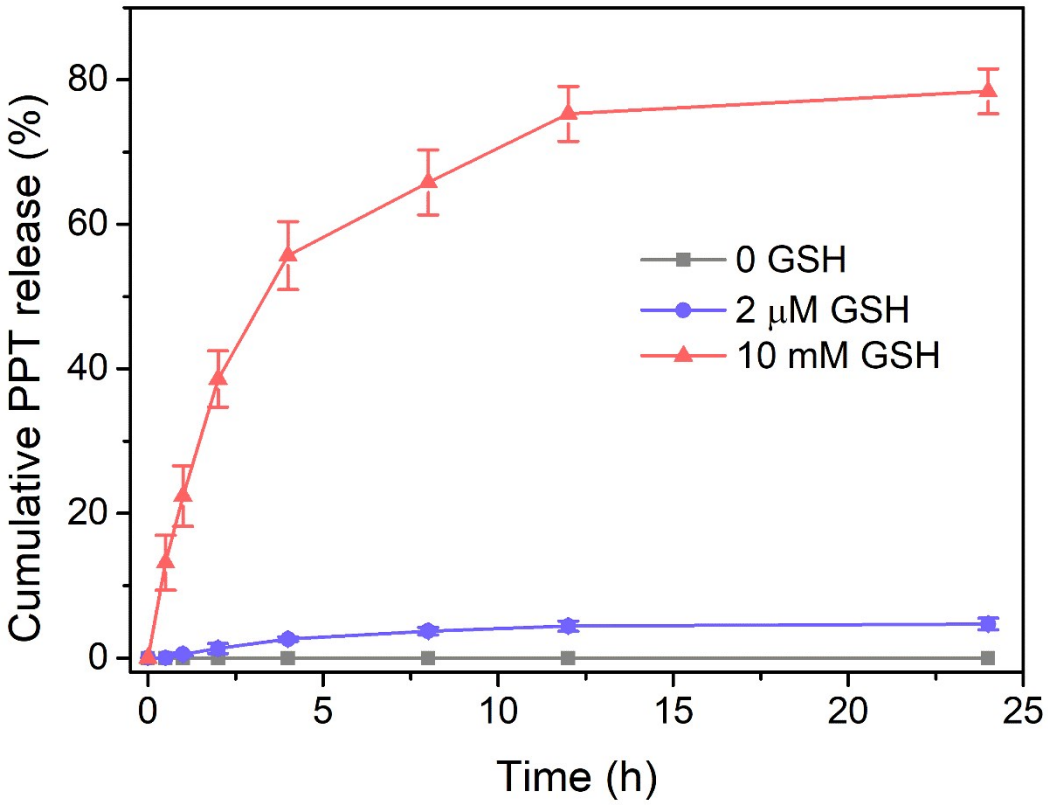

Fig. S6 PPT release profiles of DMA-NPs in PBS at pH 7.4 containing 0, 2 and 10 mM GSH. Data are presented as mean ± SD, *n* = 3.

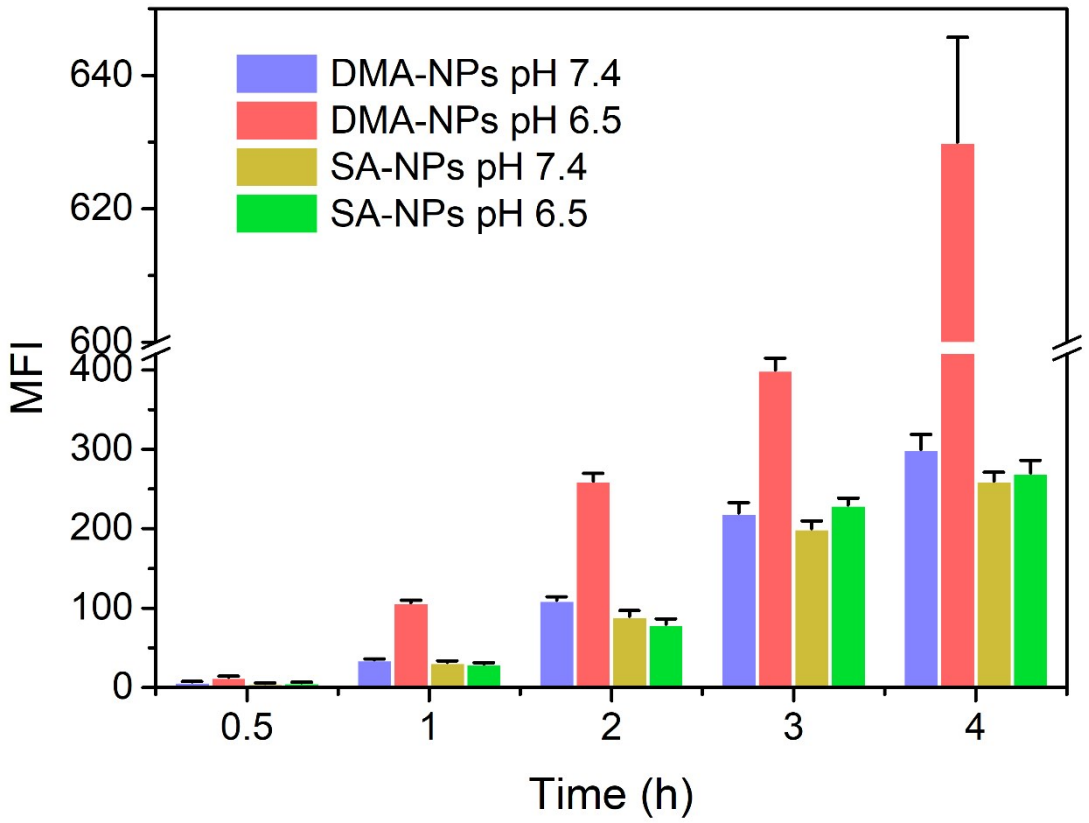

Fig. S7. Quantitative determination of cellular uptake by FCM. A549/PTX cells were treated with DMA-NPs and SA-NPs at pH 7.4 or 6.5 for different time-periods and analyzed using FCM. Data are presented as mean ± SD, *n* = 6.

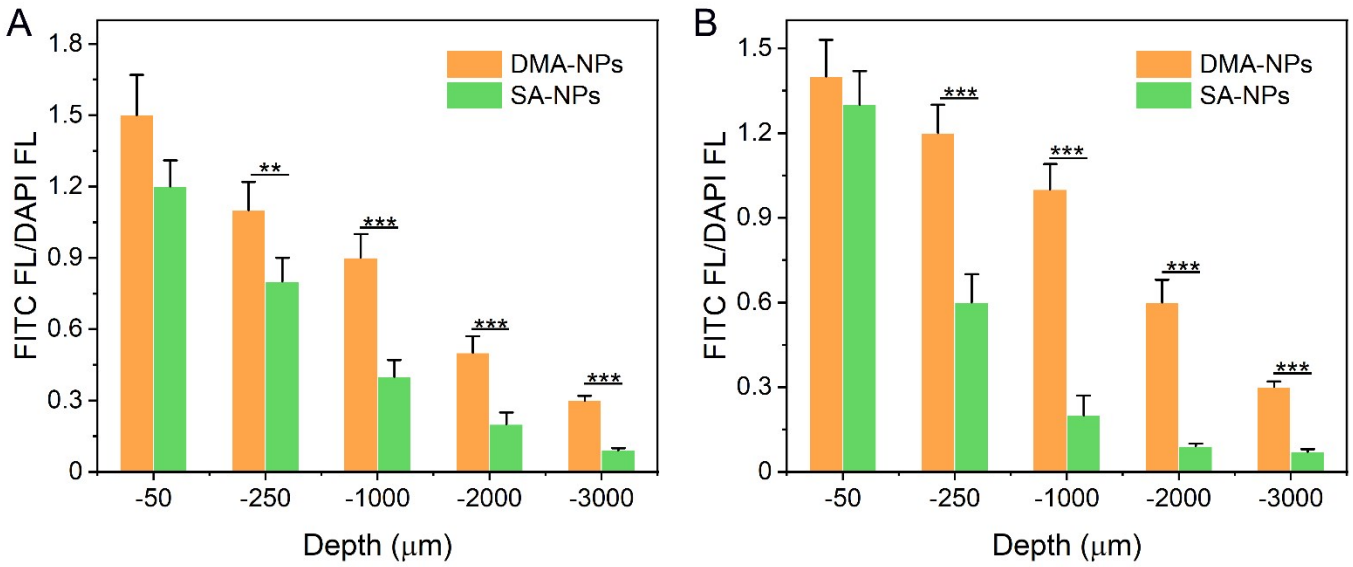

Fig. S8 The ratio of FITC and DAPI fluorescence intensity (FL) in each tumor section. (A) Intratumorally injection; (B) tail vein injection. Data presented as mean ± SD, *n* = 6. ** $p < 0.01$, *** $p < 0.001$.

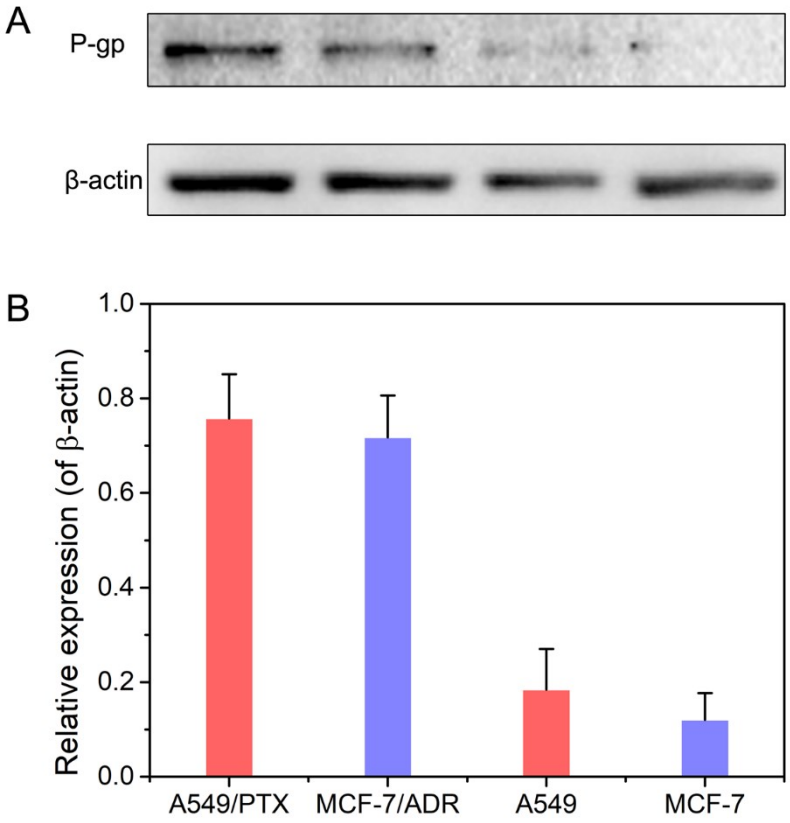

Fig. S9 P-gp expression in A549/PTX, A549, MCF-7/ADR, and MCF-7 cells, determined via western blot using β-actin as an internal control. Data are presented as mean ± SD, *n* = 3.

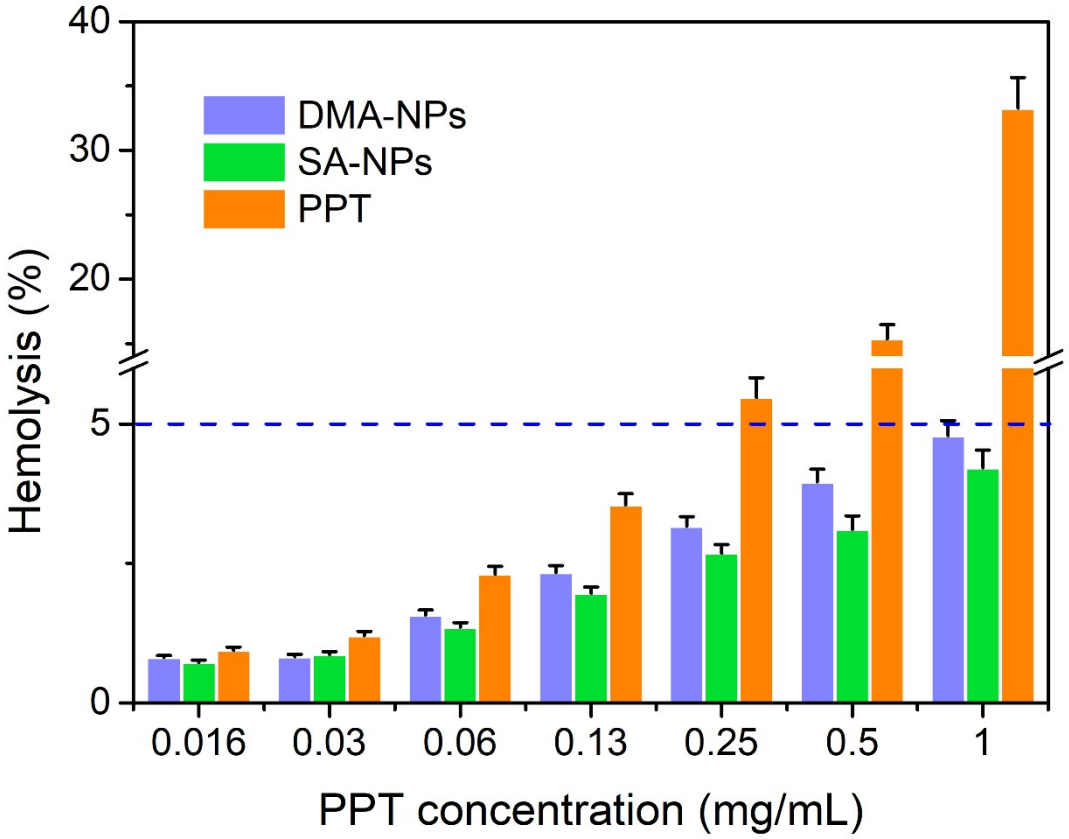

Fig. S10 Hemolytic activities of PPT, DMA-NPs, and SA-NPs on mouse red blood cells. Double-distilled water and PBS were used as the positive and negative controls, respectively. Data are presented as mean ± SD, *n* = 6.