Supporting Information

for

Development of poly (*p*-coumaric acid) as a self-anticancer nanocarrier for efficient and biosafe cancer therapy

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Experimental section

Materials: DTX was purchased from MedChemExpress. p-CA, thionyl chloride ($SOCl_2$), and dimethyl sulfoxide (DMSO) were bought from Aladdin (Shanghai, China). Pyridine was supplied by Tianjin Zhiyuan Chemical Reagent co. LTD (Tianjin, China) and purified by standard methods before use. 1,2-distearoyl-sn-glycero-3-phosphoethano-lamine-N-poly (ethylene glycol) 2000 (DSPE-PEG 2k) was purchased from Avanti Polar Lipids. Thiazolyl blue tetrazolium bromide (MTT), crystal violet, and High-performance liquid chromatography (HPLC)-grade acetonitrile were purchased from Sigma. The fluorescence dyes including, Hoechst 33342, Lysotracker Red1,1′-dioctadecyl-3,3,3′,3′ tetramethylindotricar-bocyanine iodide (DiR), 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (Dil) and coumarin 6 (C6) were supplied by Thermo Fisher Scientific. Rat anti-mouse CD31 antibodies were obtained from Abcam. Annexin V-FITC/PI Apoptosis Kit was provided by Multi Sciences (Hangzhou, China). Cell Cycle and Apoptosis Analysis Kit and Mitochondrial membrane potential assay kit with JC-1 were purchased from Beyotime® Biotechnology (Shanghai, China). Dulbecco's modified eagle medium (DMEM), trypsin-EDTA, penicillin-streptomycin (P/S), fetal bovine serum (FBS), phosphate-buffered saline (PBS) were purchased from Gibco.

Cell lines and animals: CT26 cells were obtained from American Type Culture Collection (ATCC), barring special circumstances, incubated in DMEM containing 10% FBS and 1% P/S and maintained at 37℃, under 5% CO_2. BALB/c mice (female, 4-5 weeks, 18-22 g) and SD rats (female, 8-10 weeks, 220-250 g) were provided by the Laboratory Animal Center of Sun Yat-sen University. All animal procedures in the study were approved by the Experimental Laboratory Animal Committee of Sun Yat-sen University ([2019] No.114) and conducted strictly in conformity with the guidelines of the Experimental Laboratory Animal Committee of Sun Yat-sen University.

Synthesis and characterization of PCA polymer: The PCA polymer was synthesized by the rapid polycondensation of *p*-CA. The details of the procedure were as follows: first, 10 mL of pyridine was constantly stirred in an ice bath (0~4℃) for precooling.

After stirring for 5 min, 10 mmol of $SOCl_2$ was dropwise added into pyridine, followed by stirring in an ice bath for another 15 min. Next, 5 mmol of *p*-CA was added to react with $SOCl_2$ at 25 ℃ for 1 h. Finally, the mixture was precipitated in ultrapure water, and dilute hydrochloric acid was added to remove the remaining pyridine. After washing with ultrapure water several times, the final product was freeze-dried to obtain the brown powder.

Various standard methods including ^{1}H-nuclear magnetic resonance (^{1}H-NMR; Bruker Avance III 400, Germany), Fourier transform infrared spectroscopy (FTIR, Bruker, Germany), differential scanning calorimeter (DSC, Netzsch, Germany), gel permeation chromatography (GPC; Agilent, USA), and UV-Vis spectrophotometer (Beckman, Germany) were applied to characterize the chemical structure and physical properties of PCA polymer. Additionally, to investigate the *in vitro* degradation of the polymer, PCA (10 mg/mL in DMSO) was dropwise added to stirring PBS buffer with different pH values (5.0, 6.8, and 7.4). Next, the samples were maintained in a thermostatic shaker at 37 ℃ and under 100 rpm for 7 days and 0.5 mL of solution was withdrawn at predetermined time points. Finally, the released *p*-CA was extracted by methanol and detected by high-performance liquid chromatography (HPLC; Agilent Technologies, USA) under a mobile phase of water (0.1% phosphoric acid)/methanol=30/70 and UV detection wavelength of 308 nm.

Preparation and characterization of NPs: Blank NPs (PCA NPs) and DTX@PCA NPs were both prepared by the nanoprecipitation method. Briefly, PCA polymer, DTX, and DSPE-PEG 2k were separately dissolved in DMSO at the same concentration of 10 mg/mL and mixed in a specific volume ratio. Then 600 μL of the mixture was dropwise added into 9 mL of ultra-pure water under constant stirring. The NPs solution was ultra-filtered twice using an ultrafiltration centrifugal tube (Millipore, MWCO 10k Da). After removing DMSO and the free molecules, the concentrated NPs solution was suspended in PBS for further characterization and use. Transmission electron microscopy (TEM; JEOL Ltd, Japan) was applied to visualize the morphology of NPs and dynamic light scattering (DLS; Zetasizer Nano-ZS90, Malvern, UK) was used to measure the particle size. To evaluate the stability of NPs, PCA NPs and DTX@PCA

NPs dispersed in PBS and PBS containing 10% FBS were measured by DLS at prearranged time intervals for seven days.

Drug loading capacity of DTX@PCA NPs: The drug loading capacity of DTX@PCA NPs was determined by HPLC. Briefly, DTX@PCA NPs were dissolved in acetonitrile and the NP structure was completely broken by ultrasound to release the encapsulated DTX. Next, the sample solution was centrifuged at 12000 rpm for 10 min. The DTX content was detected under a mobile phase of water/ acetonitrile=35/65 and UV detection wavelength of 230 nm. The drug loading capacity (DLC) and drug encapsulation efficiency (DLE) were calculated using the following formulae:

$$\text{DLC(wt\%)} = \frac{\text{amount of encapsulated drug}}{\text{amount of nanoparticles}} \times 100\%$$

$$\text{DLE(wt\%)} = \frac{\text{amount of encapsulated drug}}{\text{amount of feeding drug}} \times 100\%$$

Drug release behavior of DTX@PCA NPs: The *in vitro* drug release behavior of DTX@PCA NPs was investigated using the dialysis method. Briefly, 1 mL of DTX@PCA NPs solution was transferred into a dialysis bag (MWCO 3500 Da) and then immersed in PBS containing 0.1% Tween 80 with different pH values (5.0, 6.8, and 7.4). The drug release process was performed in a thermostatic shaker with a temperature of 37 ℃ and a constant shaking speed of 100 rpm. At prearranged time intervals, 1mL of sample solution was withdrawn, followed by the supplement of the corresponding release medium. Finally, the released DTX was determined by HPLC and expressed as the percentage of the cumulative amount.

Hemolysis test: A hemolysis test was performed to evaluate the hematotoxicity of PCA NPs as nanocarriers. Briefly, fresh whole blood obtained from SD rats was centrifuged at 1,000 rpm for 5 min. Next, the plasma and buffy coat layer were discarded, and the remaining red blood cells (RBCs) were further washed using PBS till the supernatant became clear. Finally, the concentrated RBCs were diluted to 2% in PBS. Thereafter, 1 mL of PCA NPs dispersed in PBS, with concentrations ranging from 31.25 to 1000 μg/mL, was mixed with an equal volume of RBC suspension. The RBC suspension mixed with pure PBS (0% hemolysis) and ultrapure water (100% hemolysis) was set as

negative control and positive control, respectively. All the samples were placed in a constant temperature oscillator with a temperature of 37 °C and a constant shanking speed of 100 rpm. After incubation for 3 h, the mixture was centrifuged at 1,000 rpm for 5 min to collect the supernatant. Finally, the absorbance of each group was measured at 541 nm using a microplate reader. The hemolysis percentage was calculated as follows:

$$\text{Hemolysis\%} = \frac{Ab_{sample} - Ab_{negative\ control}}{Ab_{positive\ control} - Ab_{negative\ control}} \times 100\%$$

***In vitro* cytotoxicity study:** The *in vitro* biocompatibility of PCA NPs and cytotoxicity of free DTX and DTX@PCA NPs on CT26 cells were both determined by MTT assay. Briefly, CT26 cells at a density of 5×10^3 cells/well were seeded in 96-well plates and incubated overnight. Then the medium was replaced by a fresh medium containing DTX or DTX@PCA NPs at different concentrations. After incubation for 24 h, 20 μL of MTT (5 mg/mL in PBS) was added to incubate with the cells for another 4 h. Then the medium was abandoned and DMSO was added to dissolve the formazan crystal produced by the living cells. After complete shaking for 5 min, the plates were placed in a microplate reader (BioTek Synergy4, USA) to measure the absorbance at a wavelength of 490 nm. The cell viability was calculated using the following formula:

$$\text{Cell viability(\%)} = \frac{Ab_{sample} - Ab_{blank}}{Ab_{control} - Ab_{blank}} \times 100\%$$

Cell apoptosis study: The apoptosis effect of nanocarrier and DTX formulations on CT26 cells was examined by a flow cytometer. The CT26 cells at a density of 3×10^5 cells/well were seeded on 6-well plates and incubated overnight. Then the medium was removed and a fresh medium containing PCA NPs, DTX, or DTX@PCA NPs at an equivalent DTX dose of 5 μg/mL was added to incubate with cells for 24 h. Next, the cells were gently digested using trypsin without ethylenediaminetetraacetic acid (EDTA) and collected by centrifugation. After washing with PBS twice, the cells were stained with Annexin V-FITC/PI according to the instructions of the Kit. Finally, the cell apoptosis was analyzed by flow cytometry (BD FACSCalibur, US).

Mitochondrial membrane potential assay: To investigate the influence on

mitochondrial membrane potential, CT26 cells were cultured in 6-well plates at a density of 1×10^5 cells per well for 24 h. Next, cells were incubated for another 12 h with PCA NPs, DTX (5 μg/mL) and DTX@PCA NPs (at a DTX dose of 5 μg/mL). The cells incubated with culture medium were served as control. After incubation, the cells were washed with PBS and collected. Next, cells of each sample suspended in 0.5 mL culture medium were mixed with 0.5 mL pre-prepared JC-1 dyeing working solution and cultured at 37 ℃ for 20 min. After washing twice using pre-cooling JC-1 dyeing buffer, the cells were resuspended in dyeing buffer and analyzed by flow cytometry.

Cell cycle study: To investigate the effect of nanocarrier and DTX formulations on cell cycle, CT26 cells plated on 6-well plates were respectively treated with PCA NPs, DTX or DTX@PCA NPs at an equivalent DTX dose of 5 μg/mL for 24 h, and the cells only treated with culture medium were set as the control group. Next, all the cells of each group were collected and washed twice using cold PBS. After fixation in 70% cold ethanol at 4 °C for another 24 h, the cells were centrifuged and washed by cold PBS again. Finally, the cells were stained following the protocol of the Cell Cycle and Apoptosis Analysis Kit and were analyzed by flow cytometry.

Wound-healing assay: Wound-healing assay was conducted to investigate the lateral migration ability of cells. Briefly, CT26 cells at a density of 1×10^6 cells/well were seeded on 6-well plates with three straight lines under the bottom of each well. The medium was abandoned till the bottom was completely covered by CT26 cells. Next, a pipette tip was applied to perpendicularly draw two parallel scratches on the monolayer cells, followed by the careful removal of cell debris by washing with PBS twice. Next, fresh medium containing PCA NPs, DTX, or DTX@PCA NPs at an equivalent DTX dose of 5 μg/mL was added and five selected fields of scratches in each group were immediately photographed by an inverted microscope (Olympus, Japan). 24 h post incubation, the same fields were photographed once again.

Transwell assay: Transwell assay was performed to investigate the vertical migration ability of cells. In this study, the matrigel was employed to mimic the extracellular matrix, which was diluted in serum-free medium at a ratio of 1:8 at 4 ℃. Then 100 μL of diluted matrigel was added into the upper chamber of the 24-well transwell plate,

followed by the incubation at 37 ℃ for half an hour. Next, the medium was removed and CT26 cells at a density of 5×10^3 cells/well were seeded into the upper chamber, in which the fresh medium was serum-free, containing PCA NPs, DTX, or DTX@PCA NPs at an equivalent DTX dose of 5 μg/mL. Meanwhile, the lower chamber was treated with fresh medium with 10% FBS, which was used to act as a chemoattractant. After incubation for 24 h at 37 °C, the cells migrated to the lower surface of the membrane were fixed in methanol for 15 min and stained with crystal violet solution (5 mg/mL in PBS) for 30 min. After washing by PBS twice and air-drying at room temperature, five random fields of the stained cells were photographed using an inverted microscope.

Cellular internalization and uptake study: Coumarin 6 (C6) was used as a fluorescence probe to label the PCA NPs and track the cellular internalization behavior of NPs on CT26 cells. CT26 cells at a density of 1×10^5 cells/dish were seeded on flat glass-bottom Petri dishes and incubated overnight at 37 °C. Then the culture medium was removed and replaced by serum-free medium containing C6-loaded PCA NPs (C6@PCA NPs, at a C6 dose of 0.4 μg/mL). After incubation for 1, 4, and 8 h, the cells were washed twice with pre-cooling PBS and stained with 1 mL Hoechst 33342 (5 μg/mL) for 15 min at 37 ℃. Next, Hoechst 33342 was removed and the cells were washed twice again. Similarly, 1 mL Lysotracker Red (75 nM) was added to incubate with cells for 30 min at 37 ℃. Finally, the cells were maintained in PBS, followed by the imaging of confocal laser scanning microscopy (CLSM, ZEISS, Germany).

Cellular uptake of PCA NPs on CT26 cells was determined by flow cytometer analysis. Likewise, CT26 cells at a density of 1×10^5 cells/well were seeded on 6-well plates and incubated overnight. Then C6@PCA NPs at a C6 dose of 0.2 μg/mL was added to incubate with cells for 0.5, 1, 2, 4, and 8 h. After washing with precooling PBS twice, the cells were directly collected and analyzed by flow cytometry.

In vitro* penetration and inhibition on three-dimensional tumor spheroids*: To better mimic the environment of solid tumors, a 3D tumor spheroid model was established to evaluate the *in vitro* penetration efficacy and inhibitory effect of DTX@PCA NPs. First, the tumor spheroids were constructed according to the following protocol: CT26 cells at a density of 5×10^3 cells/well were seeded in 96-well

low-attachment culture plates and cultured in DMEM-F12 containing 10% FBS, 1% penicillin-streptomycin, and growth factors (hEGF and bFGF, 10 ng/ml). When the size of tumor spheroids reached ~100 μm, PCA NPs, DTX and DTX@PCA NPs at an equivalent DTX dose of 5 μg/mL were separately added to treat tumor spheroids. The group treated with culture medium was set as a control group. The morphology of tumor spheroids in each group was photographed at 0, 24, and 48 h respectively. For the penetration study, C6 was used to label the NPs. Next, the free C6 solution and C6@PCA NPs at a C6 dose of 0.4 μg/ml were added and incubated for 1 and 4 h. Thereafter, the tumor spheroids were softly washed twice with PBS, fixed with 4% paraformaldehyde for 15 min, and finally transferred to flat glass-bottom Petri dishes for observation of CLSM.

Pharmacokinetics study: Next, to investigate the *in vivo* pharmacokinetics, SD rats (n = 3) were intravenously administrated with DTX or DTX@PCA NPs at an equivalent DTX dose of 5 mg/kg. At predetermined time points, the blood was withdrawn from the retro-orbital plexus to collect the plasma. Thereafter, DTX content in the plasma was analyzed by ultra-performance liquid chromatography/tandem mass spectrometry (UPLC-MS/MS, Thermo, USA) with the following MS ionization parameters: positive ESI mode; spray voltage, 3000 V; ion source temperature, 300 °C; collision energy, 0 eV. Paclitaxel was selected as the internal standard. The analytes were quantified through using Multiple Reaction Monitoring to monitor ion transitions m/z of 830.0-549.0 (DTX) and 876.0-591.0 (PTX). Chromatography was performed using the Agilent XB-C18 column at the temperature of 30 °C and a flow rate of 0.25 mL/min (mobile phase, 0.1% formic acid: methanol = 20: 80). Ultimately, the pharmacokinetic data were analyzed using WinNonlin software (version 5.2).

***In vivo* biodistribution study:** *In vivo* biodistribution study was performed on CT26 tumor-bearing mice and DiR were served as the fluorescence probe to mimic the free drug and track the *in vivo* biodistribution of PCA NPs. Briefly, the mice were randomly divided into two groups (n=3) and intravenously injected with free DiR solution (0.4 mg/kg) and DiR-loaded PCA NPs (DiR@PCA NPs) with equivalent DiR dosage, respectively. At predetermined time intervals (1, 3, 6, 12, 24, 48, 72, and 96 h), the mice

were anesthetized with isoflurane and photographed using *In vivo* Imaging System (IVIS, PerkinElmer, USA) at an Excitation/Emission (Ex/Em) wavelength of 740/790 nm. 24 h and 96 h post injection, the mice were sacrificed and their tumor tissues and major organs (heart, liver, spleen, lung, kidney) were harvested and photographed at the same Ex/Em wavelength.

***In vivo* anticancer evaluation:** *In vivo* anticancer evaluation was conducted on CT26 tumor-bearing mice. Briefly, the mice were randomized into four groups (n=5) and intravenously administrated with normal saline, PCA NPs, DTX solution, and DTX@PCA NPs at an equivalent DTX dosage of 5 mg/kg every three days. The mice treated with normal saline were served as the control group. The body weight and tumor volume of mice were recorded every two days. Specifically, the tumor volume was calculated as follows: Volume = (Length × $Width^2$)/2. At the end of the study, all the mice were sacrificed, and their tumor tissues and major organs (heart, liver, spleen, lung, kidney) were harvested. Next, the tumor tissues were photographed and weighted. Finally, to investigate the physiological and pathological changes caused by different treatments, the tumor tissues and major organs were fixed in 4% paraformaldehyde for hematoxylin and eosin (H&E) staining and terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) assay.

Serum biochemistry: CT26-tumor-bearing mice were intravenously administered with normal saline, PCA NPs, DTX, and DTX@PCA NPs at a DTX dose of 5 mg/kg. The treatment was conducted six times at a three-day interval. Ultimately, the serum was collected from the mice for detection of biochemical parameters, such as alanine aminotransferase (ALT), aspartate transaminase (AST), urea nitrogen (BUN), creatinine (CRE), and creatine kinase (CK).

Statistical analysis: The statistical analysis of data was conducted using SPSS software. Two groups were compared using the two-tailed Student's t-test. One-way ANOVA was applied to analyze multiple groups. Significant difference was displayed as $*p \leq 0.05$, $**p < 0.01$ or $*** p < 0.001$

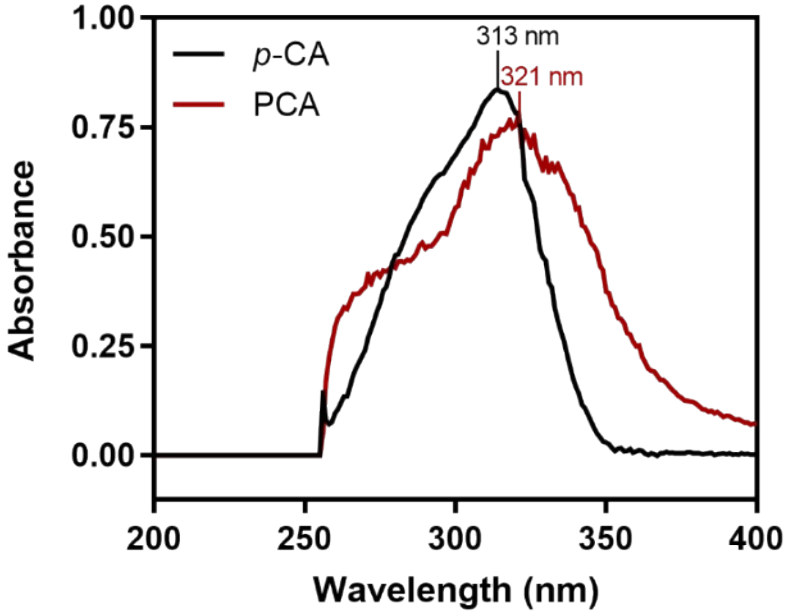

Fig. S1. UV absorption spectra of PCA polymer and *p*-CA.

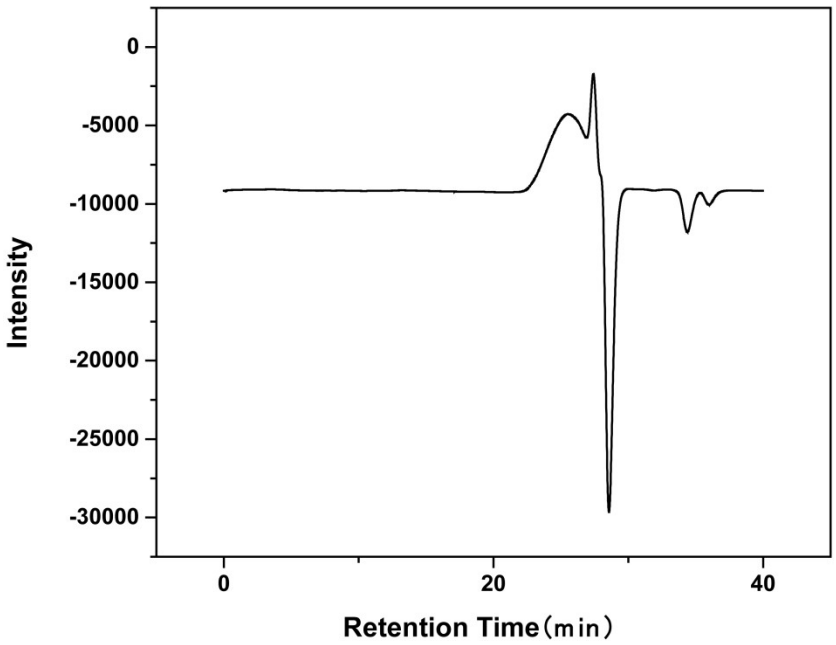

Fig. S2. The GPC origin trace of PCA polymer.

Table S1. Molecular weight information of PCA polymer

Polymer	Mn	Mw	PDI
PCA	17191	19434	1.1305

Mn: Number-average molecular weight; Mw: Weight-average molecular weight; PDI: Polydispersity index (Mw/Mn).

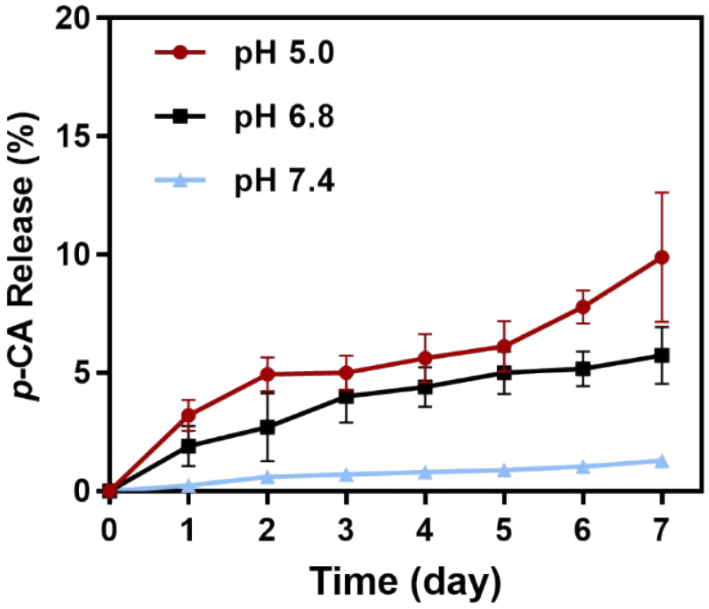

Fig. S3. Release of *p*-CA monomer from PCA polymer in PBS with different pH values (5.0, 6.8, and 7.4) within 7 days.

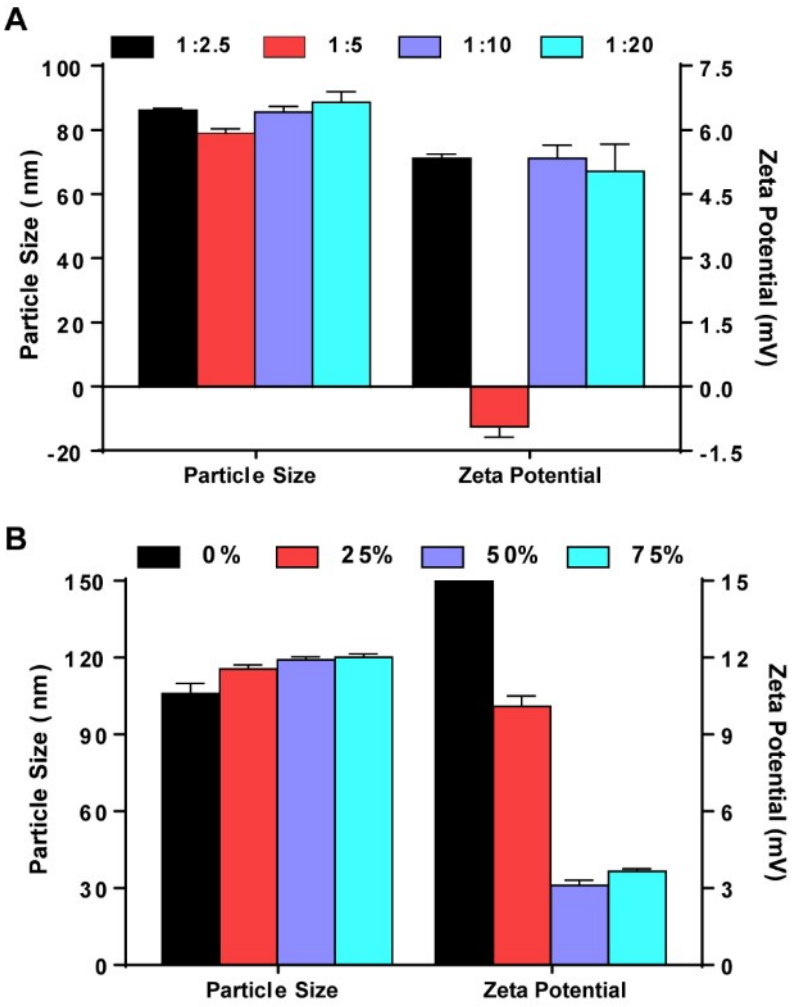

Fig. S4. (A) The influence of DSPE-PEG 2k content on properties of PCA NPs. (B)The influence of drug/carrier ratio on properties of DTX@PCA NPs.

Table S2. The influence of drug/carrier ratio on DLC and DLE of DTX@PCA NPs.

Ratio	1:2.5	1:5	1:10	1:20
DLC (%)	1.2±0.10	5.57±0.22	2.94±1.4	2.13±0.53
DLE (%)	4.2±0.2	33.4±1.3	32.3±1.5	44.7±1.1

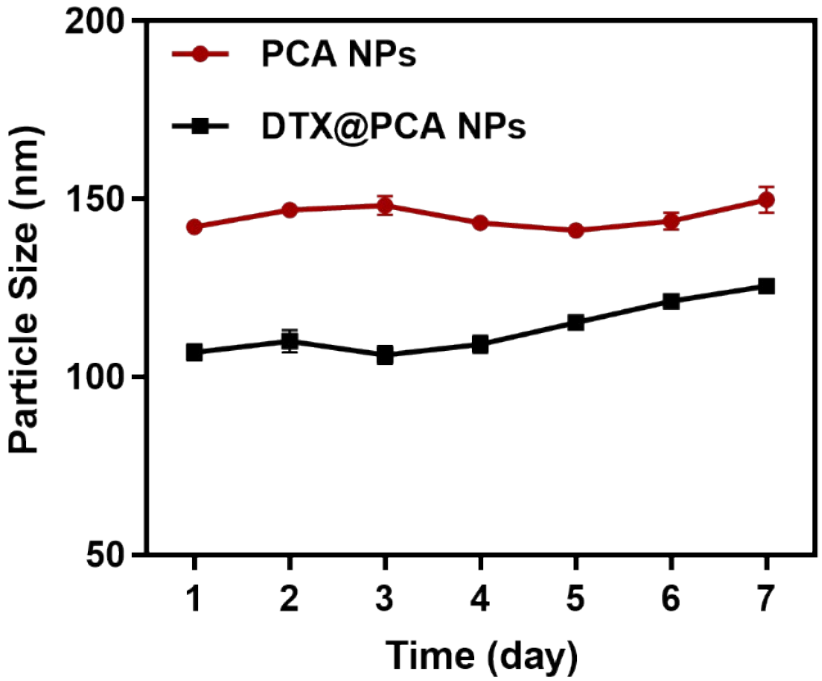

Fig. S5. Particle size change of PCA NPs and DTX@PCA NPs in PBS containing 10% FBS.

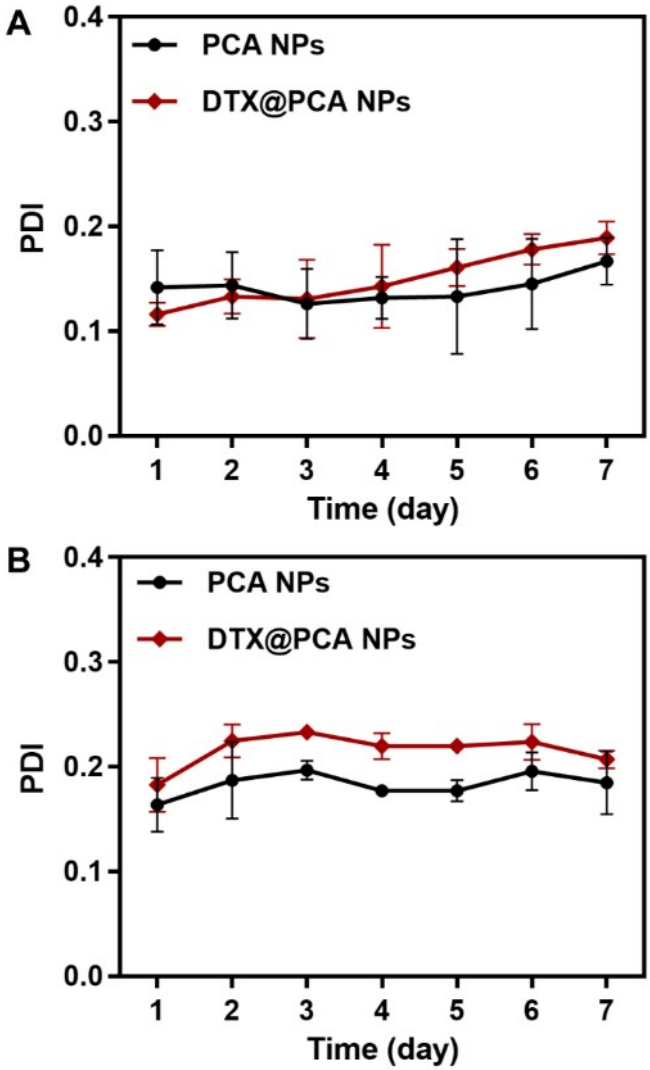

Fig. S6. Change in PDI of PCA NPs and DTX@PCA NPs in (A) PBS and (B) PBS containing 10% FBS.

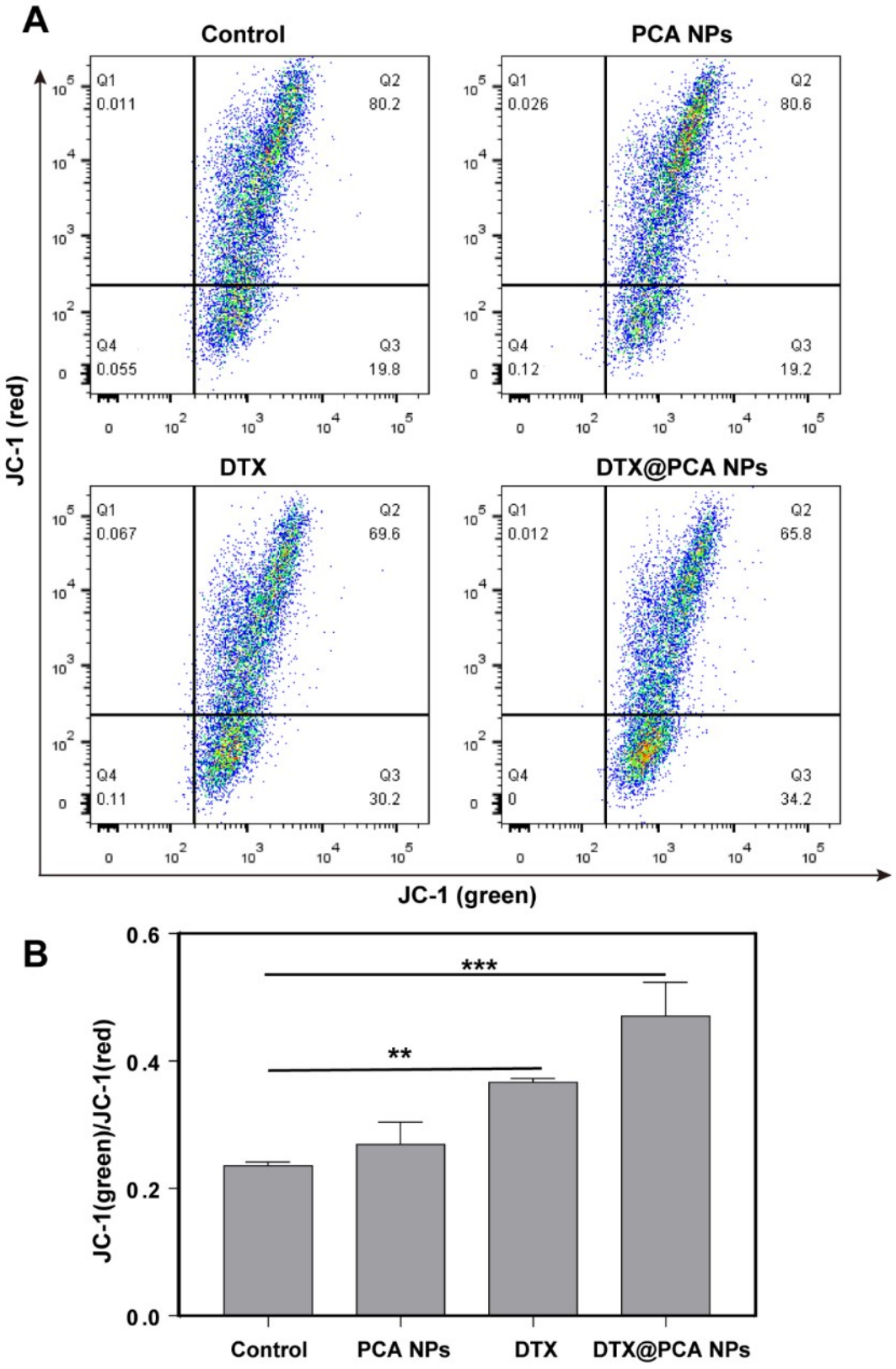

Fig. S7. (A) Mitochondrial membrane potential of CT26 cells treated with PCA NPs, DTX, and DTX@PCA NPs at an equivalent DTX concentration of 5 μg/mL. (B) Florescence analysis of mitochondrial membrane potential.

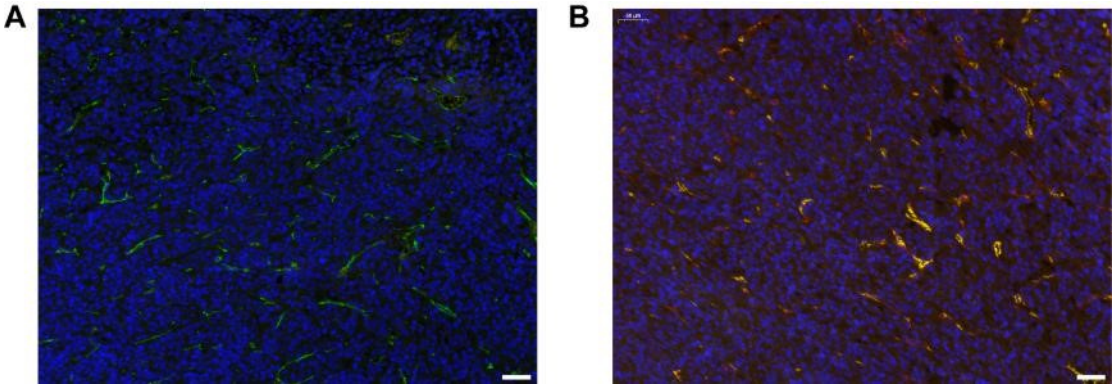

Fig. S8. Representative pictures of tumor after injection with Dil and Dil@PCA NPs for 6 h. Scale bar= 50 μm.

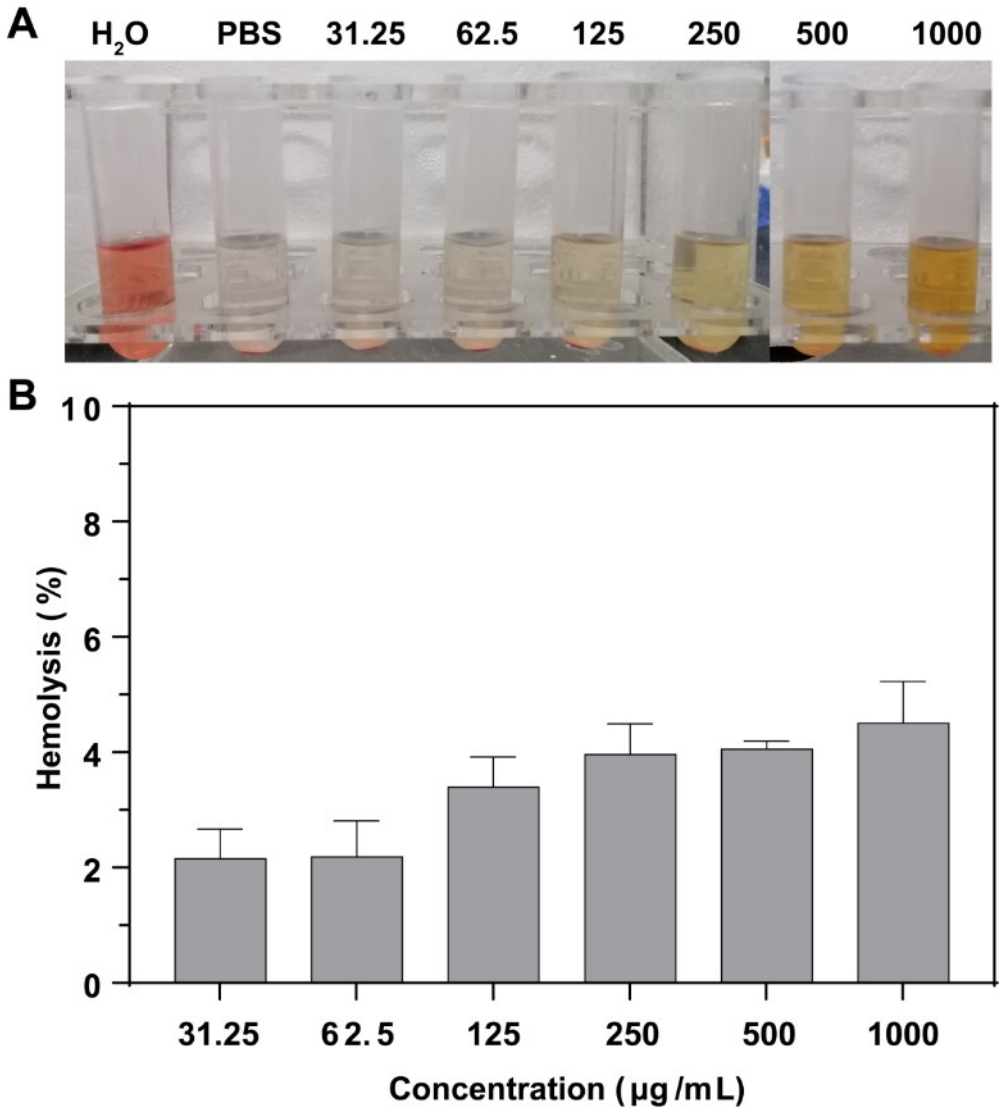

Fig. S9. (A) Picture of RBCs treated with PCA NPs across different concentration range. (B) Quantitative analysis of hemolysis rate.

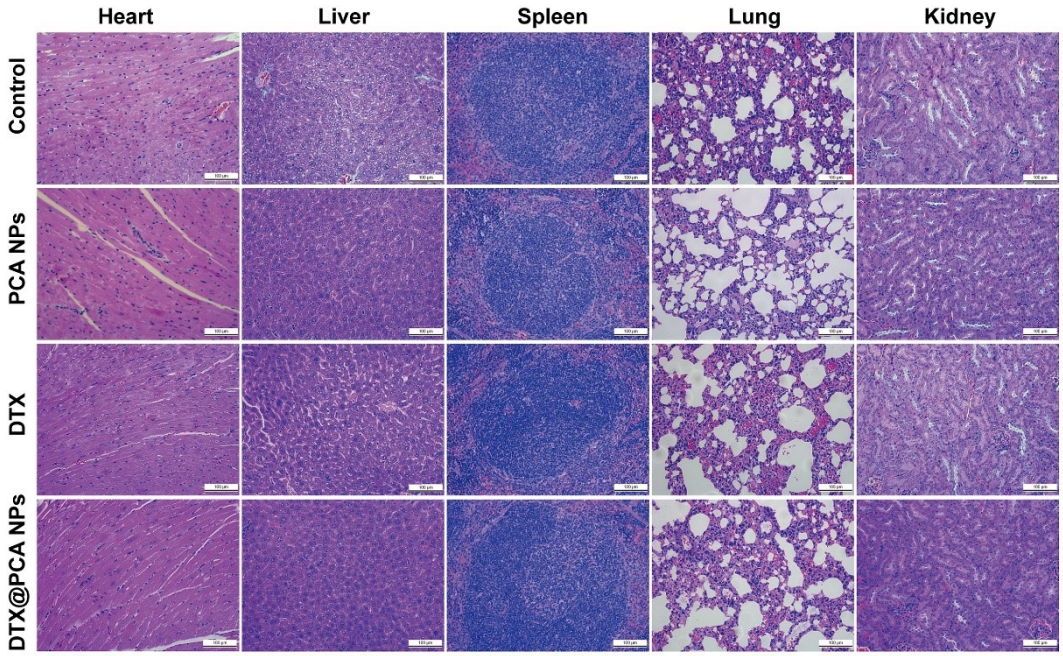

Fig. S10 *In vivo* biosafety evaluation. H&E staining images of the major organs excised from mice. Scale bar = 100 μm.

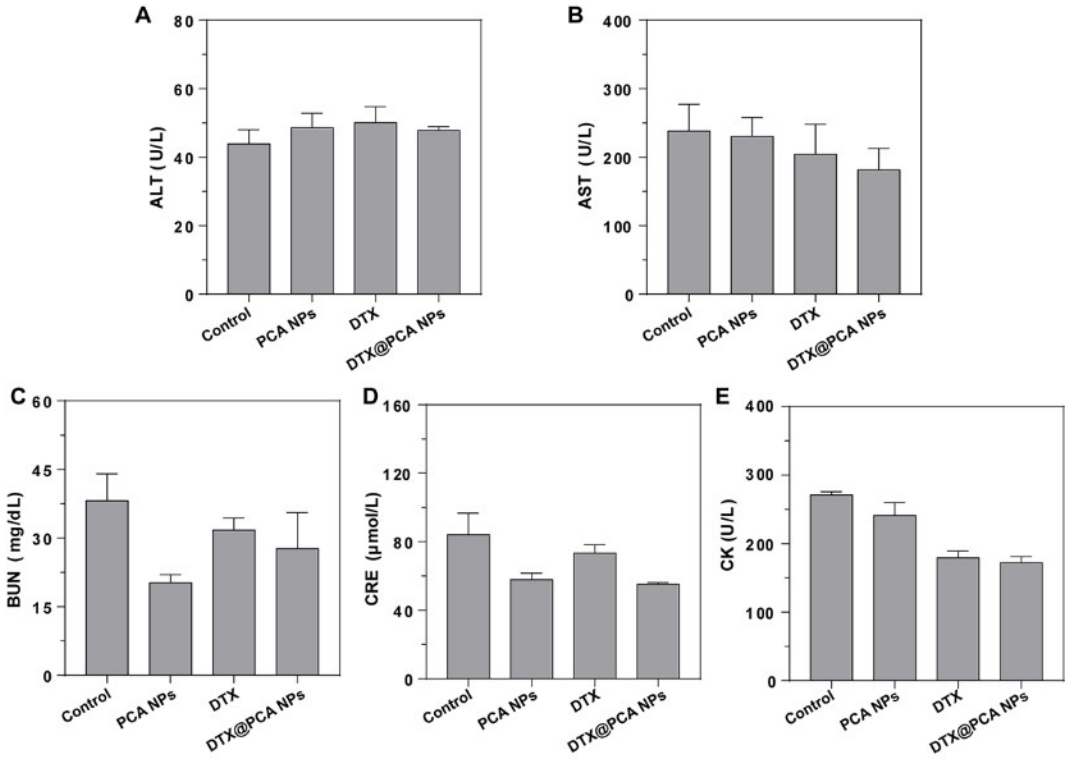

Fig. S11. Serum biochemistry analysis of (A) ALT, (B) AST, (C) BUN, (D) CRE, and (E) CK.