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Supporting Information

Small-sized copolymeric nanoparticles for tumor penetration and intracellular drug release

Chun-Nan Zhu,* Liu-Yuan Zang, Dong-Yun Zheng, Hui-Min Cao and Xiao-Jun Liu

College of Biomedical Engineering, Hubei Key Laboratory of Medical Information Analysis and Tumor Diagnosis & Treatment, and Key Laboratory of Cognitive Science (State Ethnic Affairs Commission), South-Central University for Nationalities, Wuhan 430074, P. R. China

*Corresponding author: Chun-Nan Zhu (E-mail: cnzhu@mail.scuec.edu.cn)

Materials

Cyclic carbonates (AC) is synthesized according to the literature.[1] Methoxy poly(ethylene glycol) (mPEG, M_n 2,000) and cystamine dihydrochloride are obtained from Sigma-Aldrich. Glutathione (GSH) is purchased from Aladdin. 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) is purchased from Amresco. Fetal bovine serum (FBS) and Dulbecco's Modified Eagle's Medium (DMEM) are purchased from Invitrogen Corp. HeLa cells are incubated in DMEM containing 10% FBS and 1% antibiotics (penicillin-streptomycin, 10,000 U/mL) at 37 ºC and a humidified atmosphere containing 5% CO_2. Other reagents are of analytical grade and purified by general methods.

Measurement

^{1}H-NMR spectra are recorded on a Bruker Avance III spectrometer using $CDCl_3$ as the solvent. UV-vis absorption spectra are recorded on a Shimadzu UV-2550 spectrophotometer. Fluorescence spectra are recorded by a PerkinElmer LS55 fluorescence spectrophotometer. Gel permeation chromatography (GPC) analysis is performed on an Agilent PL-GPC 50 system. DMF is used as the eluent at a flow rate of 0.3 mL/min. 20 Microliters of 1.0% (w/v) sample solution is injected for each analysis. Dynamic light scattering (DLS) measurements are carried out on a Malvern Zetasizer Nano ZS instrument. The nanoparticle aqueous solution (0.5 mg/mL) is passed through a 0.45 μm pore-sized syringe filter before DLS measurement. Transmission electron microscope (TEM) observations are conducted on a JEM-2100 (HR) electron microscope at an acceleration voltage of 200 kV. An average value is determined by three repeated measurements for each sample.

Synthesis of mPEG-*b*-PAC

mPEG-*b*-PAC is synthesized in toluene *via* ring-opening polymerization[2]. Briefly, toluene is added into a vessel containing mPEG, AC and zinc bis[bis(trimethylsilyl)amide] (1 wt‰ of AC). Then the vessel is immersed into an oil bath at 80 ºC under stirring for 24 h. The reaction mixture is dissolved in dichloromethane, then the filtrate is concentrated, dissolved in tetrahydrofuran, and dialyzed (MWCO 3,500 Da) in distilled water. The resulting solution is then lyophilized to obtain mPEG-*b*-PAC.

Preparation of DPP

The DPP nanoparticles are prepared through dialysis method. The copolymers (30 mg), DOX·HCl (3 mg) and triethylamine (30 mL) are dissolved in 4 mL of DMF, and then 4 mL of distilled water or cystamine dihydrochloride aqueous solutions are added dropwise under stirring at room temperature. After being stirred for another 12 h, the solution is transferred in a dialysis tube (MWCO 3,500 Da) dialyzing against distilled water for 24 h and is refreshed every 4 h to remove the residual DOX. The obtained solution is then filtered and lyophilized. DOX-loading content is analyzed by dissolving the DPP in DMF and then measured by a UV spectrophotometer at 485 nm. Drug-loading content (DLC) and entrapment efficiency (EE) are calculated as follows:

DLC (wt. %) = (weight of loaded drug/weight of drug-loaded nanoparticles) × 100 %

EE (%) = (weight of loaded drug/weight of drug in feed) × 100 %

Stability Measurement

For TEM analysis, the DPP solutions are treated with or without GSH for 24 h, and then the solution is dropped onto the surface of formvar-carbon film-coated copper grids. Excess solution is quickly wicked away with a filter paper. All grids are finally stained by 2 wt % phosphotungstic acid. The stability of DPP against 500 times dilution at 37 ºC is studied by measuring their size distribution using DLS. The

size change of crosslinked DPP in response to 10 mM GSH at 37 ºC is also monitored by DLS.

DOX Release from DPP

The release of DOX from the DPP is also investigated using dialysis method. 3 mg of DPP (1 mg/mL) is transferred into a dialysis tube (MWCO 3,500 Da), which is then dialyzed against 25 mL of phosphate buffered solution (PBS, 0.1 M, pH 7.4) containing different concentrations of GSH (0 mM and 10 mM) at 37 ºC. At desired time intervals, 3 mL of dialysate is taken out and an equal volume of fresh media is complemented. The amount of released DOX is determined by fluorometry (excitation at 480 nm and emission recorded at 555 nm). The DOX release experiments are conducted in triplicate and the results are presented as the average values.

***In Vitro* Cytotoxicity assay**

The cytotoxicity assay is performed with HeLa cells by MTT assay. HeLa cells are firstly seeded in a 96-well culture plate at a density of 6000 cells/well in 100 μL of DMEM containing 10% FBS at 37 ºC in a 5% CO_2 atmosphere for 24 h, and then exposed to DPP at a series of concentration for another 48 h. Then, 20 μL of MTT solution (5 mg/mL) is added to each well and further incubated for 4 h. After that, the media is replaced by 150 μL of DMSO to dissolve the formazan crystals formed by proliferating cell. The concentration of the proliferating cells in each well is confirmed by the absorbance of solvent at 570 nm using a microplate spectrophotometer (Thermo Varioskan^{TM} LUX). The relative cell viability is calculated according to the following equation:

Cell viability (%) = $(A_{sample}\text{-}A_0)/(A_{control}\text{-}A_0) \times 100$

Where A_{sample} is obtained in the presence of nanoparticles extract solutions and A_0 was obtained with complete DMEM. The results are presented as the average values of three runs.

Confocal Laser Scanning Microscopy

Live cell confocal microscopy is used to image the DPP uptake into HeLa cells. Hoechst 33342 water stock solution (blue molecular probe) is used to stain nucleus. HeLa cells are seeded at a density of 1×10^5 cells per well into biohousing chamber slide dishes loaded with a 25 mm diameter slide on cover-glass slides. After incubation for 24 h, the DPP solutions (with 5 mg/L DOX concentration) are added to each well and further incubated for 8 h. The medium is removed and the cells are washed three times with PBS. Then the nuclei are stained with 10 μL of Hoechst 33342 (2 μg/μL) at 37 ºC for 15 min. Finally, the cells are washed three times again with PBS and incubated with 200 μL of DMEM. The fluorescence is observed with a confocal laser scanning microscope (Nikon C1-Si TE2000, Japan) and the fluorescence signals of DOX and Hoechst 33342 staining are excited at 488 nm and 405 nm, respectively.

***In vivo* and *ex vivo* imaging**

All the animal experiments are performed complying with NIH guidelines for the care and use of laboratory animals and the experimental protocol are approved by the Institutional Animal Care. The female BALB/c nude mice (6-week-old) are random allocated to each group, and HeLa cells (1×10^7/well) suspended in 100 μL of DMEM are inoculated subcutaneously into the hind leg of each mice. Caliper measurements are utilized for estimating the tumor size of the nude mice, and the transplanted tumor grows until a volume about 100 mm^3, 200 μL of Cy5-labelled DPP (1 mg mL^{-1}) are injected into the mice through tail vein injection. Near-infrared imaging is carried out at predetermined time points (2 h, 4 h, 8 h and 12 h) using *in vivo* spectrum imaging system (excitation filter, 630 nm; emission filter, 670 nm). After i.v. injected with PBS, free DOX and DPP, animals are sacrificed at 12 h, the tumors are excised for

ex vivo imaging and analysis. The values are expressed as mean ± S.D. (N = 3).

***In vivo* anti-tumor efficiency**

The HeLa-xenografted mice models are established according to the above method. When the volume of transplanted tumor reaches to 50-70 mm^3, the mice are divided into three groups (N = 6) randomly and intravenously injected by PBS, free DOX and DPP (200 μL, 2.5 mg DOX /kg body weight) *via* the tail vein every other day (days 1, 3, 5, 7, 9 and 11). During the treatment process, the tumor volumes and body weight are measured by caliper every other day. The values are expressed as mean ± S.D. (N = 6).

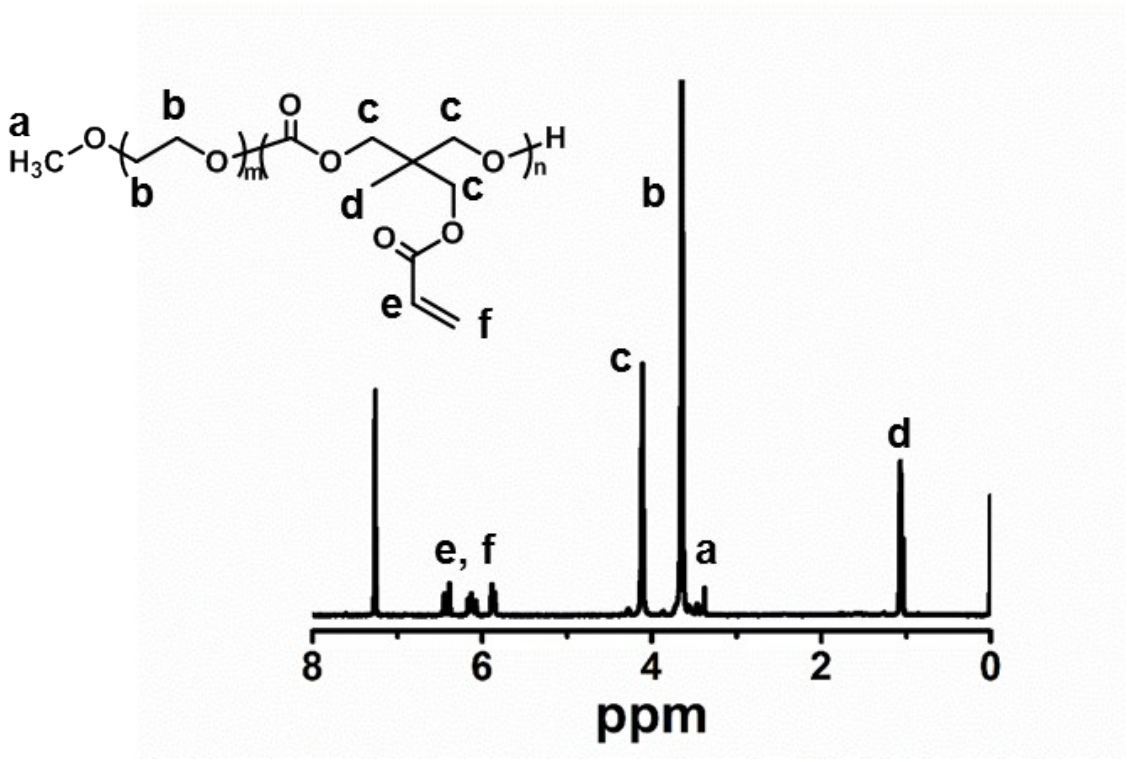

Fig. S1 ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of mPEG-*b*-PAC.

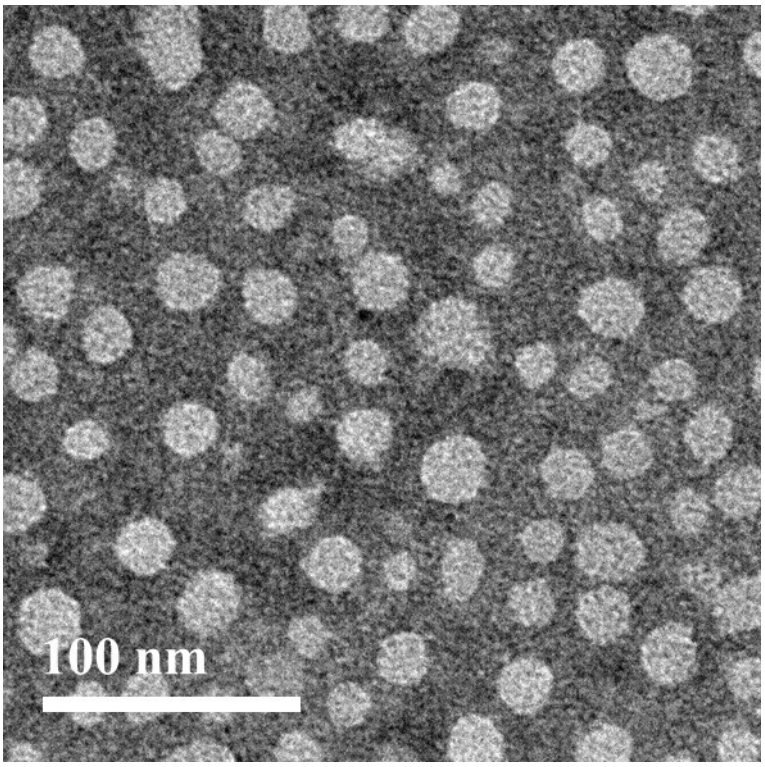

Fig. S2 TEM image of DPP in PB (0.01 M, pH 7.4) at 24 h.

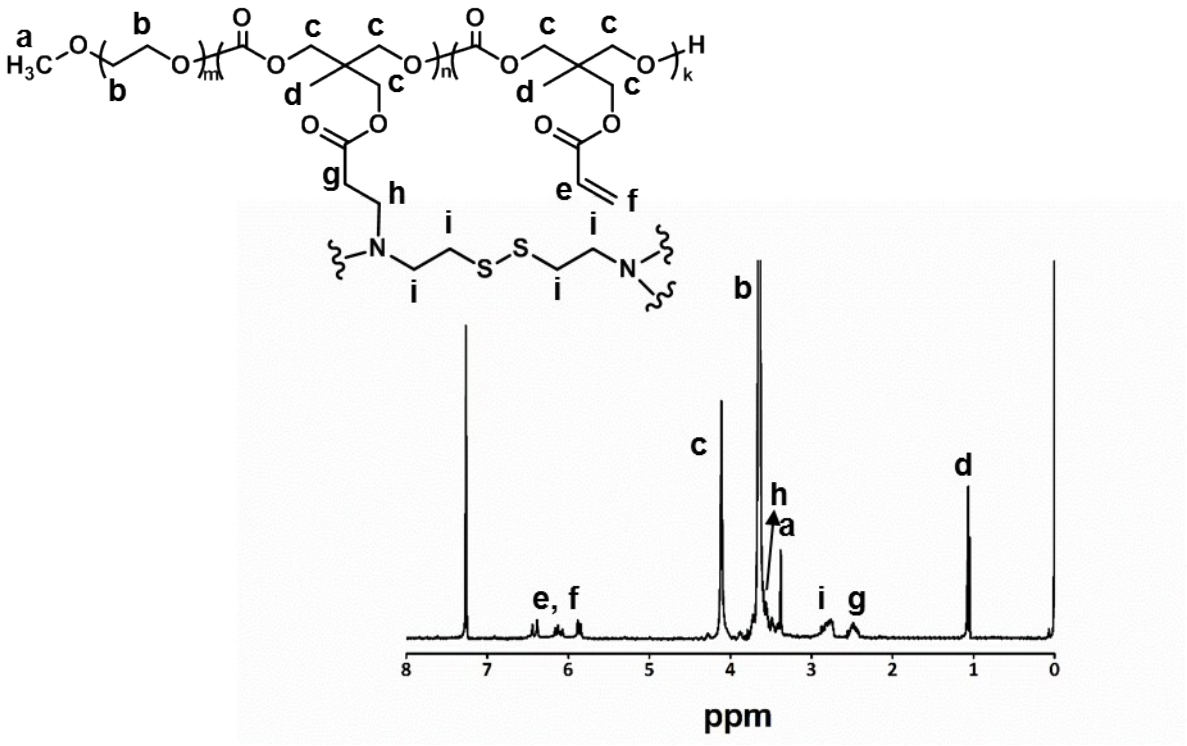

Fig. S3 ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of crosslinked copolymer.

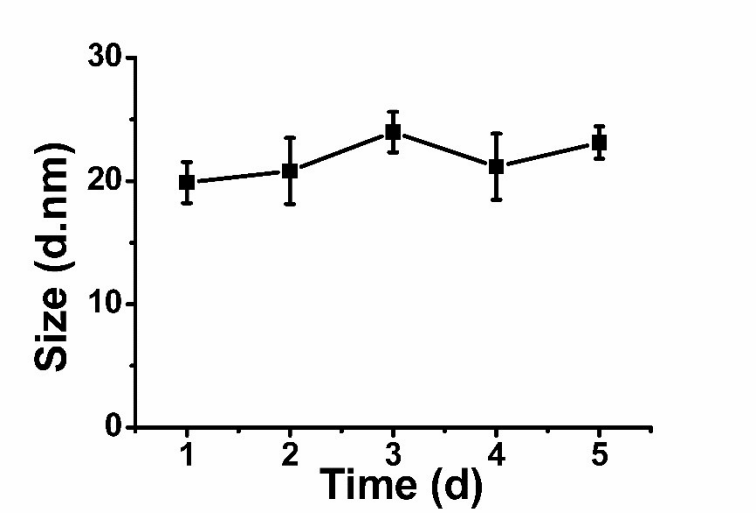

Fig. S4 Change in the particle size of DPP in PB (0.01 M, pH 7.4) at different time points measured by DLS.

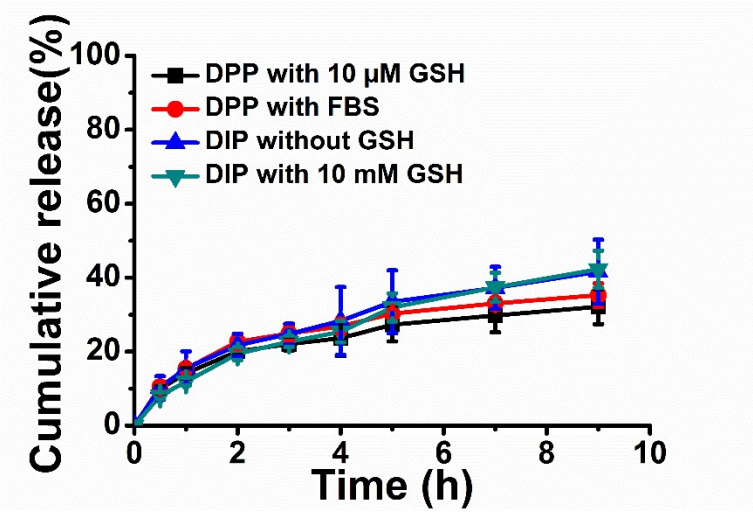

Fig. S5 *In vitro* drug-release profiles from the DPP in PB with 10 μM GSH or 5% v/v fetal bovine serum (FBS), and the DIP in PB without GSH or with 10 mM GSH at 37 °C, which is prepared through the reaction of mPEG-*b*-PAC with 1,6-hexanediamine and DOX encapsulation. Date are shown as mean ± S.D. (n=3).

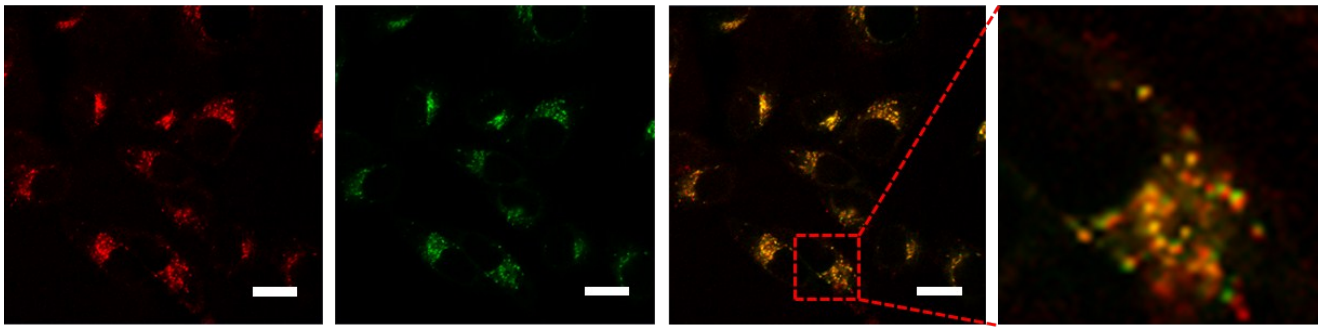

Fig. S6 CLSM images of HeLa cells incubated with DPP for 4 h, lysosomes are labeled with LysoTracker Green DND-26. Scale bar: 20 μm.

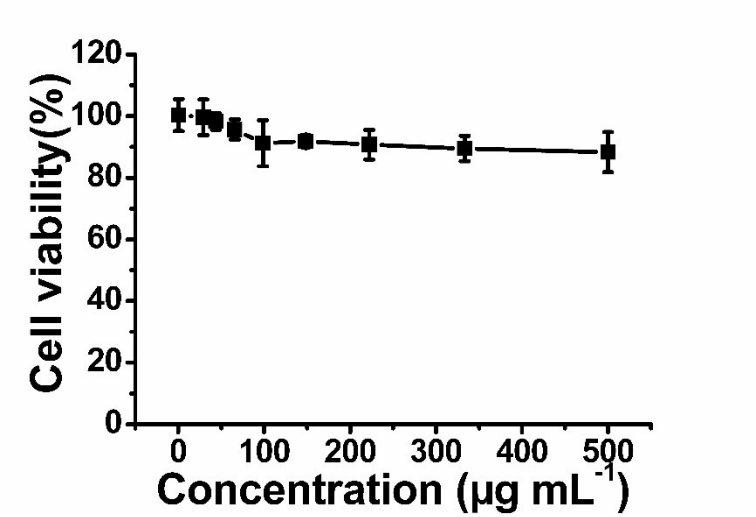

Fig. S7 Cytotoxicity of DPP without DOX-loading against HeLa cells. Date are shown as mean ± S.D. (n=3).

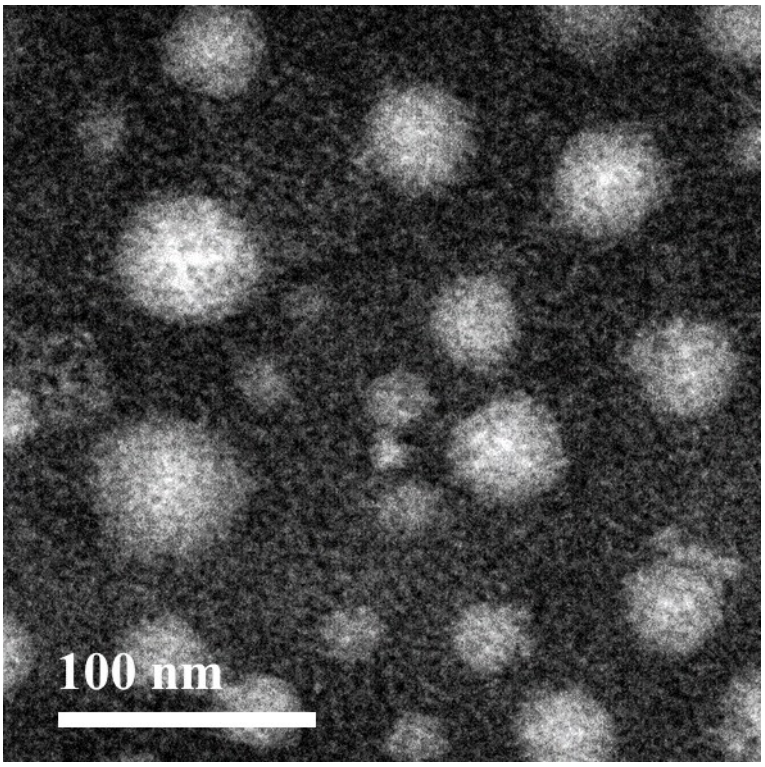

Fig. S8 TEM image of CDPP in PB (0.01 M, pH 7.4).

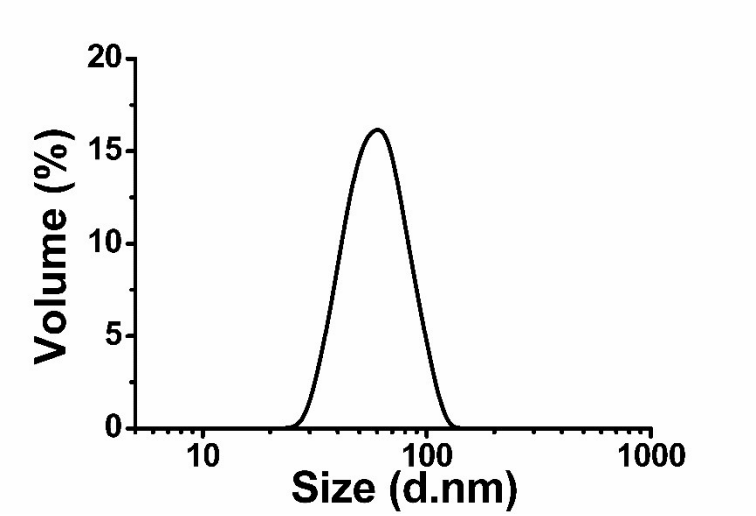

Fig. S9 Particle size of the CDPP in PB (0.01 M, pH 7.4) measured by DLS.

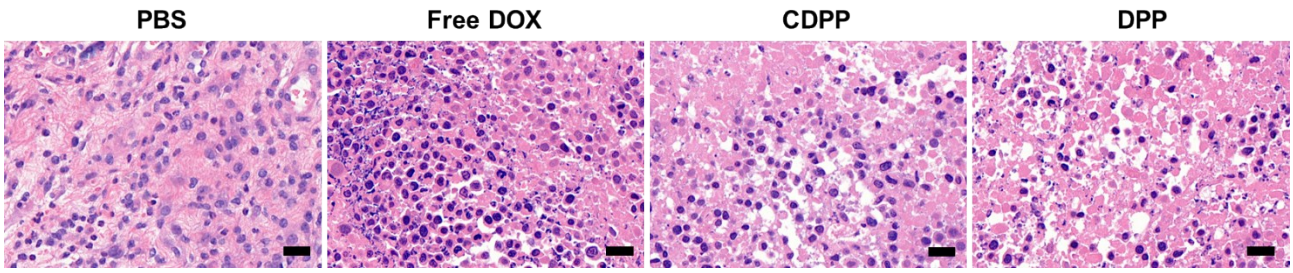

Fig. S10 Histopathology evaluation of the tumor collected at 11 d post-injection. Scale bar: 20 μm

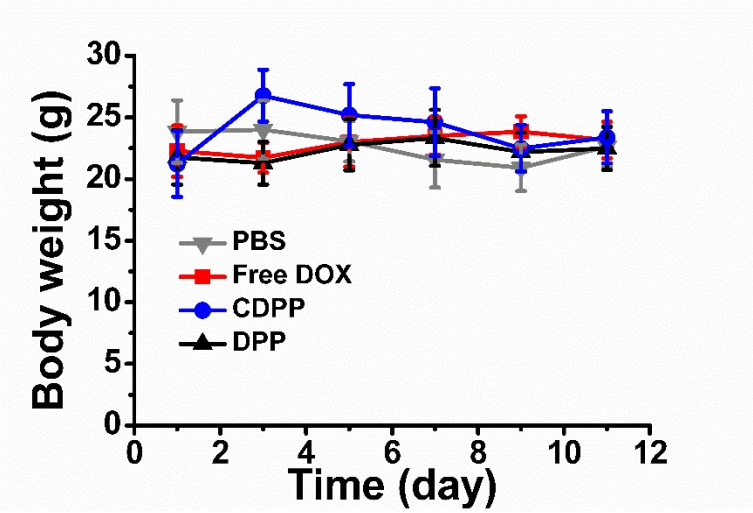

Fig. S11 Body weight changes of the PBS, free DOX, CDPP and DPP groups over the treatment process. Values are expressed as means ± S.D. (N = 6).

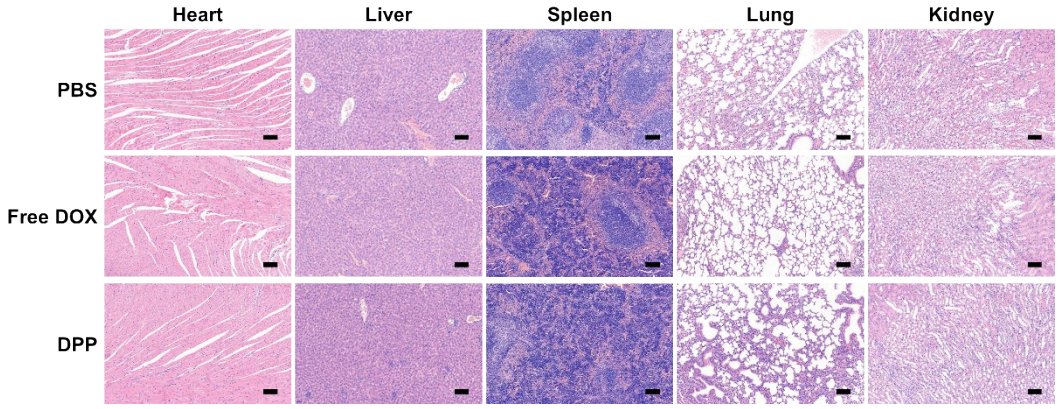

Fig. S12 Histopathology evaluation of the heart, liver, spleen, lung, and kidney collected at 11 d post-injection. Scale bar: 100 μm

References

1. W. Chen, H. Yang, R. Wang, R. Cheng, F. Meng, W. Wei and Z. Zhong, *Macromolecules,* 2010, **43,** 201-207.

2. F. Oroojalian, M. Babaei, S. M. Taghdisi, K. Abnous, M. Ramezani and M. Alibolandi, *J. Controlled Release,* 2018, **288,** 45-61.