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Supplementary information

Size-Changeable and Multi-Responsive Nanoplatform for Deep Tumor Penetration and Targeted Combinatorial Radio/Chemotherapy

Fatima Zohra Dahmani,[a,b] Danni Zhong,[b] Aboubaker El G. Dahmani,[d] Yuchen Qi,[b] Tingting Xie,[b] Bo Zhou,[b] Wanli Li,[b] Ke Yao,[a,e] Lei Li[,c], and Min Zhou[*, a,b,f,g]*

[a]Eye Center, the Second Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou 310009, China

[b]Institute of Translational Medicine, Zhejiang University, Hangzhou 310009, China

[c]Shanghai Key Laboratory of Regulatory Biology, Institute of Biomedical Sciences, School of Life Sciences, East China Normal University, Shanghai 200241, China.

[d]Jiangsu Key Laboratory of Drug Screening, China Pharmaceutical University, Nanjing 210009, China

[e]Zhejiang Provincial Key Lab of Ophthalmology, Hangzhou, China

[f]Key Laboratory of Cancer Prevention and Intervention, National Ministry of Education, Zhejiang University, Hangzhou 310009, China

[g]State Key Laboratory of Modern Optical Instrumentations, Zhejiang University

* Address correspondence to

Email: zhoum@zju.edu.cn

Email: lli@bio.ecnu.edu.cn

Methods

Preparation of IR780/HPDAu nanoparticles

IR780/HPDAu nanoparticles were prepared using dialysis method. 100 µL of IR780 solution in DMSO (400 µg/mL) was added to 3 mL of HPDAu solution in distilled water (5 mg/mL). The mixture was then stirred for 3 h then sonicated for 30 min using a probe ultrasonicator. Thereafter, the obtained solution was dialyzed against deionized water for 24 h followed by centrifugation (5000 rpm for 10 min), filtration through 0.45 µm syringe filter and freeze-drying.

In Vitro Drug Release and Responsiveness of HPDAu nanoparticles

The *in vitro* responsiveness of HPDAu nanoparticles to the tumor microenvironment attributes was assessed upon incubation in mimicking conditions. Firstly, the change in particle size and zeta potential of HPDAu was assessed before and after incubation with HAase (1:1, v:v) at pH 7.4 and 6.5 using DLS. Besides, the DOX release rates of HPDAu were determined upon incubation in different conditions using fluorescence spectroscopy. 1 mL of HPDAu (500 µg/mL DOX) with or without HAase (1 mg/mL) was added into a dialysis tube (3500 MWCO) then incubated in 150 mL of phosphate buffer saline (pH 7.4, 10 mM) or acetate buffer (pH 6.5 and pH 5.5, 10 mM) in a shaking water bath (100 rpm, 37 °C).[1] At fixed time points, 200 µL of each sample was withdrawn then DOX concentrations were determined using a microliter plate reader (SpectraMax M5, Molecular Devices, USA) at excitation/emission wavelengths of 480/595 nm. All assays were performed in triplicate.

Cellular Uptake Study

4T1 cells were cultured in 10% FBS-DMEM medium (supplemented with 100 U/ml penicillin and 100 μg/mL streptomycin) at 37 °C in a 5% CO_2 humidified atmosphere. The cellular uptake of HPDAu was visualized using Operetta high-content imaging system (PerkinElmer, USA). 4T1 cells (1x 10^4 cells) were plated in ninety six-well plates and further cultured overnight. The cells were then incubated with DOX, HPDAu, HAase (1mg/mL)+HPDAu for 6 h or 12 h (at equivalent DOX concentration of 1 μg/mL). The blocking experiment was performed after 1 h pretreatment with free HA (1 mg/mL), the cells were then treated with HPDAu as described above. After a predetermined time of incubation (6 h and 12 h), the cells were washed twice with PBS, stained with Hoechst 33342 for 15 min, washed again with PBS, then imaged at 20x magnification. The fluorescence intensity in each well was determined by analyzing the DOX channel images using Harmony software.

***In Vitro* Apoptosis Detection**

The apoptosis induction of HPDAu combined with RT was evaluated using Annexin V-FITC/PI apoptosis detection kit (Yeasen Biotech Co., Shanghai, China). 4T1 cells (5 x 10^5 cells/well) were plated in six-well plates then allowed to adhere overnight. The cells were treated with PBS, DOX, AuNPs, HPDAu (1 μg/mL of DOX, 4 μg/mL of Au) for 4 h, then washed with PBS and incubated in fresh medium. Thereafter, RT, DOX+RT, AuNPs+RT and HPDAu+RT groups were treated with X-ray irradiation (4 Gy), followed by 12 h incubation. For apoptosis evaluation, all of the cell groups were performed according to the manufacturers' protocols and analyzed using flow

cytometry (CytoFLEX, Beckman-Coulter, Pasadena, CA, USA). All assays were performed in triplicate.

MTT Assay

The *in vitro* cytotoxic effect of HPDAu was evaluated using MTT assay. 4T1 cells (5 x 10^3 cells/well) were plated in ninety six-well plates then allowed to adhere for 24 h. The cells were treated with HPDAu (1 μg/mL of DOX, 4 μg/mL of Au) for 4 h, then irradiated with different X-ray doses (0, 2, 4, 6 Gy) and further incubated for 48 h. Cells treated with X-ray irradiation only (RT) were used as control. 10 μL of MTT was added to each well followed by 4 h incubation. Formazan precipitates were then dissolved in 150 μL DMSO and the absorbance was recorded using SpectraMax M5 microtiter plate reader (570 nm). All assays were performed in sextuplicate.

The cell viability of immortalized primary human hepatocytes (HepLL) and neonatal mouse cardiac fibroblasts (NMCF) upon treatment with HPDAu was evaluated using the same assay. HepLL and NMCF cells were cultured in 10% FBS-DMEM medium (supplemented with 100 U/ml penicillin and 100 μg/mL streptomycin) at 37 °C in a 5% CO_2 humidified atmosphere. The cells (1 x 10^4 cells/well) were seeded in ninety six-well plates for 24 h, then incubated with different concentrations of DOX and HPDAu (0.1, 0.5, 1, 5, and 10 μg/mL of DOX), respectively. 10 μL/well of MTT solution was then added followed by the same protocol as described above.

Table S1. Physicochemical characterization of different nanoformulations. Data are presented as mean±SD (n = 3).

	Mean particle size (nm)	**PDI**	**Zeta potential**
PAMAM	6.9±0.80	0.328	+32.43±2.41
PD	5.4±1.37	0.122	+19.57±0.12
AuNPs	10.93±1.24	0.226	+26.7±1.21
HPDAu	97.04±5.20	0.120	-20.9±2.35

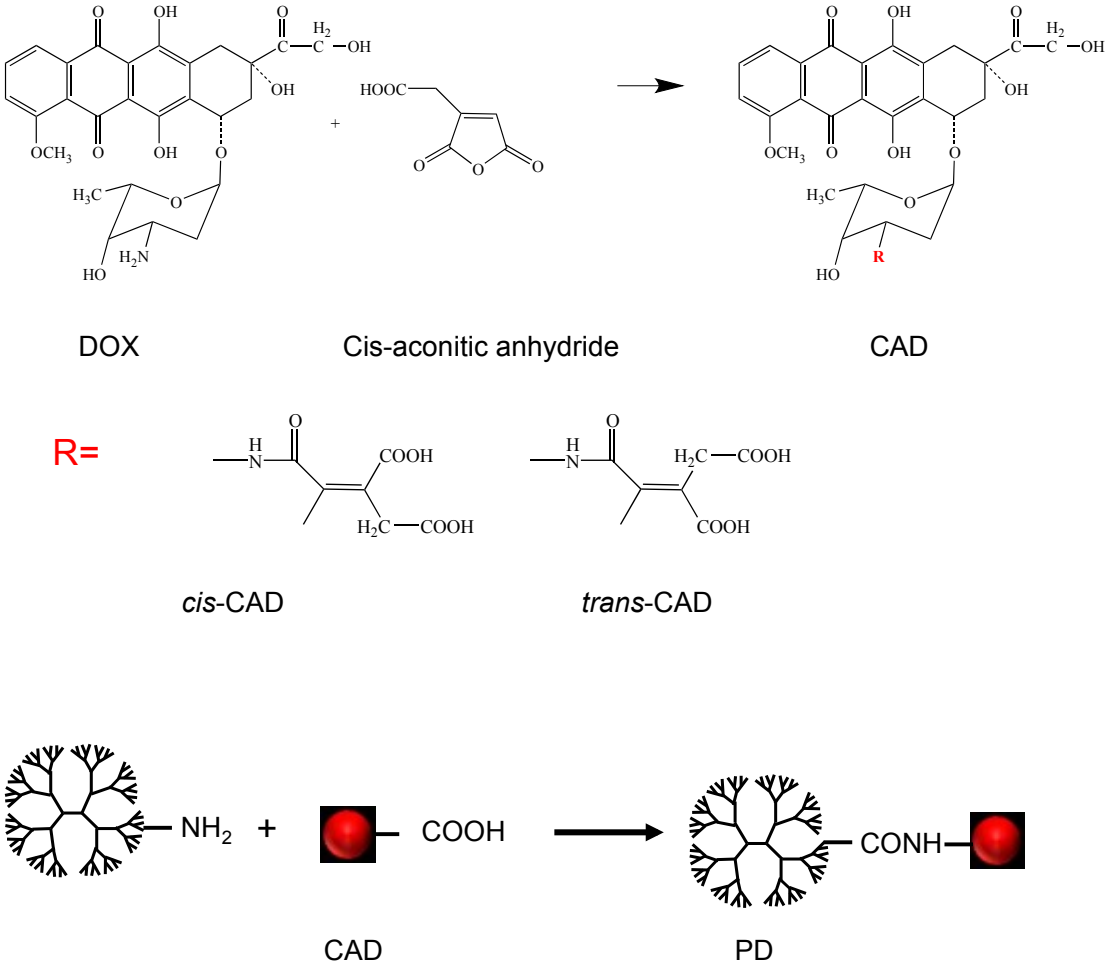

Fig. S1 Synthesis route of *cis*-aconityl-doxorubicin CAD (A) and DOX-PAMAM (PD) conjugate (B).

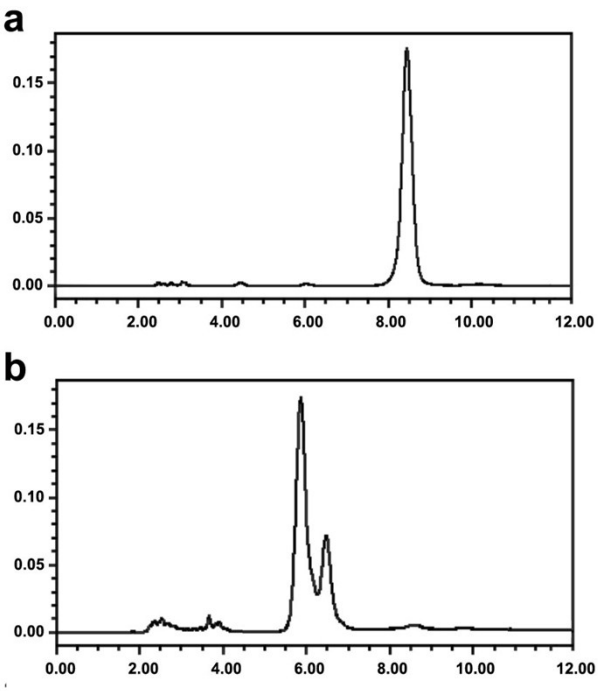

Fig. S2 High performance liquid chromatography (HPLC) chromatograms of (a) DOX solution (Rt 8.53 min) and (b) reaction mixture after 24 h (CAD1: Rt 5.91, CAD2: Rt 6.67). *Cis*-aconityl-doxorubicin (CAD).

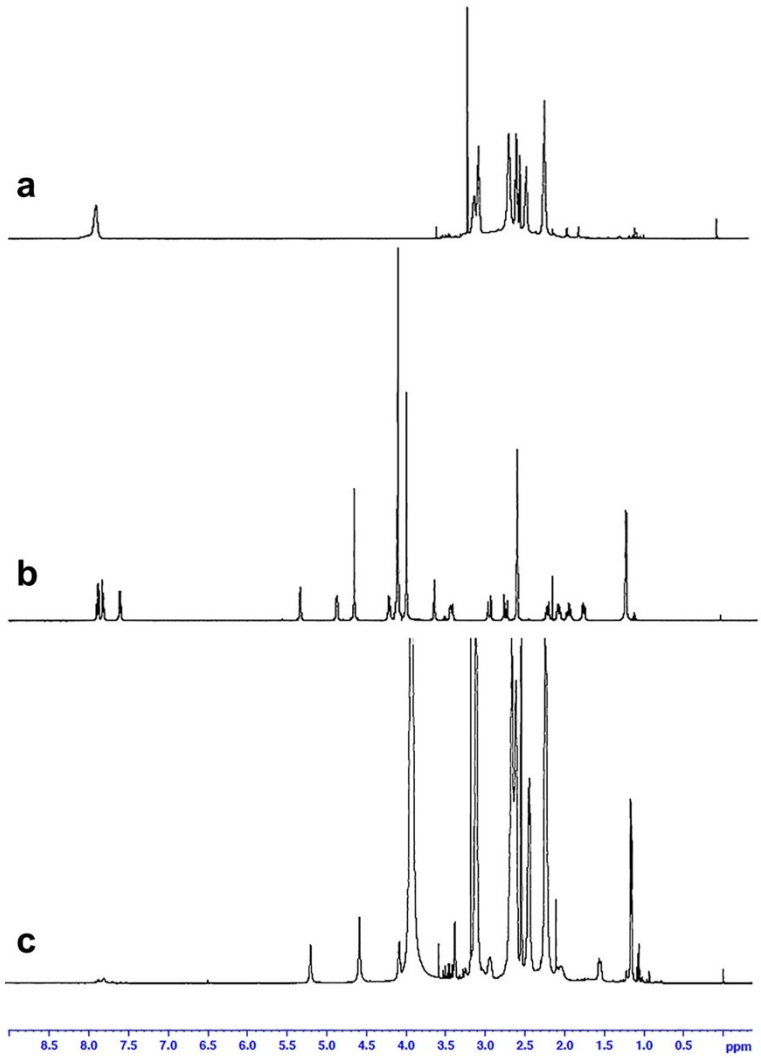

Fig. S3 Proton nuclear magnetic resonance (^{1}H NMR) spectra of (a) PAMAM (DMSO-d6), (b) DOX (DMSO-d_6), (c) DOX-PAMAM (PD) conjugates (DMSO-d_6:D_2O, 1:1).

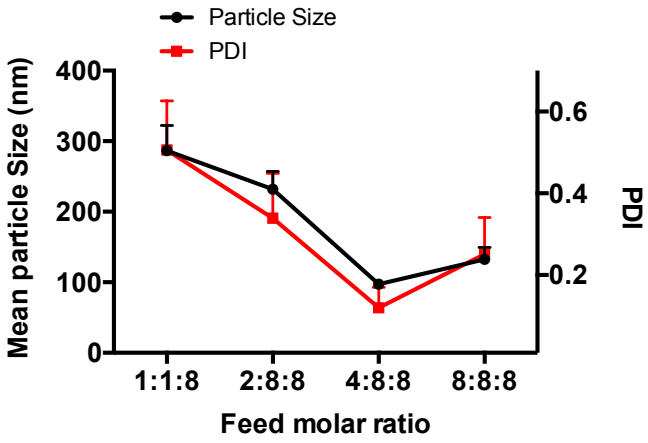

Fig. S4 Particle size distribution of HPDAu nanoparticles as a function of EDC:ADH:HA molar ratio.

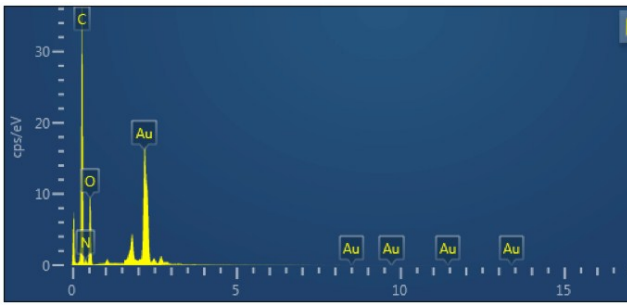

Fig. S5 Energy-dispersive X-ray spectroscopy (EDS) analysis of HPDAu nanoparticles shows characteristic gold peaks.

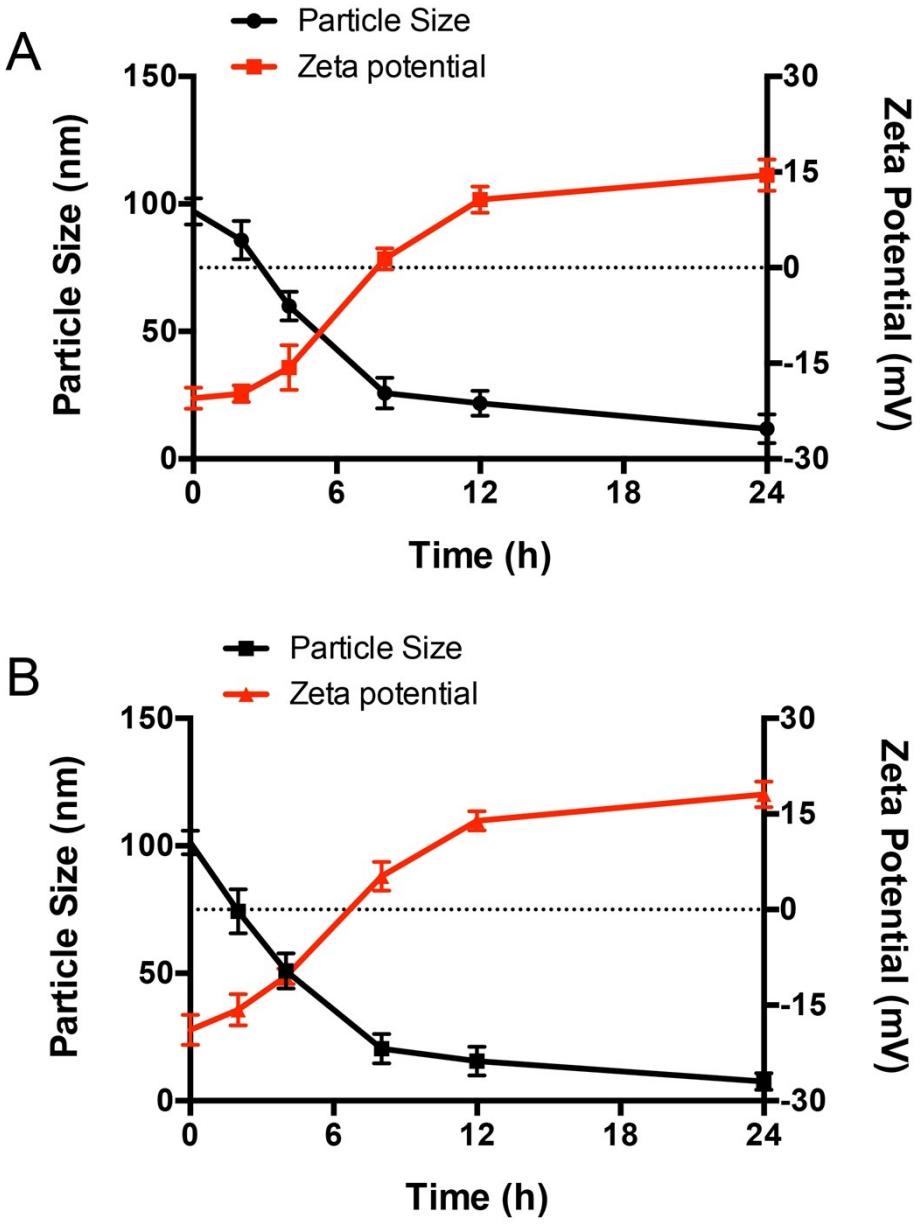

Fig. S6 Change in particle size and zeta potential of HPDAu incubated with hyaluronidase (HAase) at pH 7.4 and 6.5, respectively, as a function of time.

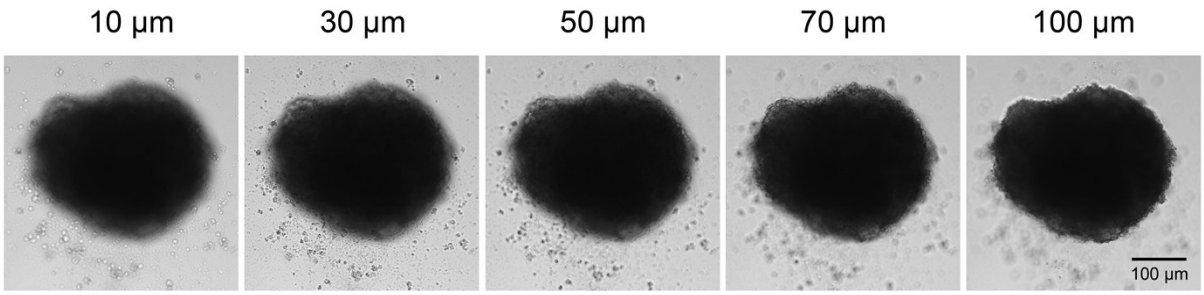

Fig. S7 Bright-field Z-stack confocal images of 4T1 multicellular spheroids (control group), acquired from the surface top towards the equatorial plane at 20 μm intervals. The scale bars indicate 100 μm in length.

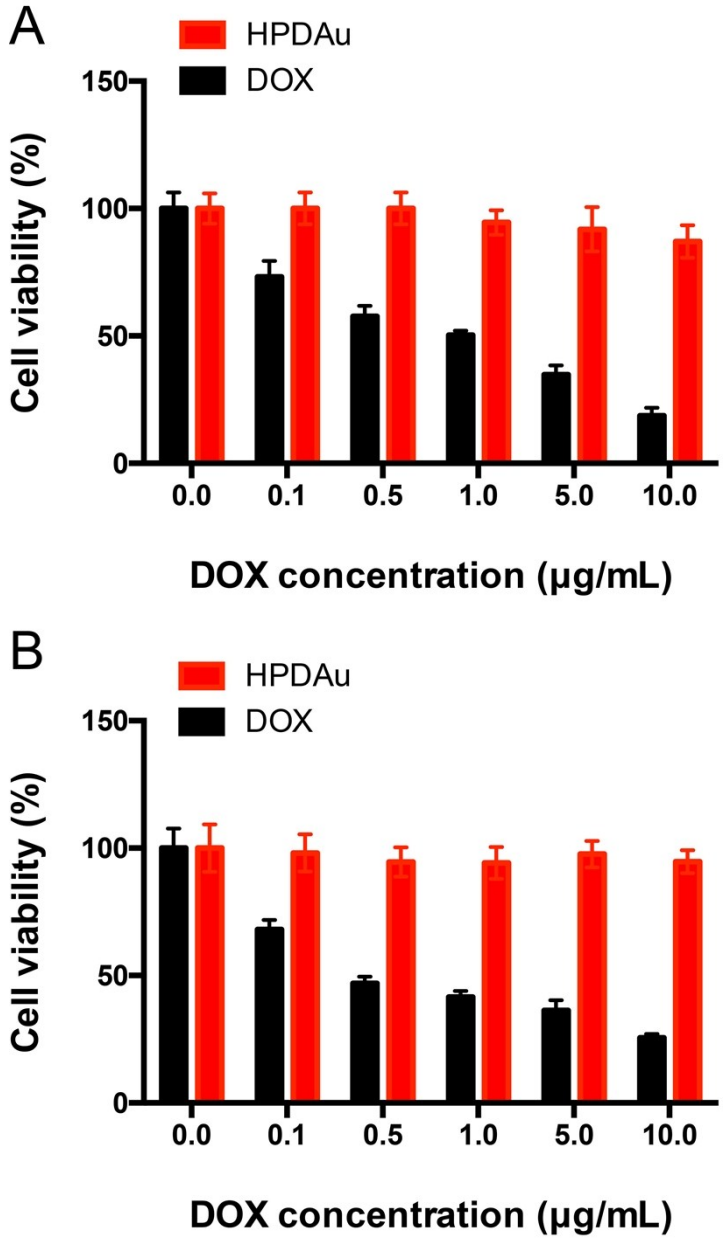

Fig. S8 *In vitro* cytotoxic effect of HPDAu nanoparticles at different DOX concentrations towards (A) HepLL and (B) NMCF cells after 48 h incubation. All data are presented as mean±SD, (n = 6).

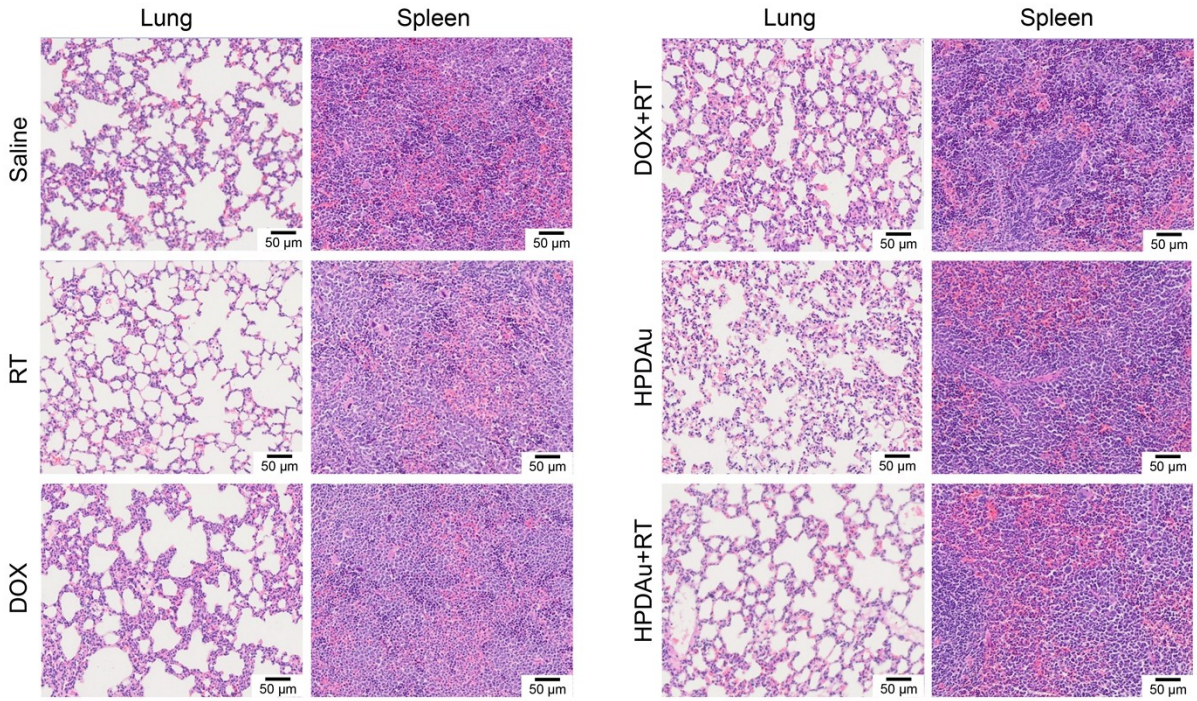

Fig. S9 Histopathological examination of lungs and spleen from different groups: saline (control), radiotherapy (RT), DOX, DOX plus RT (DOX+RT), HPDAu, and HPDAu plus RT (HPDAu+RT). The scale bars indicate 50 μm in length.

References

1. T. Jiang, R. Mo, A. Bellotti, J. Zhou and Z. Gu, *Adv. Funct. Mater.*, 2014, **24**, 2295-2304.