Detachable Nanoparticle-Enhanced Chemoimmunotherapy Based on Precise Killing of Tumor Seeds and Normalizing the Growing Soil Strategy

Lei Wang,[†,‡,§] Kaili Ding,[†] Cuixia Zheng,[†] Huifang Xiao, [†] Xinxin Liu,[†] Lingling Sun,[†] Rida Omer, [†]Qianhua Feng,[*,†,‡,§] Zhenzhong Zhang[*,†,‡,§]

[†]School of Pharmaceutical Sciences, Zhengzhou University, 100 Kexue Avenue, Zhengzhou 450001, China

[‡]Collaborative Innovation Center of New Drug Research and Safety Evaluation, Henan Province, Zhengzhou 450001, China

[§]Key Laboratory of Targeting Therapy and Diagnosis for Critical Diseases, Henan Province, Zhengzhou 450001, China

Materials and Methods

The Hyaluronic acid (molecular weight: ~3.4 KDa) was purchased from Freda Biochem Co., Ltd. (Shandong, China). N-(3-Dimethylaminopropyl)-N`-ethylcarbodiimide hydrochloride (EDC) and N-Hydroxysuccinimide (NHS) were purchased from Aladdin Bio-chem Technology Co., Ltd (Shanghai, China). 3-diethylaminopropyl isothiocyanate was purchased from Macklin Bio-chem Technology Co., Ltd. The peptide (Gly-Arg-Val-Gly-Leu-Pro-Gly 98.84%) was synthesized and analyzed using high-performance liquid chromatography and electrospray ionization mass spectrometry by ChinaPeptides (Shanghai, China). Recombinant Human MMP-2 (rhMMP-2) was purchased from PeproTech.

Anti-CD45-APC-Cy7, anti-CD11b-BB515, anti-CD11c-BV605, anti-F4/80-BV421, anti-Gr-1-PECy7, anti-CD86-APC-R700, abti-CD103-BV510, anti-CD40-PE, anti-CD3-BV510, anti-CD8-PerCP-Cy5, anti-CD4-PE and

anti-FOXP3-BV421 were all purchased from BioLegend. Antibodies for Calreticulin (CRT) was purchased from Bioss Biotech Co., Ltd (Beijing, China). ELISA kits for IL-10, IL-12, IL-6, IFN-γ and TNF-α analysis were purchased from Multi Sciences (Lianke) Biotech Co., Ltd.

Synthesis of HA-grafted DOX prodrug

Preparation of HA-grafted DOX prodrug according to previous method.[1] First, HA was dispersed in formamide. Then, a specified amount of N-(3-Dimethylaminopropyl)-N`-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were respectively dissolved in formamide, and added them into the above HA solution with the ratio of COOH:EDC:NHS = 1:5:5, followed by stirring this mixture at room temperature for 6 h for HA activation. Subsequently, DOX•HCl was put into above solution, and the mixture was further stirred at room temperature for 24 h. Finally, unreacted free DOX•HCl, EDC and NHS were removed by dialysis. The product was obtained by freeze-dried and characterized through ^{1}H NMR (Avane II 500 MHz, Bruker, Switzerland) and FT-IR (Thermo Scientific, Nicolet 6700) (Figure S1 and S2, Supporting Information).

Synthesis of MMP-2-DEAP

The MMP-2 peptide Gly-Arg-Val-Gly-Leu-Pro-Gly (1 mmol) was reacted with DEAP (10 mmol) in DMSO (10 mL) containing TEA (1 mL) and pyridine (0.1 mL) under 35℃ for 36 h, finally producing MMP-2-DEAP. After the reaction, the resulting solution was dialyzed using a dialysis membrane (Spectra/Por MWCO 3.5 kDa; Spectrum Lab., USA) against ® fresh water for 2 days. After the end of the dialysis,

the liquid in the dialysis bag was freeze-dried, and then the chemical structure was confirmed by ^{1}H NMR (Figure S3 and S4).

Synthesis of DOX@HA-MMP-2-DEAP

Firstly, HA-grafted DOX prodrug was put into 10 mL of formamide to make it completely dissolved. A specified amount of EDC and NHS were respectively dissolved in formamide, and added them into the above solution, followed by stirring this mixture at room temperature for 6 h, and then ethylenediamine was added into the mixture drop by drop. Next, the solution was reacted at room temperature for 24 h. Subsequently, free reactants were removed through analysis. Finally, the activated prodrug products were obtained after lyophilization.

Secondly, the MMP-2-DEAP was dissolved in formamide, and the EDC and NHS were added into the solution with a certain proportion stirring for 12 h. Next, the prodrug products were added into the solution, followed by reacting 24 h. The resulting solution was purified by dialysis to remove unreacted free reactants.

Evaluation of the pK_b for DOX@HA-MMP-2-DEAP

The p*K*b value of DOX@HA-MMP-2-DEAP was obtained by an acid-base titration method. DOX@HA-MMP-2-DEAP was dissolved in deionized water (1 mg/mL) and the pH was adjusted to 12 with 1 M NaOH. The solution was titrated by the dropwise addition of 0.5 M HCl solution to obtain the pH profile. The average pH value from triplicate titrations was plotted against the volume of added HCl solution.

Preparation & Characterization of DOX@HA-MMP-2-DEAP/CXB

CXB loaded micelles were made by dialysis.[2] DOX@HA-MMP-2-DEAP was

put in formamide with CXB at a molar ratio (DOX@HA-MMP-2-DEAP: CXB) of 5:1 stirring to make them mix evenly. Then, the PBS was added to the formamide solution drop by drop until the solution appeared turbid. Dialysis using water and a dialysis bag (MWCO 14,000) was done for 24 h. The solution was finally filtered using a 0.45 μm filter to get rid of unloaded CXB.

To investigate tumor acidity and MMP-2-induced switch the morphology and the surface charge of nanoparticle, the nanoparticles were incubated with MMP-2 (1 ug/mL) at pH = 6.5 or 7.4 for 4 h, followed by the determination of hydrodynamic diameter and surface charge of the nanoparticles by DLS, transmission electron microscopy (TEM) and zeta-potential measurements, respectively.

The hydrodynamic diameter and the stability of the nanoparticles (1 mg/mL) in PBS solution (pH 7.4) were measured by dynamic light scattering (DLS, Nano-ZS ZEN3600, Malvern). The assembled morphology of DOX@HA-MMP-2-DEAP (1 mg/mL) was directly observed *via* TEM (*HT7700*). The loading ratio of and encapsulation efficiency of CXB by DOX@HA-MMP-2-DEAP/CXB nanoparticles were determined by HPLC (Waters, USA) with a ultraviolet–visible detector at 250 nm. They were calculated using following formulas: Loading ratio = (weight of loaded CXB)/(total weight of DOX@HA-MMP-2-DEAP and CXB)×100%; Encapsulation efficiency = (weight of loaded CXB)/(weight of initially added CXB)×100%.

To evaluate the release profile of CXB from the nano-assembly, DOX@HA-MMP-2-DEAP was dissolved in 1 mL of PBS at different pH in presence

or absence of rhMMP-2 (1 μg/mL), transferred to a dialysis bag (molecular weight cutoff: 8,000-14,000 Da), and dialyzed against 9 mL of PBS solution containing 0.1% SDS at 37°C with stirring at a speed of 100 rpm/min. At different time intervals, the amount of released CXB and DOX in the dialysis buffer was detected by HPLC. The accumulative percentage of CXB and DOX was calculated.

Cell culture

4T1 cell were cultured in RPMI 1640 medium. All culture medium contained 10% FBS and 1% antibiotics (penicillin–streptomycin, 10,000 U/mL), and all cells were incubated at a humidified atmosphere with 5% CO_2, 37°C.

Intracellular uptake of doxorubicin prodrug *in vitro* using CLSM and flow cytometry analysis

To exploit the receptor-mediated selective intracellular uptake of doxorubicin prodrug *in vitro*, 4T1 cancer cells were seeded into 12 well tissue culture plates (1×10^5 cells/well) and incubated overnight. To validate the HA backbone's role in helping cellular uptake of micelles, 4T1 cells were pre-cultured with free HA prior to the incubation with particles to saturate cellular HA receptors. Results from CLSM (Leica TCS SP8) and flow cytometry analysis (BD Accuri C6) both exhibited a remarkably decreased intracellular fluorescence intensity of HA@DOX, which therefore demonstrated HA-dependent, specific endocytosis.

Evaluation of cytotoxicity

4T1cells were seeded into 96-well plates at a density of 7×10^3 cells each well in the presence of different concentrations of HA, DOX, or HA@DOX. After treatment

for 24 h or 48 h, cells were incubated with CCK-8 agent for 1 h at 37℃ and the absorbance value (OD) at the wavelength of 450 nm was measured using a microplate reader (Synergy H1, BioTek).

To study the biocompatibility of blank nanoparticles (HNPs), the cells (HUVEC, 4T1) were seeded into 96-well plates at a density of 7×10^3 cells each well in the presence of different concentrations of HNPs treatment for 24 h, and then the relative viability of cells was examined by MTT assay as described above.

Induction of immunologic cell death (ICD) with the vesicles

To determine chemotherapy-induced ICD of the tumor cells, surface expression of calreticulin (CRT) and extracellular release of HMGB1 secretion were examined *in vitro*. For flow cytometric analysis of cell surface expose of CRT, 4T1 cells were seeded into the 6-well plate (3×10^5 cells/well) and the next day the cells were incubated with HNPs, DHPD, HPDB or DHPDB for 8 h. DHPDB was pre-incubated with MMP-2 in a buffer of pH 6.5 for 4 h before added into the cell culture medium. The cells were then harvested, washed twice with PBS, incubated with FITC-conjugated anti-CRT antibody for 30 min. Washing with PBS to remove free antibody before flow cytometric examination.

To detect the release of HMGB1, each group of culture supernatants were collected and the concentration of HMGB1 in the supernatant was measured using an HMGB1 ELISA kit, according to the manufacturer's instructions, respectively.

Preparation of DCs

In vitro differentiation of DCs was performed with primary bone marrow cells

from female C57BL/6 mice at 6-8 weeks of age using the induced DCs protocol.[3] DCs were harvested 6 days after the start of the culture and used for experiments.

***In Vitro* DC activation with tumor cell supernatant**

4T1 cells were plated at 3×10^5 cells/mL in 6-well plates were cultured in RPMI 1640 medium supplemented with 10% FCS, 50 μM beta-mercaptoethanol and penicillin/streptomycin and incubated with HNPs, DHPD, HPDB, DHPDB. After 24 h, each group-conditioned supernatant was transferred onto the harvested DCs in 12-well plates. Activation of DC was determined after 24 h using anti-CD86-FITC, anti-CD40-APC antibody or anti-CCR7- Alexa Fluor 488 and then analyzed by flow cytometry.

PGE_2 level analysis *in vitro*

Briefly, 4T1, RAW264.7 and NIT-3T3 cells were cultured in 96 well-plate overnight. After RAW264.7 and NIT-3T3 cells stimulation with LPS and TGFβ, respectively, the medium was replaced with fresh one. Then, the cells were incubated for 24 h with CXB of different concentrations respectively. 4T1 cells were incubated with HNPs, DHPD, HPDB, DHPDB for 24 h. The amounts of PGE_2 in the supernatants were measured using a PGE_2 ELISA Kit (Cayman Chemical) according to the manufacturer's instructions. All samples were conducted in triplicate.

Western blot analysis

The experimental steps are the same as above. After cells incubated for 24 h, the treated cells were collected to measure the COX-2 protein levels by western blot analysis.[4]

CXCL12 level analysis

NIT-3T3 cells were seeded in 96 well-plate overnight. After, stimulated by TGF-β for 24 h, the medium was replaced with fresh one. Then, the cells were incubated with HNPs, DHPD, HPDB, DHPDB for 24 h. The amounts of CXCL12 in the supernatants were measured using a CXCL12 ELISA Kit according to the manufacturer's instructions. All samples were conducted in triplicate.

***In Vivo* Biodistribution and Pharmacokinetics studies**

The DHPD/IR783 NPs were prepared according to the loading method above. Subsequently, 4T1 tumor-bearing BABL/c mice were *i.v.* injected with free IR783 and DHPD/IR783, respectively, when the tumor size reached 200 mm^3. The fluorescent intensity and the distribution were measured by an IVIS spectrum *in vivo* imaging system (IVIS, PerkinElmer, USA) at scheduled time points. Moreover, the ex vivo biodistributions of DHPDB and free DOX in tumor and other major organs gave a similar operation step. In addition, following intravenous injections in SD mice and collecting blood samples at various predetermined times, the pharmacokinetic profiles were recorded.

***In vivo* antitumor activity assay**

The antitumor effect was performed using a 4T1 murine TNBC tumor model. To establish the animal tumor model, 1×10^6 4T1 cells in 100 μL of PBS were subcutaneously injected into the 6-week old mice at the right flank. The tumor-bearing mice were randomly divided into six groups (n = 5) when the tumor volume reached 100 mm^3. The mice were then treated with PBS, DHPD, HPDB,

DHDAB or DHPDB at an DOX dose of 5 mg/kg and CXB dose of 10 mg/kg, respectively for 5 times at an interval of 2 days. The tumor sizes and body weight were monitored every 2 days for 15 days, and the relative tumor volume was calculated as V_t/V_0 (V_t was the tumor volume on t; V_0 was the tumor volume on 0). The main organs after treatments harvested for H&E staining. The tumors from different groups of mice were harvested for H&E staining and immunofluorescence staining. The PGE_2 levels of tumor tissues were examined by ELISA.

Intratumoral infiltration of immune cells

To study the immune cells, the tumor xenografts were harvested 3 times post treatment and cut into small pieces, immersed in the solution of 1 mg/mL collagenase IV and 0.2 mg/mL DNase I for 45 min at 37°C. The single cell suspension was stained with fluorescent-labeled antibody according to manufacturer's protocols. To examine the intratumoral infiltration of CD103 DCs ($CD11c^+CD103^+$) and the matured DCs ($CD11c^+CD40^+CD86^+$), cells were stained with anti-CD11c-BV605, anti-CD103-BV510, anti-CD40-PE and anti-CD86-APC-R700 antibodies according to manufacturer's protocols. For the analysis of CTLs ($CD3^+CD4^-CD8^+$), the T lymphocytes were stained with anti-CD3-BV510, anti-CD4-PE and anti-CD8-PerCP-cy5 antibodies according to manufacturer's protocols. For macrophage analysis, cells were stained with anti-CD11b-BB515, anti-F4/80-BV421 and anti-CD206-AF647. $CD11b^+F4/80^+CD206^+$ was defined as M2 phenotype macrophages. To analyze the frequency of T regs ($CD3^+CD4^+Foxp3^+$), the lymphocytes were stained with anti-CD3-BV510, anti-CD4-PE, and

anti-Foxp3-BV421 according to the manufacturer's protocols. The cells were analyzed by flow cytometric measurement (BD FACS Canto^{TM}). For the analysis of MDSC ($CD11b^+Gr\text{-}1^+$), the cells were stained with anti-CD11b-BB515 and anti-$Gr\text{-}1^+$-PE-cy7 antibodies according to manufacturer's protocols.

Analysis of T cells and DCs in dLNs

To examine the T lymphocytes and DCs in dLNs, the dLNs were harvested and ground gently to obtain a single cell suspension. Then the single cells were stained with fluorescent-labelled antibody according to the manufacturer's protocols. For the analysis of $CD8^+$ T cells and $CD4^+$ T cells, T lymphocytes in the spleen were stained with anti-CD3-BV510, anti-CD8-PerCP-cy5, anti-CD4-PE according to the manufacturer's protocols. For DCs analysis, cells were stained with anti-CD11c-BV605, anti-CD103-BV510, anti-CD40-PE and anti-CD86-APC-R700 antibodies according to manufacturer's protocols.

Cytokine secretion in the sera

To examine the levels of IFN-γ, TNF- α, IL-10, IL-12 and IL-6 cytokines in blood, blood from mice treated with different therapeutics was collected and centrifuge to obtain sera. Cytokines in the sera were measured using an ELISA kit according to the manufacturer's instructions.

Investigation of overall survival and lung metastasis

For 4T1 breast metastasis tumor model, 4T1 cells (1×10^6 in 100 μL) were subcutaneously injected into the second right breast of female BALB/c mice (n = 5). When the tumor reached to 100 mm^3, mice were randomly divided into 6 groups. The

mice were treated with PBS, HNPs, DHPD, HPDB, DHDAB and DHPDB, respectively. At the 24th d after treatments, the mice were sacrificed. Three lungs of each group were dissected, and metastatic nodules on the surface of lungs were isolated, counted and imaged. To assess the overall survival, tumor-bearing mice were treated as described above (n = 5), and monitored.

Statistical Analysis

Results are given as Mean ± S.D. One way analysis of variance (ANOVA) was used to determine the significance of the difference. Statistical significance was set at ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.01$).

Reference

(1) Gao, N.; Yang, W.; Nie, H.; Gong, Y.; Jing, J.; Gao, L.; Zhang, X., Turn-on theranostic fluorescent nanoprobe by electrostatic self-assembly of carbon dots with doxorubicin for targeted cancer cell imaging, in vivo hyaluronidase analysis, and targeted drug delivery. *Biosens Bioelectron* **2017,** *96*, 300-307.

(2) Xiao, H.; Song, H.; Yang, Q.; Cai, H.; Qi, R.; Yan, L.; Liu, S.; Zheng, Y.; Huang, Y.; Liu, T.; Jing, X., A prodrug strategy to deliver cisplatin(IV) and paclitaxel in nanomicelles to improve efficacy and tolerance. *Biomaterials* **2012,** *33* (27), 6507-6519.

(3) Mayer, C. T.; Ghorbani, P.; Nandan, A.; Dudek, M.; Arnold-Schrauf, C.; Hesse, C.; Berod, L.; Stuve, P.; Puttur, F.; Merad, M.; Sparwasser, T., Selective and efficient generation of functional Batf3-dependent $CD103^{+}$ dendritic cells from mouse bone marrow. *Blood* **2014,** *124* (20), 3081-3091.

(4) Ye, H.; Wang, K.; Wang, M.; Liu, R.; Song, H.; Li, N.; Lu, Q.; Zhang, W.; Du, Y.; Yang, W.; Zhong, L.; Wang, Y.; Yu, B.; Wang, H.; Kan, Q.; Zhang, H.; Wang, Y.; He, Z.; Sun, J., Bioinspired nanoplatelets for chemo-photothermal therapy of breast cancer metastasis inhibition. *Biomaterials* **2019,** *206*, 1-12.

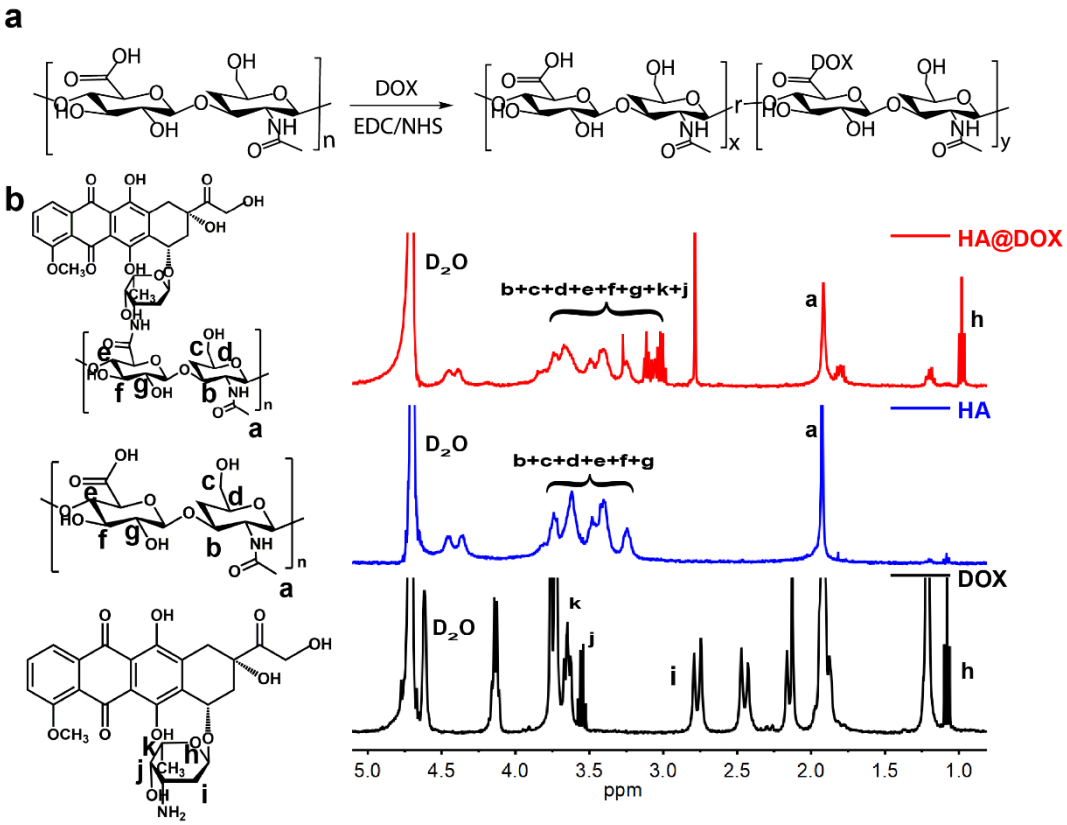

Figure S1. a) The synthetic route of HA@DOX. b) ^{1}H NMR spectrum of DOX, HA and HA@DOX.

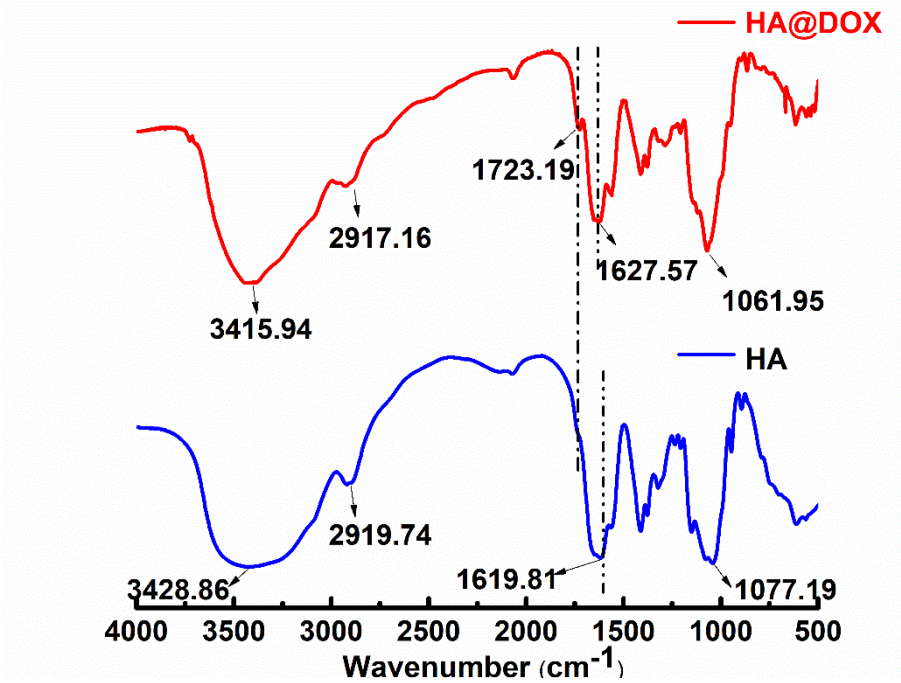

Figure S2. FTIR spectra of HA and HA@DOX. The absorption peak at 1723.19 cm^{-1} was assigned to the characteristic stretching vibration of the carbonyl group in DOX, indicating that DOX was successfully introduced to HA.

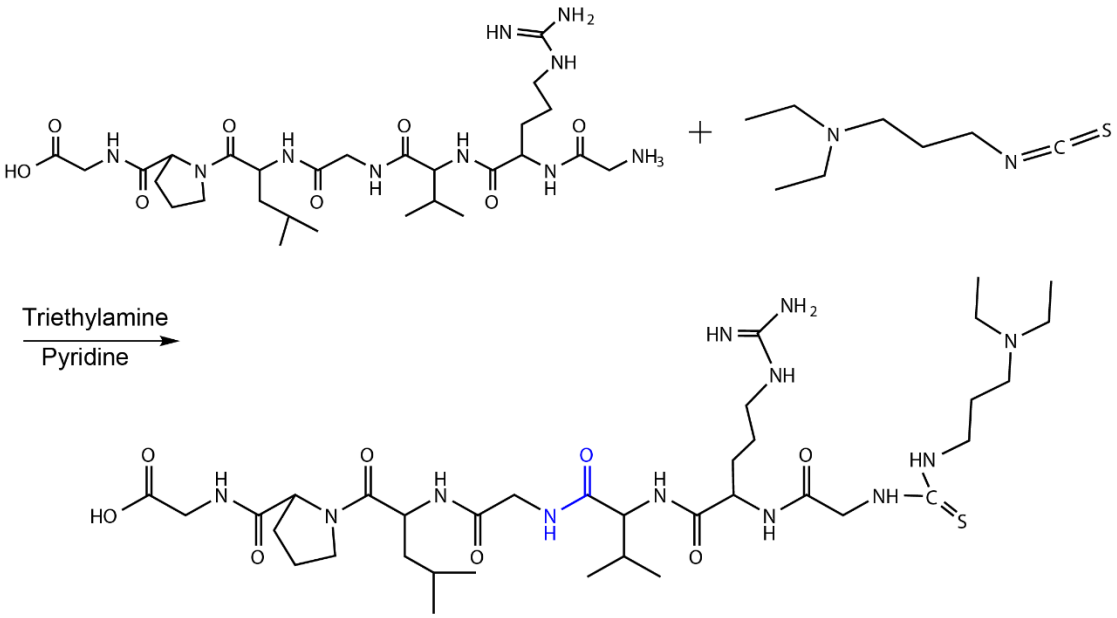

Figure S3. The synthetic of MMP-2-DEAP.

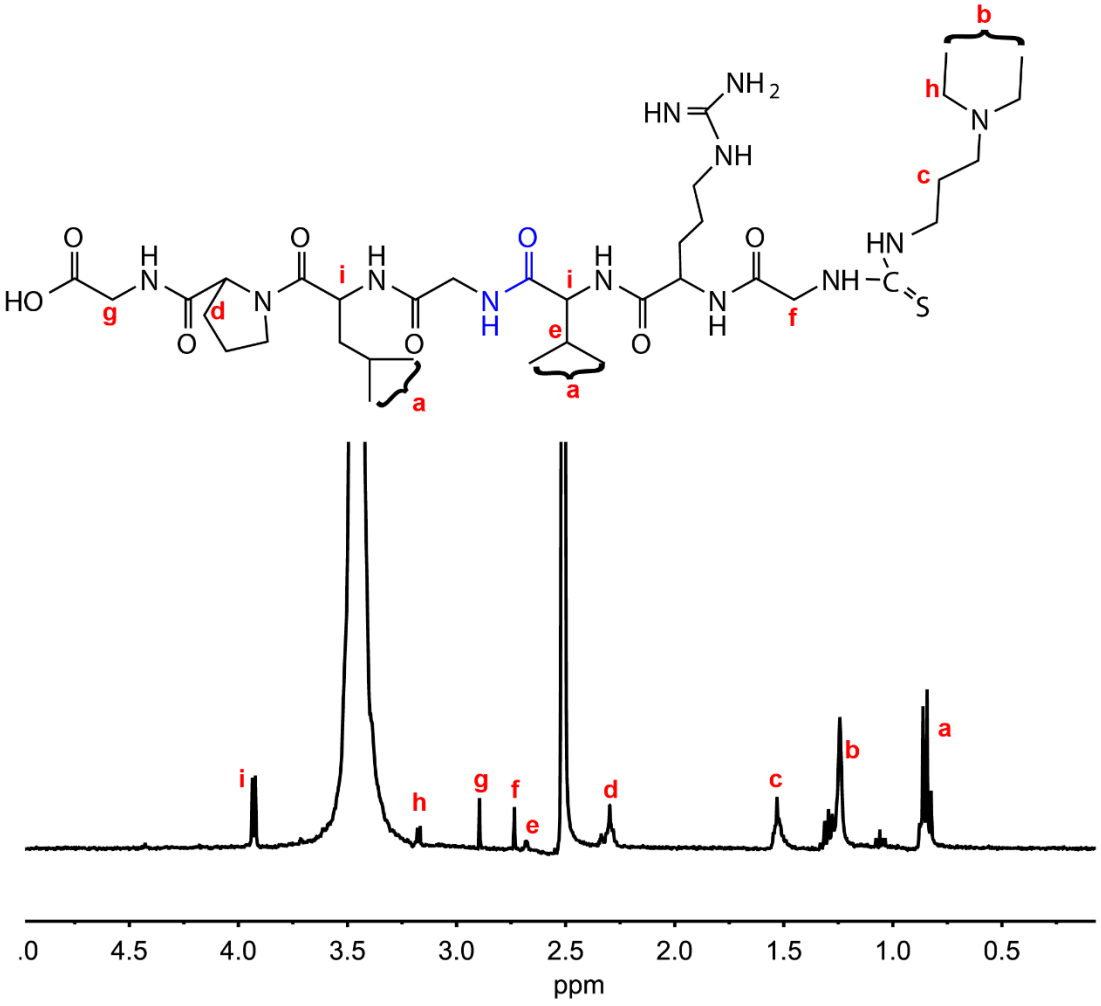

Figure S4. The ^{1}H-NMR spectrum of MMP-2-DEAP.

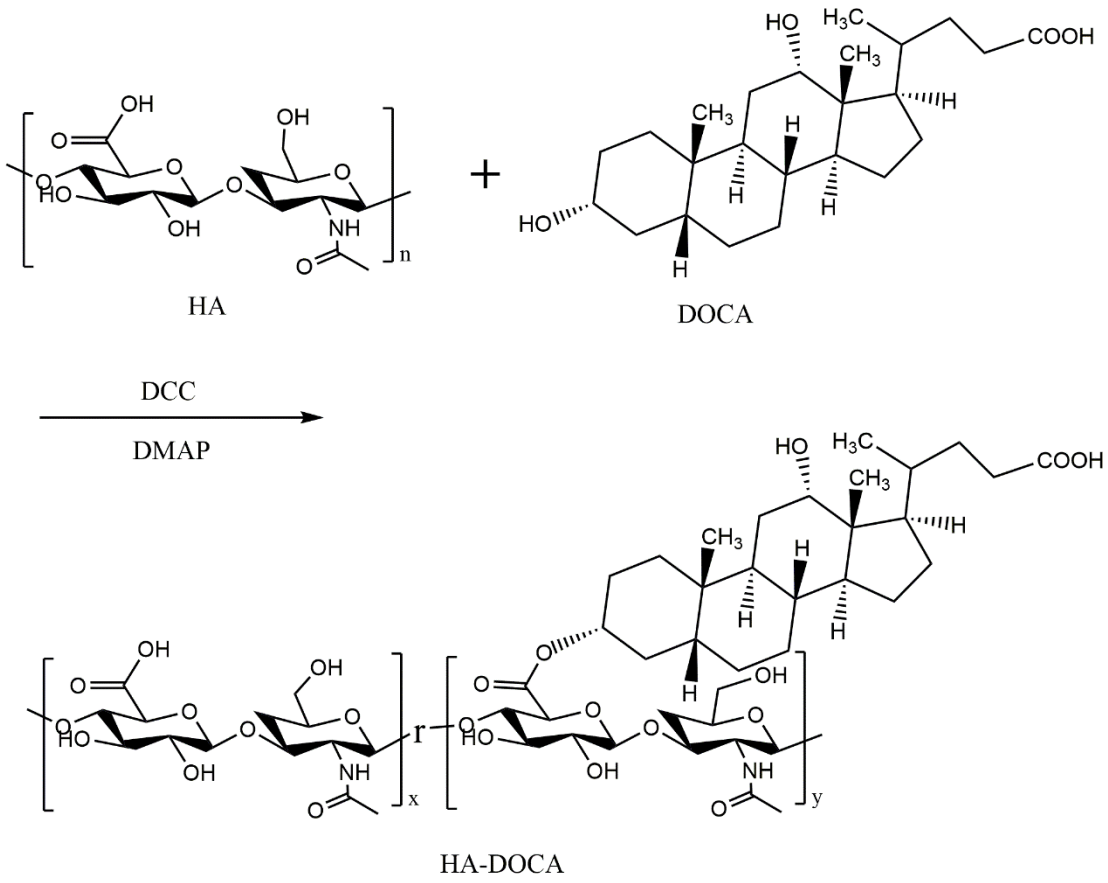

Figure S5. The synthetic of HA-DOCA.

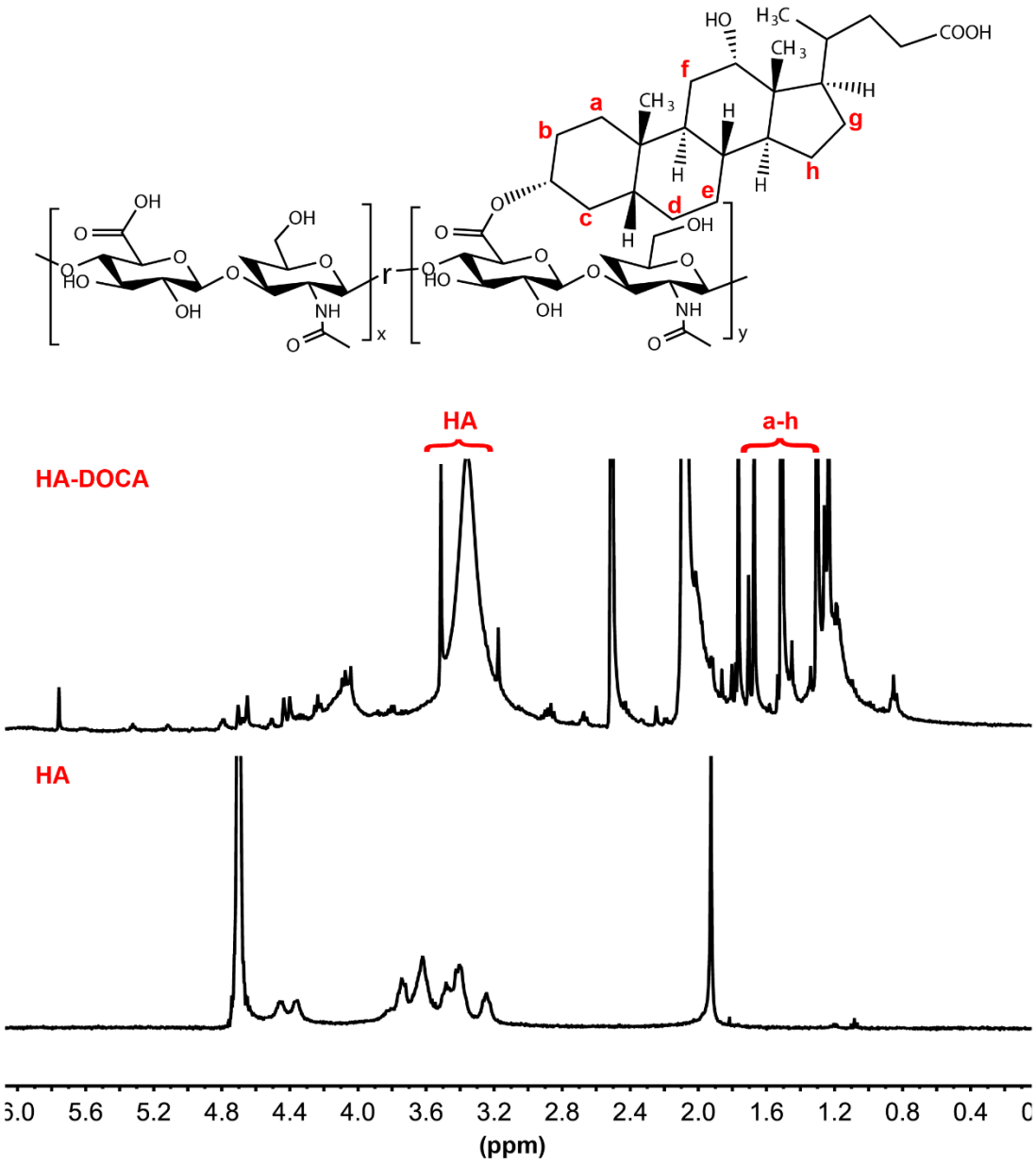

Figure S6. ^{1}H NMR spectrum of HA-DOCA and HA.

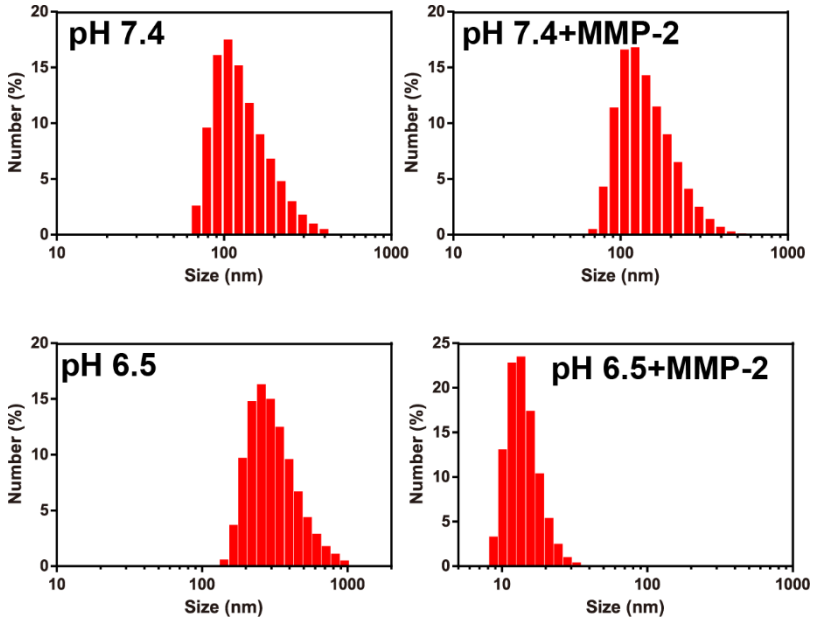

Figure S7. The hydrodynamic size of DHPD nanoparticles at different conditions

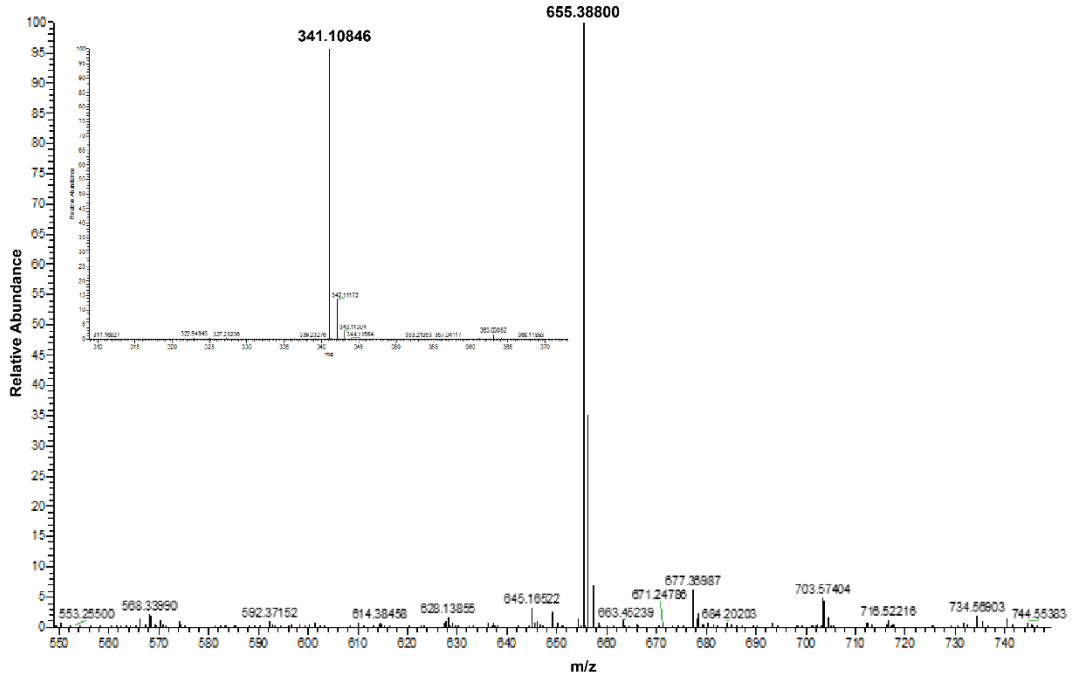

Figure S8. ESI-MS spectrum of GPLGVRG after incubation in a buffer solution of MMP-2.

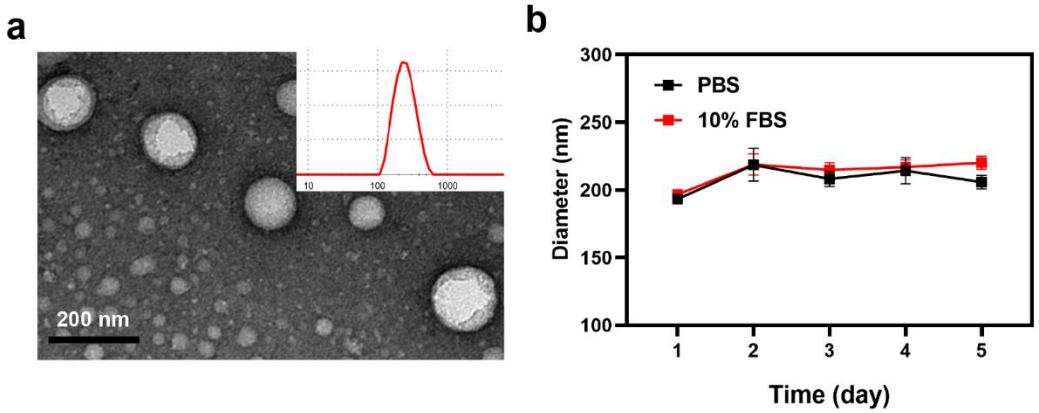

Figure S9. a) TEM images and the hydrodynamic size of DHPDB nanoparticles; b) The stability of nanoparticles in PBS or 10%FBS.

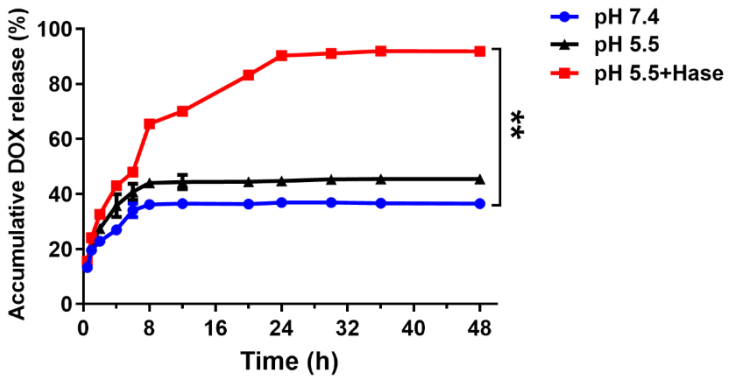

Figure S10. DOX release profiles of HA@DOX at different conditions (*$P < 0.05$; ** $P < 0.01$).

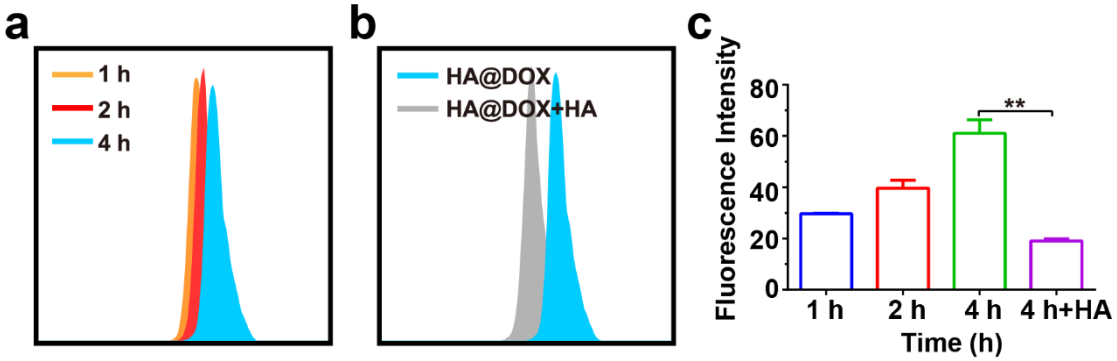

Figure S11. a) The cellular uptake of HA@DOX in 4T1 cells was measured by flow cytometry. b) Cellular uptake in 4T1 cells with or without preincubation with 10 mg/mL HA by flow cytometry. c) The fluorescence intensity of the cellular uptake of HA@DOX in CLSM observation (*$P < 0.05$; ** $P < 0.01$).

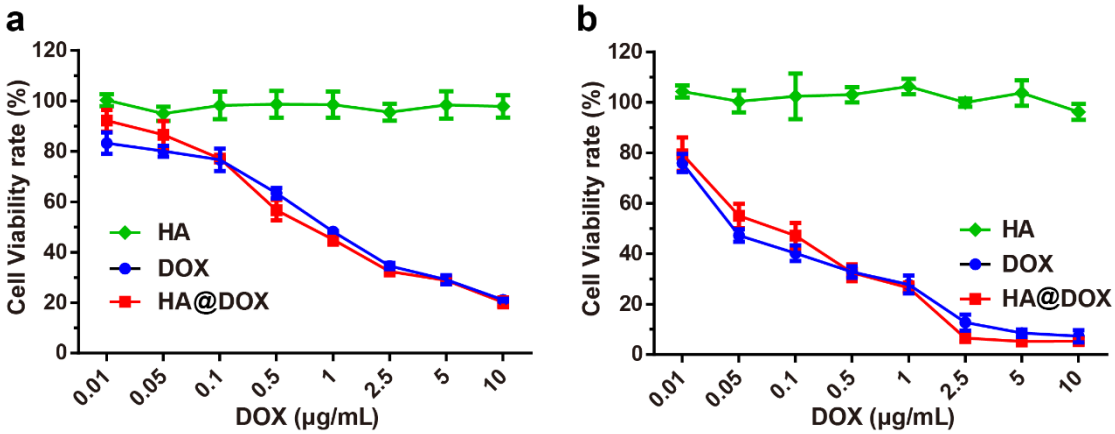

Figure S12. a) and b) The cell viability of free DOX and HA@DOX at 24 h and 48 h respectively.

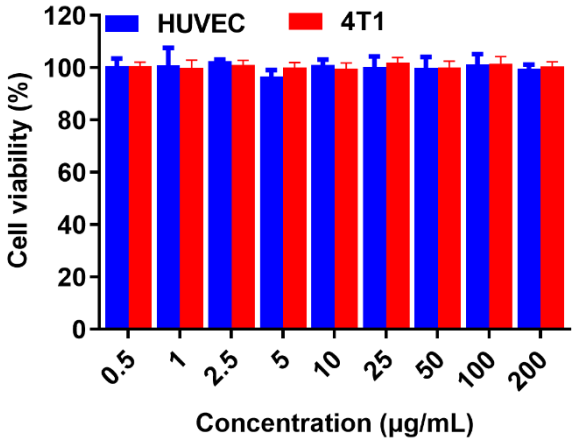

Figure S13. The cytotoxicity of blank nanoparticles in HUVEC and 4T1 cells

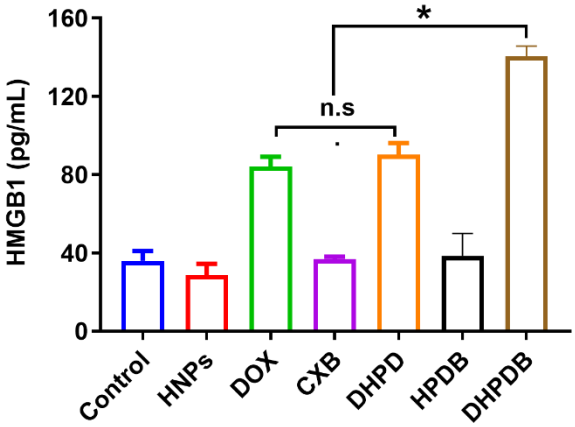

Figure S14. The released HMGB1 of 4T1 tumor cells (*$P < 0.05$; ** $P < 0.01$).

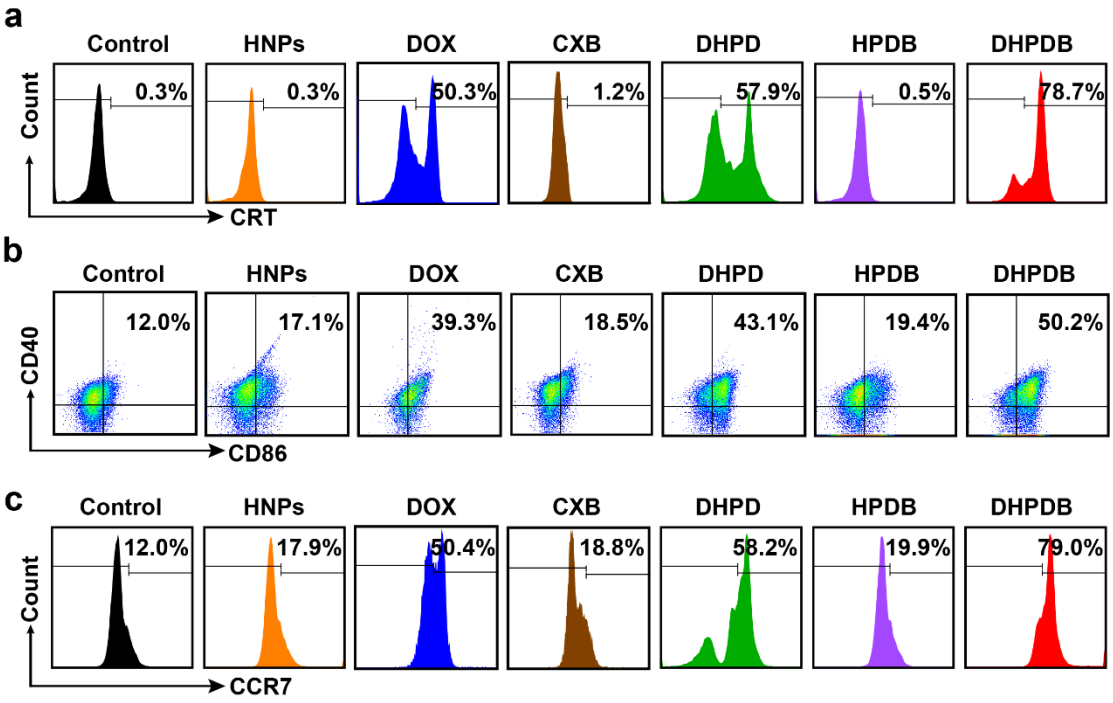

Figure S15. a) FCM examination of CRT exposure on the surface of 4T1 cells. b) and c) FCM measurement of DC maturation and CCR7 expression.

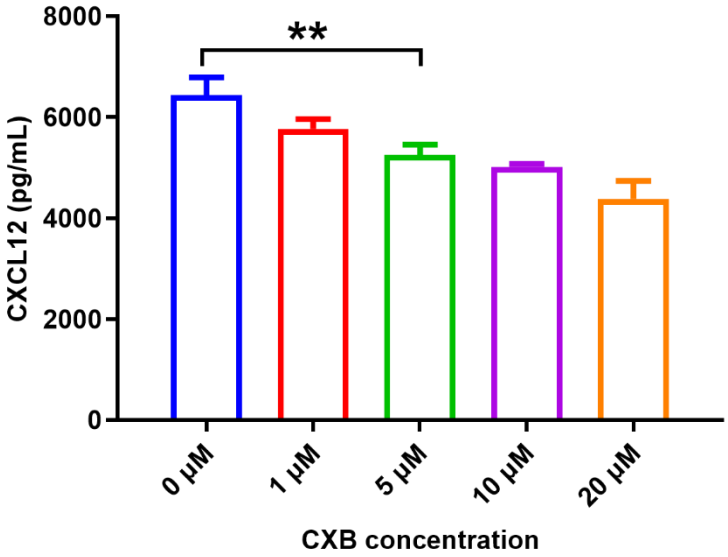

Figure S16. CXCL12 released from TGF-β-stimulated fibroblasts after being treated with CXB for 24 h (*$P < 0.05$; ** $P < 0.01$).

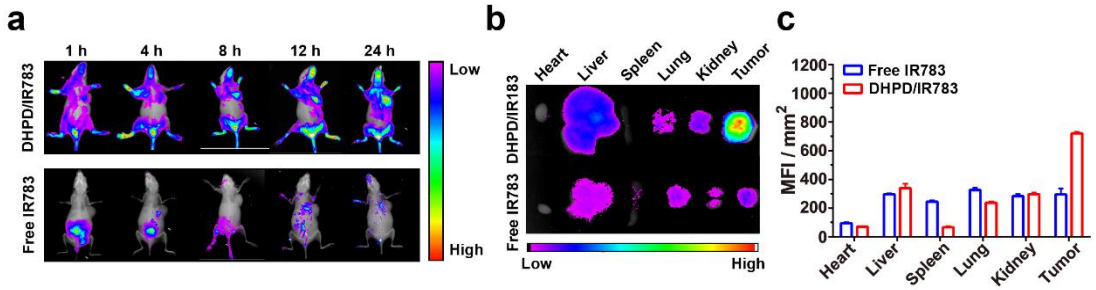

Figure S17. Tumor targeting *in vivo*. a) *In vivo* fluorescence imaging of the 4T1-bearing BABL/C mice after intravenous injection with free IR783 and DHPD/IR783 NPs at different time points. b) Ex vivo fluorescence images of important tissues collected from the mice 24 h after administration and c) fluorescence intensity of the IR783 signal.

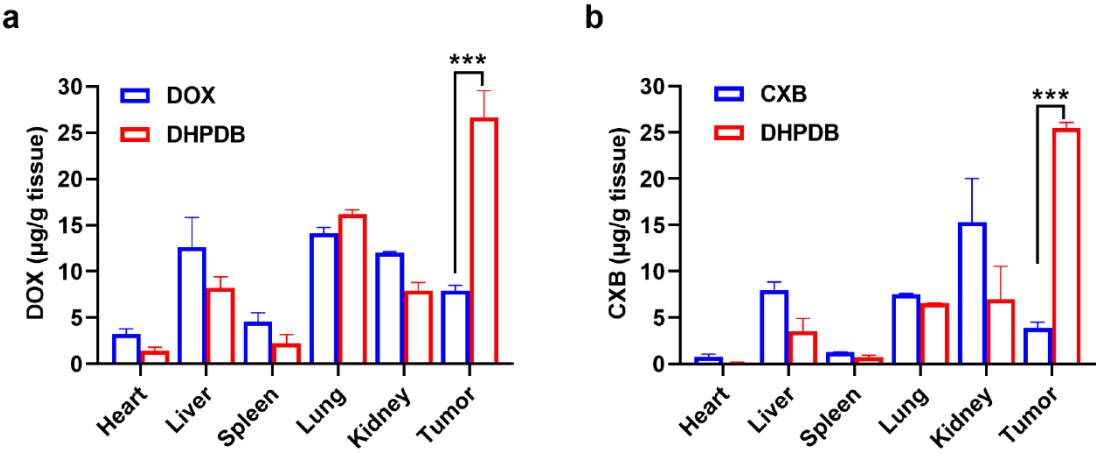

Figure S18. Biodistribution of DOX measured at 24 h post intravenous injection of free DOX and DHPDB by measuring the concentrations of DOX with HPLC (*$P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

.

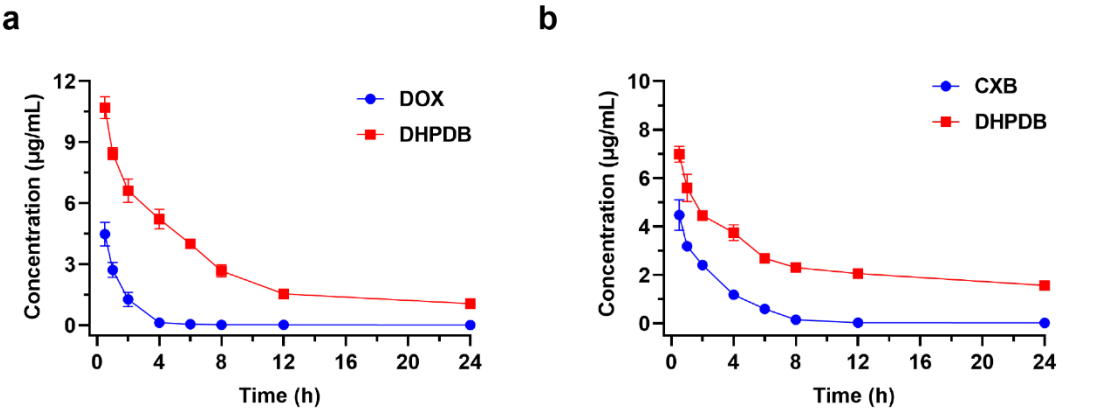

Figure S19 Representative plasma concentration-time profiles of free DOX, free CXB and DHPDB after *i.v.* injection into rats.

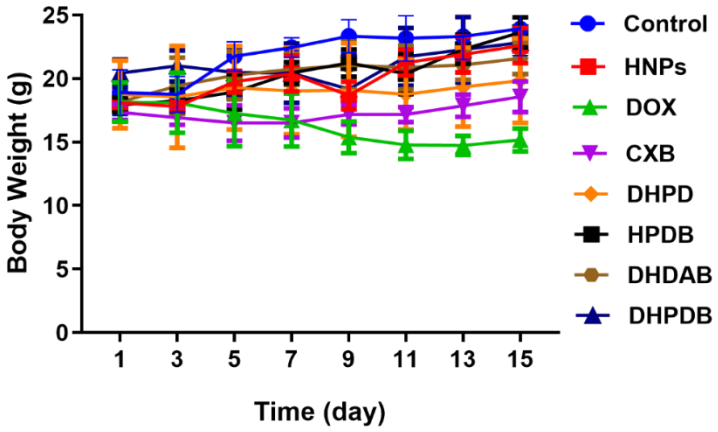

Figure S20. Body weight change of the 4T1-bearing BABL/c mice monitored during the anti-tumor therapy period.

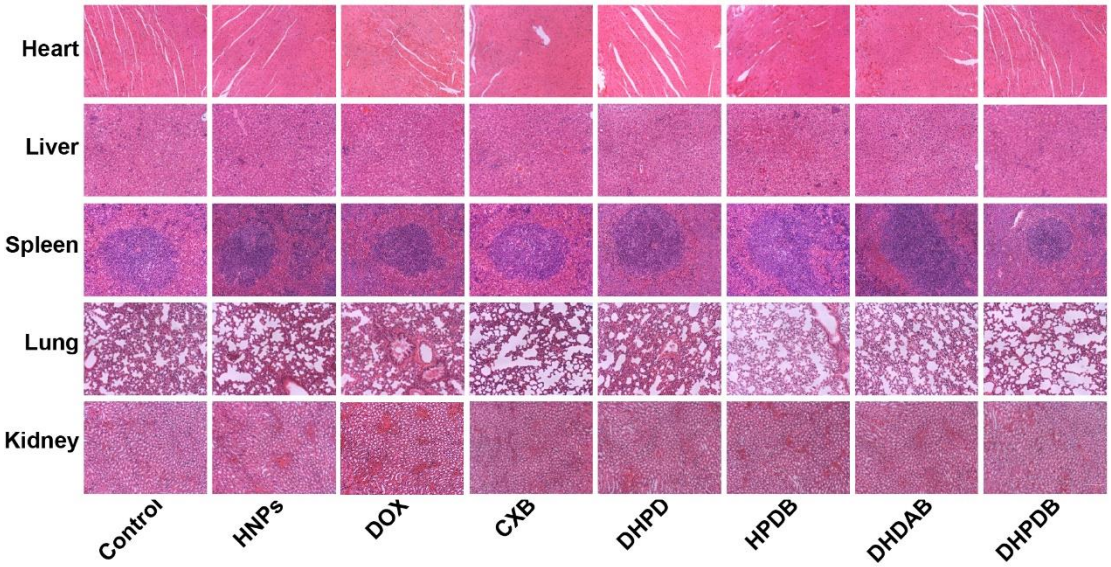

Figure S21. H&E examination of the heart, liver, spleen, lung, kidney, harvested at the end of antitumor assay (Scale bars = 100 μm).

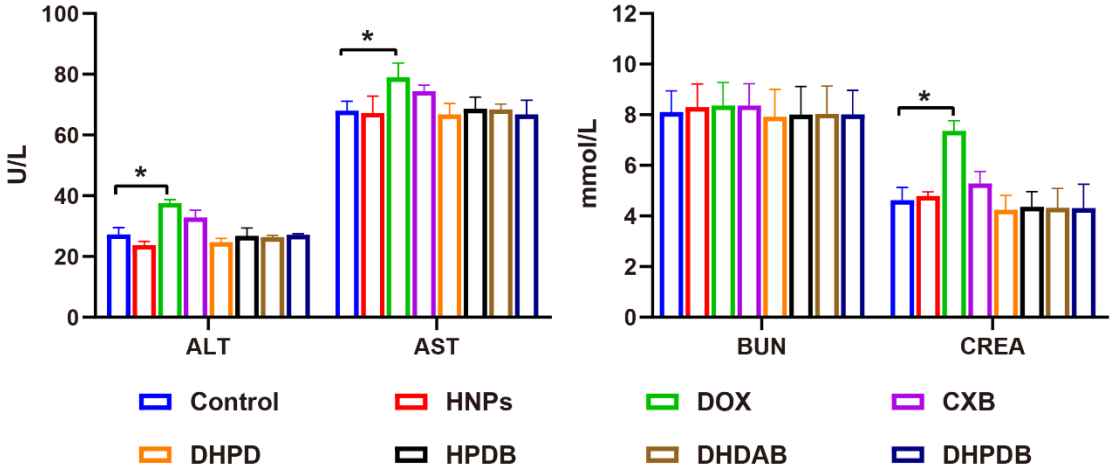

Figure S22. The biochemical analysis of the sera from the mice receiving different treatments (*P < 0.05).

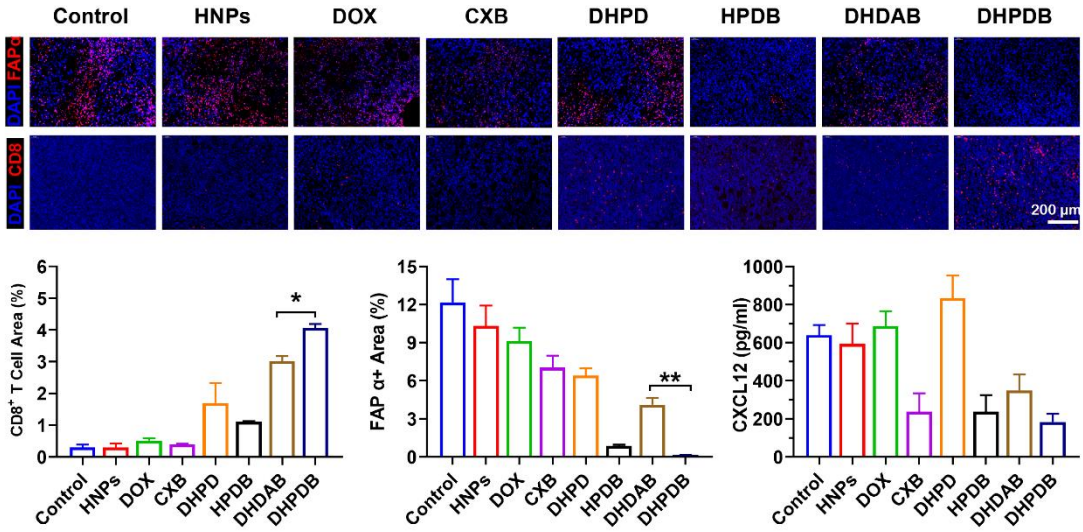

Figure S23. The expression of $CD8^+$ T cells, CAF (FAP α^+) and CXCL12 in tumors (*P < 0.05; ** P < 0.01).

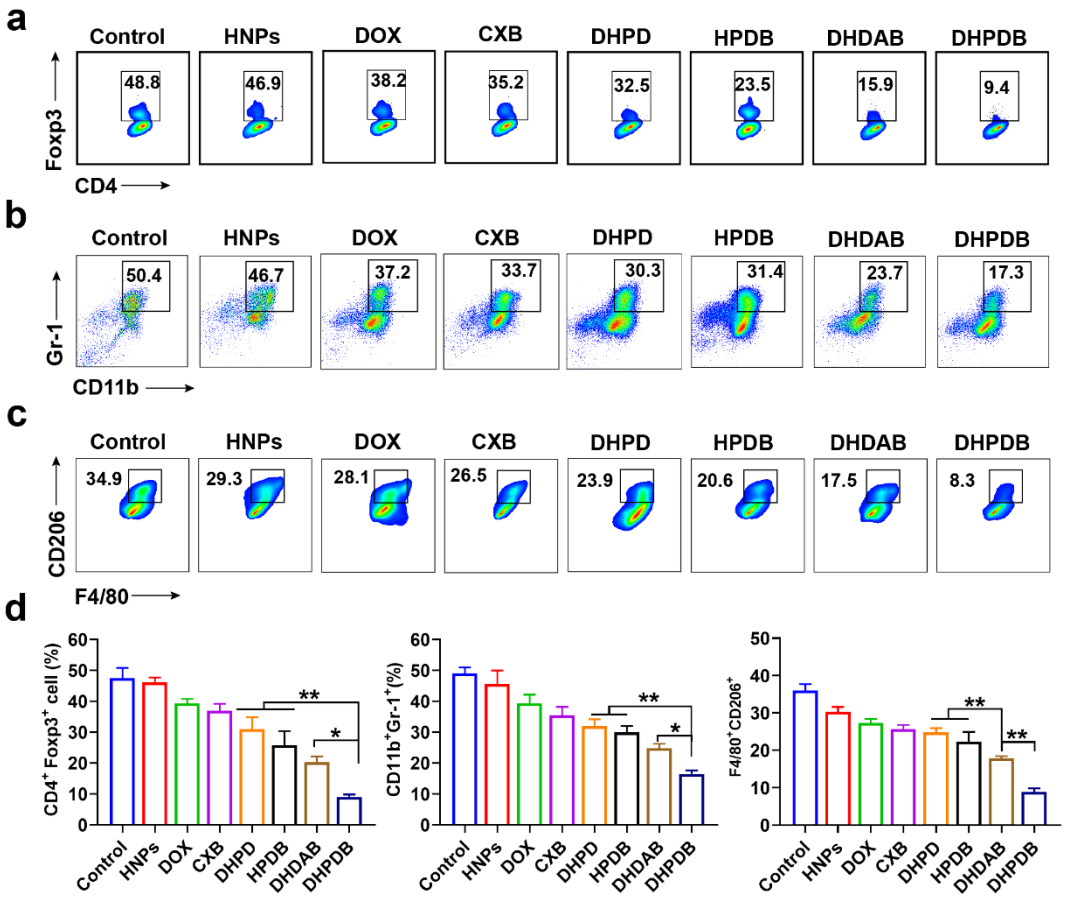

Figure S24. Representative FCM profiles of a) Tregs ($CD4^+Foxp3^+$cells), b) MDSCs ($CD11b^+Gr\text{-}1^+$cells), c) M2-TAMs ($CD11b^+F4/80^+CD206^+$cells) in 4T1 tumors. d) Quantitative analysis of Tregs, MDSCs, M2-TAMs in 4T1 tumor site (*P < 0.05; ** P < 0.01).

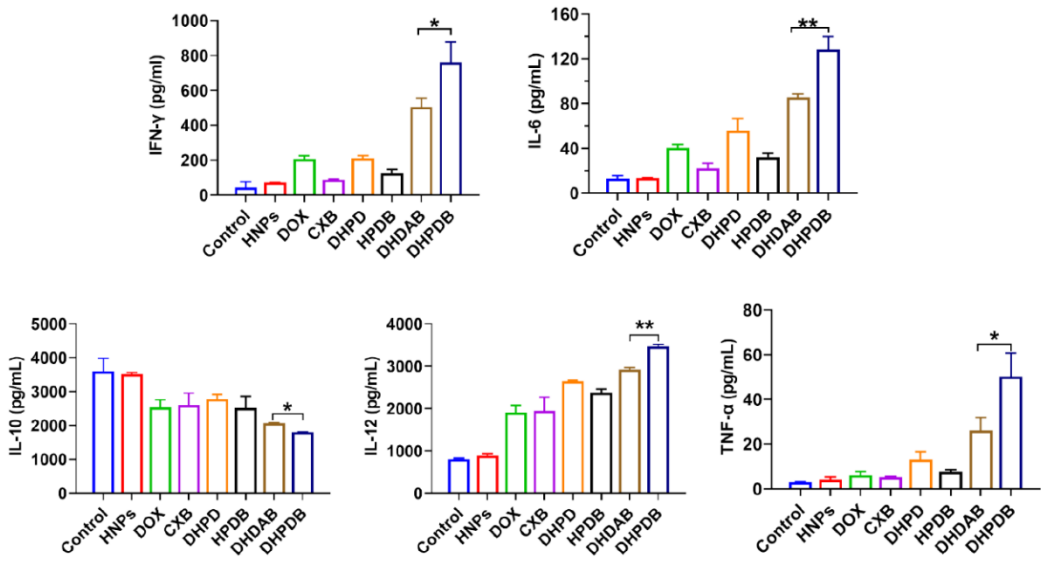

Figure S25. The cytokines IFN-γ, IL-6, IL-10, IL-12 and TNF-α in serum collected from mice after different treatments (*$P < 0.05$; ** $P < 0.01$).

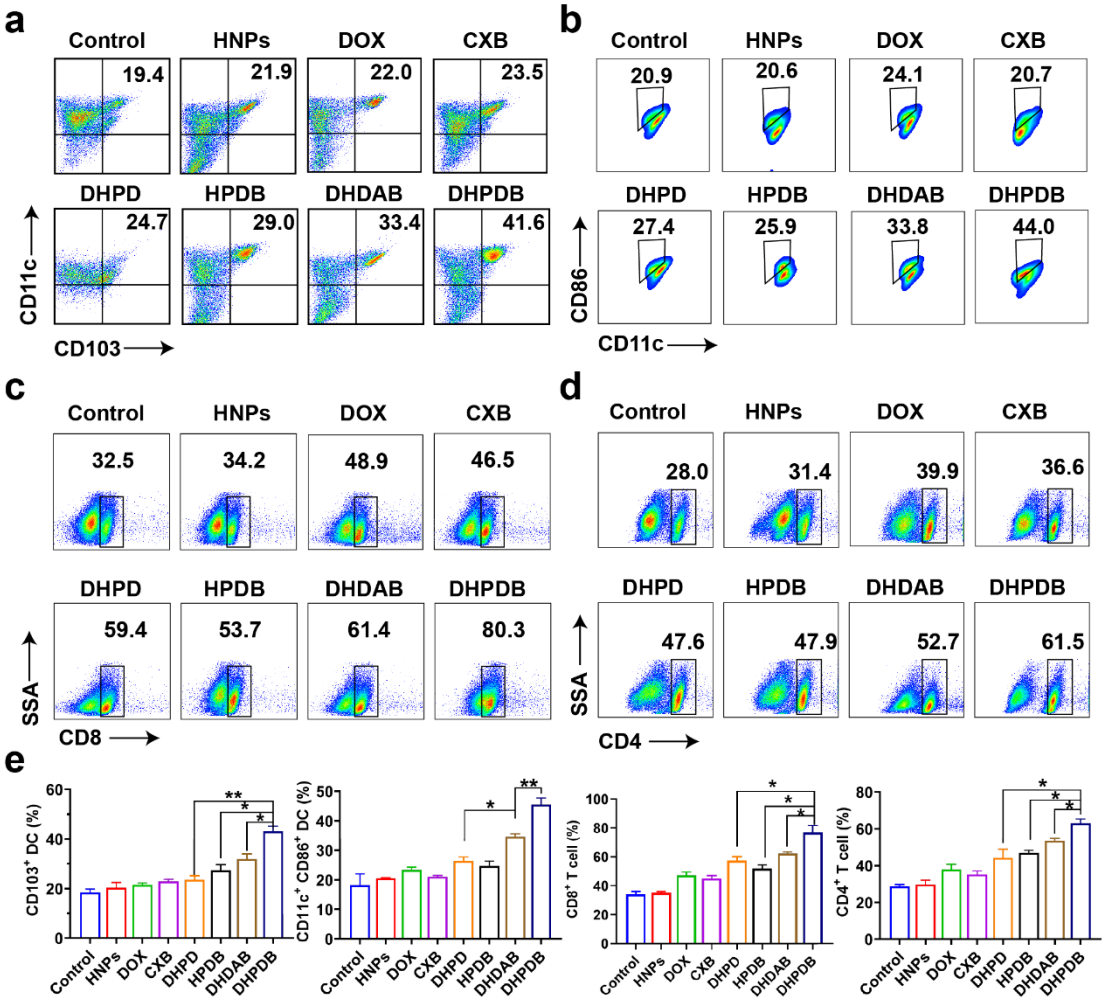

Figure S26. Percentages of a) $CD103^+$DCs, b) $CD86^+$ matured DCs, c) $CD8^+$ T and d) $CD4^+$ T cell infiltrate in dLNs. e) Quantification of DCs, matured DCs, $CD8^+$ T and $CD4^+$ T cell in

dLNs (*$P < 0.05$; ** $P < 0.01$).

Table S1. The loading efficiency of CXB and DOX in the nanoparticles

Drugs	Loading percentage [%]	Encapsulation efficiency [%]
DOX	8.3 ± 0.9%	80.9 ± 1.5%
CXB	11.4 ± 1.4%	62.9 ± 2.6%

Table S2. Pharmacokinetic parameters

Parameter	DOX	DHPDB (DOX)	CXB	DHPDB (CXB)
t1/2	0.78 ± 0.28	4.84 ± 0.76	1.79 ± 0.26	5.90 ± 0.82
AUC μg/ml*h	7.86 ± 0.72	63.93 ± 0.26	13.28 ± 0.16	51.32 ± 1.37