Electronic supplementary information **for:**

Microfluidic self-assembly of high cabazitaxel loading albumin nanoparticles

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EXPERIMENTAL SECTIONS

Materials and Methods

Materials. Human serum albumin (HSA) was purchased from Octa pharma (Vienna, Austria). CTX was obtained from Yew Biological Technology Co, Ltd (Wuxi, China). The inverted W-type microfluidic chip was designed independently and customized at Dalian Institute of Chemical Physics, Chinese Academy of Sciences (Dalian China). Fluorescein isothiocyanate (FITC), 4',6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich (St Louis, MO, USA). DiR iodide was obtained from Thermo Fisher Scientific (Waltham, MA, USA). All other reagents were of analytical grade. All the cells were obtained form American Type Culture Collection (ATCC), and cultured according to ATCC requirements. The animal procedures were performed in accordance with the Guidelines on Humane Treatment of Laboratory Animals and procedures for the care and use of laboratory animals. All animal experiments were approved by the institution animal ethics committee (Jilin University) (number 201709006).

Preparation of NPs-CTX. HSA-NPs were prepared by the MF method (MF-NPs-CTX) and BU method (BU-NPs-CTX) separately. CTX was dissolved in ethyl alcohol, and mixed with 12.5% NaCl and 200 mg/mL HSA at a volume ratio of 2:1:1 as a mixed solution. BU-NPs-CTX were prepared as described previously[1, 2]. Briefly, the mixed solution was directly injected into 65 °C deionized water under magnetic stirring for 30 seconds. After that, the solution was quickly cooled to 2-8 °C, then BU-NPs-CTX were concentrated by hollow fiber ultrafiltration. Ethanol and unencapsulated CTX were removed at the same time. For the MF method, a microfluidic system with two syringe pumps and a PDMS-glass microfluidic chip with an inverted W micromixer was used (Fig. 1), the width of the chip channel is 200μm, the height is 75μm, the groove depth of the inverted W-type passive mixer at the bottom is 35μm, and the distance between the two grooves is 50μm. Fig. 1A was shown that the microfluidic chip has three inlets and one outlet. Briefly, the mixed solution (Inner fluid) was injected from inlet 2 by a 1.0 mL glass syringe and deionized water (Outer fluid) was injected from inlet 1 and 3 by two 10.0 mL glass syringes, the flow rates of the mixed solution and deionized water were controlled by two syringe pumps respectively. The

resulting NPs (MF-NPs-CTX) were collected at the outlet and were cooled in an ice bath. The effect of shear stress on MF-NPs-CTX was investigated by varying the flow rate (flow rate of Inner fluid varied from 15μL/min to 60 μL/min) and flow ratio of deionized water and mixed solutions (varied from 10:1 to 40:1). Meanwhile, the concentration of CTX (5, 10, 15, 20 mg/mL) and the temperature of the Outer fluid (40, 50, 60, 70, 80 ℃) were also discussed in the optimization process. The synthesis of MF-NPs-CTX was repeated at least three times under every condition. After the preparation condition was optimized, MF-NPs-CTX was concentrated as the same method with BU-NPs-CTX. Then both of the NPs were freeze-dried.

Particle size and zeta potential of NPs. Both NPs (MF-NPs-CTX and BU-NPs-CTX) were re-dissolved with deionized water and were applied to the determination of particle size and zeta potential by Zetasizer Nano particle size analyzer (Nano-ZS ZEN3600, Malvern, UK). The morphologies of the two NPs were observed by scanning electron microscopy (SEM, JSM-6700F, JEOL, Tokyo, Japan). Namely, one drop of deionized water-dissolved NPs was added to the surface of a silicon wafer which was reprocessed with absolute ethanol. After NPs were air-dried, the silicon wafer was sputter-coated with platinum and detected with SEM.

Plasma stability of NPs. Plasma stability of NPs was evaluated according to the previous study [3]. Briefly, freeze-dried MF-NPs-CTX and BU-NPs-CTX were re-dissolved in 10% fetal bovine serum (FBS), then they were incubated at 37 ℃. The particle size of the NPs was measured at a set point.

DEE and DLC of NPs. DEE and DLC of MF-NPs-CTX and BU-NPs-CTX were measured by high-performance liquid chromatography (HPLC). For calculating DEE of the NPs, the concentrations of drug loaded into NPs and unencapsulated were detected respectively. For DLC, freeze-dried NPs were re-dissolved in acetonitrile and sonicated to release CTX, then the centrifugated solution was injected into HPLC. DEE and DLC were calculated using equations below [4, 5]:

$$DEE = \frac{weight\ of\ CTX\ in\ NPs\text{-}CTX}{weight\ of\ CTX\ in\ NPs\text{-}CTX + \ weight\ of\ CTX\ in\ the\ filtrate} \times 100\%$$

$$DLC = \frac{weight\ of\ CTX\ in\ NPs}{weight\ of\ NPs} \times 100\%$$

***In vitro* drug release evaluation.** Tween-CTX (CTX was formulated in Tween 80 and ethanol mixed solution according to JEVTANA®, 13% ethanol: Tween 80 = 4:1), MF-NPs-CTX, and BU-NPs-CTX were used to evaluate drug release of NPs. Two mL of Tween-CTX, MF-NPs-CTX, and BU-NPs-CTX with same CTX content were placed into dialysis bag with molecular weight cutoff of 10 kDa separately, then the dialysis bag was placed in 80 mL PBS containing 0.1% Tween 80 [6, 7]. Aliquots of samples were taken out and the same volume of release mediums was added at the specific time point. The drug's concentrations were determined by HPLC.

Cell culture and cytotoxicity evaluation of NPs. To evaluate the cytotoxicity of free-CTX, MF-NPs-CTX and BU-NPs-CTX, PC-3, HeLa, MCF-7 and A549 cells were adopted. Namely, 5000 cells per well were incubated in 96-well plate for 24 hours, then free-CTX, MF-NPs-CTX, and BU-NPs-CTX were added into the cell culture medium with varying CTX concentrations (5, 30, 60, 90, 120 and 150 μg/mL). Cell viability of the cells was performed at 48 hours after dosing.

Cellular uptake evaluation *in vitro*. Qualitative and quantitative evaluation of cellular uptake of the FITC labeled NPs (MF-FITC-NPs-CTX) were carried out by confocal microscopy and flow cytometry. HSA was first labeled with FITC as described before [8]. Namely, 0.1 mg/mL FITC was dissolved in Na_2CO_3/$NaHCO_3$ (pH=9.8) and incubated with HSA (5 mg/mL) overnight. Then FITC labeled HSA was placed into dialysis bag with molecule weight cut-off 10 kDa to remove unattached FITC. MF-FITC-NPs-CTX were prepared as the same procedure as MF-NPs-CTX separately.

Flow cytometry was used to quantify cellular uptake of FITC labeled NPs. Briefly, PC-3 and HeLa cells were seeded in 12-well plate with a density of 10^5 per well. After 24 hours of cell culture, MF-FITC-NPs-CTX were added. After incubating for 1, 2, 4 and 6 hours, then cells were collected and fixed by 4% formaldehyde. Then the fluorescence intensity was measured by flow cytometer.

To qualitatively measure the uptake of the MF-FITC-NPs-CTX, laser scanning confocal microscope (LSCM) was used. Briefly, 10^5 PC-3 and HeLa cells were seeded per dish 24 hours in advance, MF-FITC-NPs-CTX were added into the cell culture medium. Then cell culture medium was removed after MF-FITC-NPs-CTX incubating for 1,2 and 4 hours. After washing three times with PBS, the

cells were fixed with 4% paraformaldehyde for 15 minutes, then PBS was added to wash away excess paraformaldehyde. Then the cells were stained with DAPI for 10 minutes, after washing the excess DAPI with PBS, observation of cellular uptake was performed using laser confocal microscopy.

Hemolysis assay of NPs. The safety profile of the two NPs was evaluated by the hemolysis assay [9]. Briefly, different concentrations of Tween-CTX, MF-NPs-CTX, and BU-NPs-CTX were incubated with 2% red blood cells in 1.5 mL EP tube for 2 h, and red blood cells treated by normal saline and 1% Triton X-100 served as negative and positive controls, respectively. Then the tubes were centrifuged at 43× g for 5 minutes to observe hemolysis, then, 100 μL supernatant was aspirated to measure the absorbance at 560 nm using microplate reader. The hemolysis rate was calculated by the following equation.

$$Hemolysis\ Rate = \frac{Absorbance - Negative\ Group}{Positive\ Group - Negative\ Group} \times 100\%$$

In vivo **antitumor efficiency evaluation.** The PC-3 xenograft model was established on 6-8 weeks old nude mice. Briefly, 5~10×10^6 PC-3 cells were injected subcutaneously into nude mice [10]. When the average volume of tumors in nude mice reached 150 mm^3, PC-3 xenografted mice were randomly divided into 4 groups (n=5). Then, 2.0 mg/kg CTX of different preparations (NS, Tween-CTX, MF-NPs-CTX, and BU-NPs-CTX) were administered by tail vein injection every 4 days. Body weight and tumor volume measurements were taken every two days during dosing. When the tumor volume reached 1500 mm^3 or the body weight decreased by more than 1/3, the nude mice would be injected with an excess of sodium pentobarbital and then euthanized. After four administrations, the nude mice were euthanized by injection of an excess of sodium pentobarbital. after that, the heart, liver, spleen, lungs, and kidneys were taken out and stored in 4% paraformaldehyde for further *in vivo* toxicity analysis by HE staining.

The biodistribution of NPs was investigated after tumor volume reached about 600 mm^3, then two NPs (BU-NPs-DiR, MF-NPs-DiR) were injected into PC-3 xenografted mice (n=3) through the tail vein. The biodistribution images of NPs were obtained at 1, 2, 4 and 8 hours after injection using an

in vivo imaging system (λ_{ex}: 745 nm, λ_{emit}: 820 nm, Caliper Life Sciences, Hopkinton, MA, USA) [11]. After imaging, nude mice were sacrificed immediately and the heart, liver, spleen and lungs and tumors were taken out for determining fluorescence intensity.

To further verify the outcome of different CTX formulations on PC-3 tumor suppression, HeLa-bearing tumor model was established in the same way as PC-3 xenograft model and treated in the same procedure. After three administrations, HeLa-bearing nude mice were euthanized by injection of an excess of sodium pentobarbital and tumors were harvested for tumor weight measurement.

Supporting Figures:

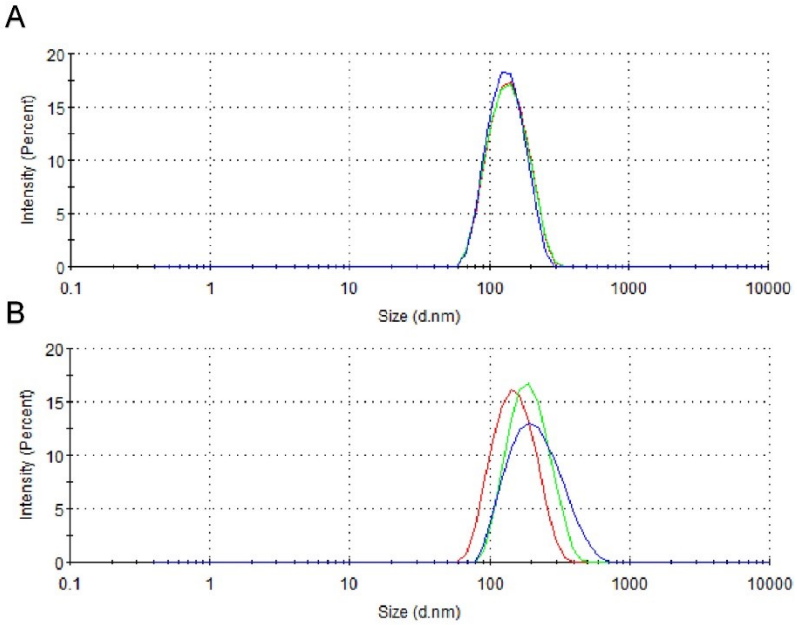

Fig. S1 Size distribution of (A) MF-NPs-CTX and (B) BU-NPs-CTX prepared under three parallel conditions using particle analyzer.

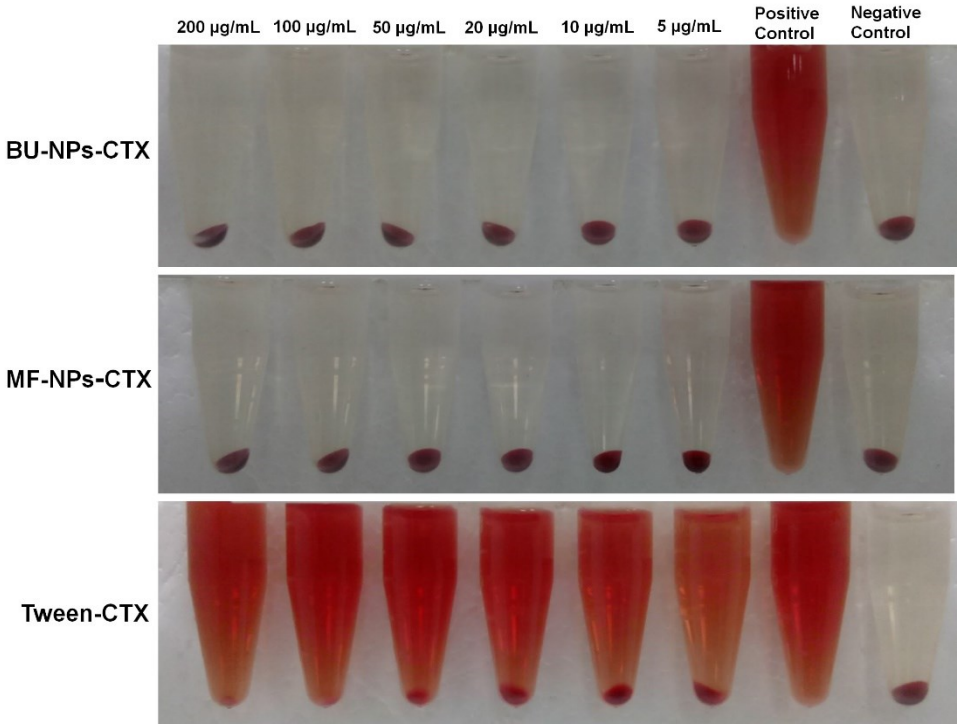

Fig. S2 Hemolysis evaluation of different CTX formulations. Obtained red blood cells were treated with different concentrations of BU-NPs-CTX, MF-NPs-CTX and Tween-CTX, normal saline and 1% Triton X-100 were used as negative and positive control.

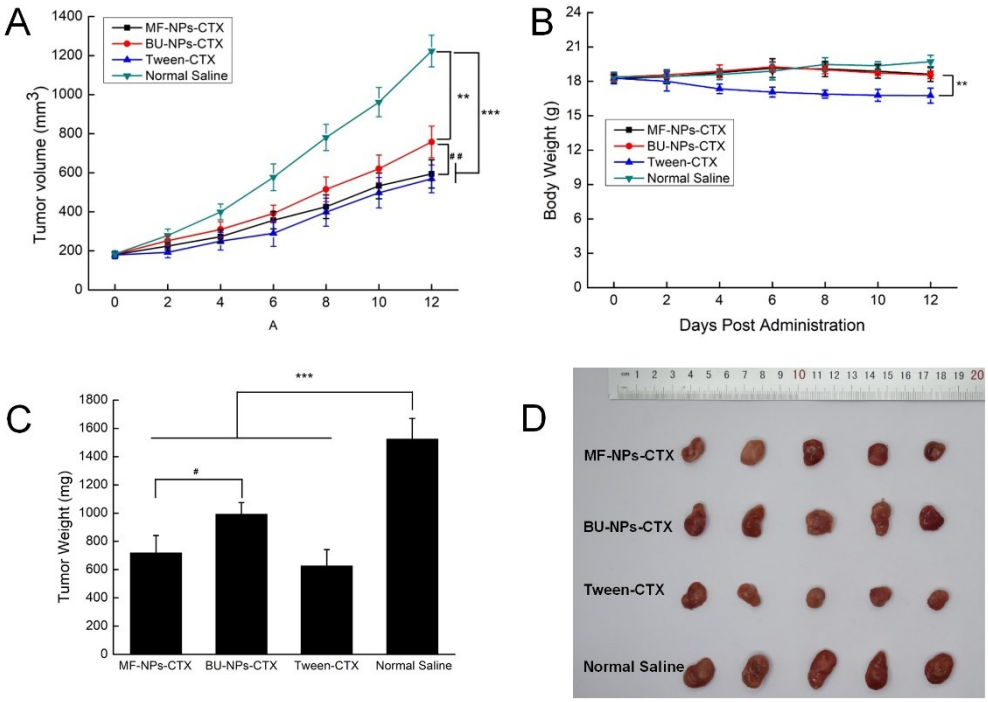

Fig. S3 *In vivo* antitumor evaluation of HeLa-bearing nude mice of MF-NPs-CTX, BU-NPs-CTX and Tween-CTX. (A) Tumor volumes vary with time after intravenous injection every four days (**P＜0.01, Student's t-test, BU-NPs-CTX versus normal saline, ***P＜0.001, Student's t-test, MF-NPs-CTX and Tween-CTX versus normal saline. ##P＜0.01, Student's t-test, MF-NPs-CTX versus BU-NPs-CTX). (B) Body weights of HeLa-bearing nude mice change with time (**P＜0.01,

Student's t-test, MF-NPs-CTX and BU-NPs-CTX versus Tween-CTX). (C) The weight of solid tumor after treatment of MF-NPs-CTX, BU-NPs-CTX and Tween-CTX and normal saline (***P＜0.001, Student's t-test, MF-NPs-CTX, BU-NPs-CTX and Tween-CTX versus normal saline. #P＜0.05, Student's t-test, MF-NPs-CTX versus BU-NPs-CTX). (D) The actual picture of solid tumor treated with different CTX formulations and normal saline.

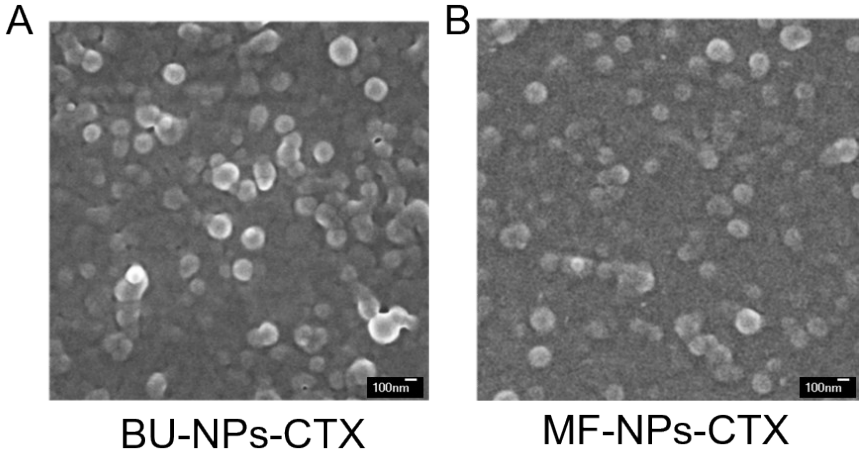

Fig. S4 Scanning electron microscope (SEM) image of (A)BU-NPs-CTX and (B)MF-NPs-CTX

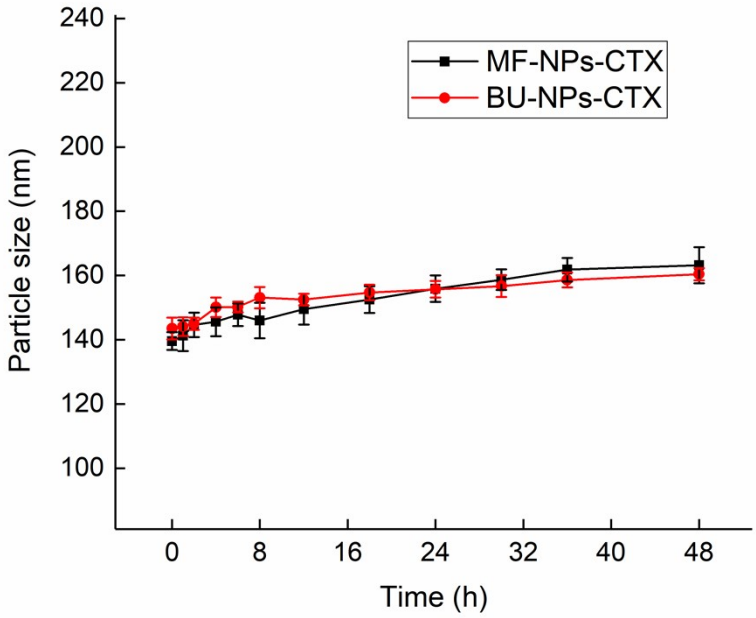

Fig. S5 Stability of BU-NPs-CTX and MF-NPs-CTX in vitro, BU-NPs-CTX and MF-NPs-CTX were dissolved in saline.

References

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