

Electronic Supplementary Information

A Redox-Sensitive Core-Crosslinked Nanosystem Combined with Ultrasound for Enhanced Deep Penetration of Nanodiamond into Tumors

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Experimental section

Reagents, cell lines and animals

NanoAmando nanodiamonds (NDs) soft hydrogel was purchased from the NanoCarbon Research Institute (Nagano, Japan). Pullulan (MW 200,000 Da) was purchased from Hayashibara Biochemical Laboratory (Okayama, Japan). Selenium powder, 4-Bromobenzyl alcohol, hydrazine hydrate, and 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCI) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Doxorubicin hydrochloride (DOX·HCl) was purchased from Aladdin Industrial Co. (Shanghai, China). Cell Counting Kit-8 (CCK-8) was obtained from Dojindo Molecular Technologies (Shanghai, China). The Annexin V-FITC/ Propidium Iodide (PI) Apoptosis Detection Kit and CAM/PI were bought from Beyotime Technology (Shanghai, China). Fetal bovine serum (FBS) was obtained from Haoyang Technology Co., Ltd. and Dulbecco's Modified Eagle's Medium (DMEM) was obtained from HyClone (high glucose, Logan, Utah, USA). All chemical reagents were analytical grade and used directly without further purification unless otherwise noted. Solutions were prepared using deionized water with a resistance of 18.5 MΩ-cm.

Human hepatocellular carcinoma cells (HepG2) and human umbilical vein endothelial cells (HUVEC) were obtained from the Chinese Academy of Sciences Cell Bank (Shanghai, China). The cells were cultured in DMEM medium supplemented with 10% FBS and 1% penicillin–streptomycin at 37 °C in a humidified 5% CO₂ atmosphere. The cells were sub-cultured when they reached 90% confluence and the cell culture medium was replaced every two days.

Female BALB/c mice, nude mice (4-6 weeks, 17-23 g) and Sprague-Dawley rats (SD, 8-10 weeks, 180-220 g) were purchased from the Laboratory Animal Center of Chongqing Medical University (Chongqing, China).

Synthesis and characterization of Pu-HBSe complex

The purified HBSe was synthesized as described in a previously reported method with slight modifications.¹ Specifically, selenium powder (1.98 g, 25 mmol) and sodium hydroxide (1.52 g, 38 mmol) were dissolved in anhydrous dimethylformamide (DMF, 100 mL). Then, 100% hydrazine hydrate (1 mL, 25 mmol) was added dropwise into the above mixture under vigorous stirring at room temperature. Next, 4-Bromobenzyl alcohol (4.675 g, 25 mmol) was added and the mixture was stirred in a 130 °C oil bath within a reflux cooler system for 4 h. After the reaction completed, the synthetic product was diluted with deionized water and extracted with ethyl acetate for twice. The organic layer was collected and dried by anhydrous sodium sulfate. The solvent was removed through rotary evaporation in a water bath at 45 °C and the purified HBSe was obtained by drying in vacuo.

The synthesis protocol for carboxymethyl pullulan (CMP) was obtained from our previous report.² 50 mg CMP and 12.4 mg EDCI were dissolved in 1.5 mL deionized water under vigorous stirring for 30 min. As a carboxyl activator, EDC was used to activate the carboxyl group on carboxymethyl pullulan for improving the coupling efficiency. Subsequently, the above mixture was dropwise added into 2 mL of HBSe solution (80.35 mg mL⁻¹ in DMF) and stirred for 5 h. The mixture was extracted with ethyl acetate and the aqueous layer was collected. Finally, the as-synthesized Pu-HBSe

was dialyzed against deionized water and lyophilized for further use.

The obtained Pu-HBSe was analyzed by nuclear magnetic resonance ($^1\text{H-NMR}$, Bruker Avance III HD 600 MHz, Switzerland) and fourier transform infrared spectroscopy (FTIR, Thermo Scientific Nicolet IS50, USA).

Synthesis and characterization of nanodiamonds-doxorubicin loaded core-crosslinked nanosystem (NDX-CCS)

NDX-CCS was synthesized by a facile two-step strategy. First, 10 mg NDs were dispersed in deionized water by a sonicator (Sonics & Materials Inc., USA) at an intensity of 60 W for 1 h (pulse duration: 40 s; pulse interval: 20 s) and mixed with 10 mg DOX-HCl at a weight ratio of 1:1. The mixture was then adjusted to a pH of ~ 7.5 with 2.5 mM NaOH to promote drug binding to the NDs. Next, the resulting NDX was collected by centrifugation (13000 rpm, 10 min), resuspended in deionized water, and ultrasonicated for an additional 10 min.³ Afterwards, 2 mL of NDX solution (5 mg mL^{-1} in deionized water) was added to the above Pu-HBSe solution and vigorously mixed. After 2 h, the NDX-loaded non-crosslinked nanosystem (NDX-NCS) was prepared, and directly irradiated for 3 h by a 25 W incandescent light bulb with a filter at 184 Lux to obtain an NDX-loaded core-crosslinked nanosystem (NDX-CCS).⁴

The loading content (LC) and encapsulation efficiency (EE) of DOX were determined by UV-vis spectroscopy (Thermo Scientific Nanodrop 2000, USA) at 480 nm and calculated according to the following equations:

$$\text{LC (\%)} = (\text{weight of loaded drug} / \text{weight of drug-loaded nanoparticles}) \times 100\%$$

$$\text{EE (\%)} = (\text{weight of loaded drug} / \text{weight of total drug}) \times 100\%$$

After being stained with sodium phosphotungstate solution, the NDX-CCS's morphology and nanostructure were observed with a transmission electron microscope (TEM, Hitachi H-7600, Japan) at an acceleration voltage of 200 kV. The hydration particle size and surface zeta potential of nanosystem were determined with a Zetasizer Nano-ZS instrument (ZEN3600, Malvern, UK). To evaluate the stability, NDX, NDX-NCS, and NDX-CCS solutions were stored in phosphate buffered saline (PBS) with 10% FBS at 37 °C and observed at predetermined time intervals by dynamic light scattering (DLS). The DOX in vitro release rates from the NDX-CCS were evaluated in five different kinds of PBS: PBS, PBS with 1 μM H_2O_2 , PBS with 100 μM H_2O_2 , PBS with 10 μM GSH, and PBS with 2 mM GSH. Briefly, NDX-CCS in dialysis bags were immersed in different kinds of PBS and constantly shaken in a 37 °C water bath at 100 rpm. The amount of DOX released was measured by UV-vis spectroscopy at various predetermined times. All the experiments were conducted in triplicate and the values were recorded as mean \pm standard deviation.

Hemolysis assay

BALB/c mice blood was centrifuged (3000 rpm, 10 min) at 4 °C and washed three times with PBS to separate the red blood cells (RBCs). For the hemolysis assay, 0.5 mL of 2% RBCs (v/v) was incubated with varying concentrations of NDX-CCS solution (10, 25, 50, 100, 200 $\mu\text{g mL}^{-1}$) at 37 °C for 4 h. The negative control was RBCs suspended in PBS and the positive control was RBCs mixed with deionized water. After centrifugation (3000 rpm, 10 min), the absorbance of supernatants at 540 nm was

analysed by a microplate reader (Infinite M200 Pro N, TECAN, Switzerland). The percentage of hemolysis was calculated as follows:

$$\text{Hemolysis (\%)} = (\text{Abs}_{\text{sample}} - \text{Abs}_{\text{negative}} / \text{Abs}_{\text{positive}} - \text{Abs}_{\text{negative}}) \times 100\%$$

Intracellular drug uptake and retention of NDX-CCS

The uptake of NDX-CCS by HepG2 cells was evaluated by confocal laser scanning microscopy (CLSM, Nikon A1⁺R, Japan). HepG2 cells (2×10^5 cells per well) were seeded in a confocal cell-culture dish for 24 h. Then, the culture medium was replaced with fresh medium containing free DOX, NDX, NDX-NCS, and NDX-CCS (DOX equivalent dose: 10 mg L^{-1}). After 4 h of incubation, the cells were washed twice with PBS, stained with Hoechst 33342 for 10 min, and observed using CLSM at the excitation wavelength of 485 nm. Furthermore, the quantitative intracellular drug uptake was measured by flow cytometry (FCM, FACS Vantage SE, Beckman Coulter, USA).

For intracellular drug retention assay, HepG2 cells were seeded into confocal cell-culture dishes and incubated until they adhered. After replacing the medium with fresh culture medium containing free DOX, NDX, NDX-NCS, and NDX-CCS (DOX equivalent dose: 10 mg L^{-1}), the cells were cultured for 4 h, washed with PBS twice, and cultured again for an additional 12 h. Finally, the fluorescence images were directly recorded by CLSM and the mean DOX fluorescence intensity was quantified by FCM.

Cytotoxicity of nanomaterials and NDX-CCS in vitro

The cytotoxicity of Pu-HBSe and NDs was investigated in HUVEC and HepG2 cells using CCK-8 assay. Cells were seeded into 96-well plates (1×10^4 cells per well) and

cultured overnight prior to treatment with the prepared nanomaterials at different concentrations (0.01, 0.1, 1, 10 and 100 mg L⁻¹). After 24 h co-incubation, the cell viabilities were tested by CCK-8 assay and the absorbance was measured using a microplate reader. The toxicities of free DOX, NDX, NDX-NCS, and NDX-CCS were explored in HepG2 cells using the same method described above. Final DOX concentrations were set as: 0.01, 0.1, 1, 10 and 100 mg L⁻¹. Six replicates were conducted for each group.

Apoptosis assay

Apoptosis of the HepG2 cells was detected using the Annexin V-FITC/ Propidium Iodide (PI) Apoptosis Detection Kit. Briefly, cells (2×10⁵ cells per well) were seeded in 6-well plates and cultured overnight. After 24 h of treatment with drug-free DMEM, free DOX, NDX, NDX-NCS, and NDX-CCS (DOX equivalent dose: 10 mg L⁻¹), the cells were collected by trypsin, stained with 5 μL of Annexin V-FITC and 10 μL of PI for 15 min, and then harvested for FCM. In addition, cells were stained with CAM/PI dye to differentiate living (green) and dead (red) cells for CLSM observation.

HepG2 tumor-bearing mice models

Female BALB/C nude mice were injected subcutaneously with HepG2 cell suspension (1×10⁷ cells in 0.2 mL per mouse) in the left dorsolateral flank. Tumors were allowed to develop until they reached a size of 80 mm³ before other in vivo experiments were carried out. The tumor volume was calculated as length × weight²/2.

In vivo biodistribution and imaging study

NDX-CCS in vivo biodistribution was visualized using fluorescence (FL) imaging

performed on an IVIS spectrum imaging instrument (IVIS Lumina III, PerkinElmer, USA). Free DOX and NDX-CCS (10 mg kg^{-1} , 0.2 mL) were intravenously injected into the HepG2 tumor-bearing mice, within a safe dosage limit, to acquire clearer imaging. Images were then obtained at 0, 4, 8, 12, and 24 h post-administration. After the 24 h scan, tumors and major organs (heart, liver, spleen, lung, kidney) were harvested for ex vivo imaging. The fluorescence imaging signal intensity within selected regions of interest (ROI) was quantified and analyzed using the Living Image Software. Finally, tumors were collected for cryosection, stained with DAPI and observed by CLSM. For quantitative in vivo biodistribution analysis. The excised tumors and major organs were washed with PBS, weighed, homogenized and diluted with 1 mL deionized water, and centrifuged at 10000 rpm . The obtained supernatant was extracted with chloroform/isopropanol ($3:1 \text{ v/v}$) and subjected to UV spectrophotometry to determine the DOX level. The corresponding DOX tissue concentrations were calculated accordingly.

HepG2 tumor-bearing mice were intravenously injected with NDX-CCS (10 mg kg^{-1} , 0.2 mL) and treated with ultrasound irradiation (1 MHz , 1 W cm^{-2} , 5 min) using a low intensity focused ultrasound instrument. The tumors were dissected and homogenized in deionized water at 4, 8, 12, and 24 h after i.v. administration. Following centrifugation, DOX supernatant was extracted with chloroform/isopropanol ($3:1 \text{ v/v}$) solvent and measured with a UV spectrophotometer. In order to perform different nanosystem biodistribution studies, free DOX, NDX, NDX-NCS, NDX-CCS, and NDX-CCS were i.v. administered to tumor-bearing mice at a DOX dose of 10 mg kg^{-1}

before ultrasound irradiation, and biodistribution assay was performed at 12 h post-administration. The remaining procedure was the same as described above.

Intratumoral drug penetration and pharmacokinetic study

For intratumoral drug penetration assessment, mice bearing 600 mm³ HepG2 tumors were randomly divided into three groups. After 24 h post-injection with free DOX, NDX-CCS, and NDX-CCS + US, respectively, 100 mm³ of the central tumor regions and whole tumors were harvested for DOX concentration evaluation. The remaining procedure was the same as described above. The amount of DOX infiltration was determined with UV spectrophotometry and the penetration index (PI) was calculated by dividing the DOX concentration in the central tumor region by the DOX concentration in the whole tumor. CLSM was performed on frozen tumor slices in order to directly visualize drug penetration following DAPI staining.

Plasma pharmacokinetics was investigated by measuring DOX concentration in blood at predetermined time intervals. The blood of SD rats was collected through the retro-orbital vein after i.v. administration at a DOX dose of 5 mg kg⁻¹. Blood samples were centrifuged (5000 rpm, 4 °C) for 10 min, and 100 µL of supernatant plasma was extracted with 900 µL of acetonitrile to precipitate all the proteins. The organic phase was detected using UV spectrophotometry to determine DOX concentration.

In vivo anti-tumor therapy and biosafety assay

For in vivo anti-tumor efficacy evaluation, female nude mice bearing 80 mm³ HepG2 tumors were randomly divided into six groups: Saline group, free DOX group, NDX group, NDX-NCS group, NDX-CCS group, and NDX-CCS + US group (DOX

equivalent dose of 5 mg kg⁻¹, ultrasound irradiation: 1 MHz, 1 W cm⁻², 5 min). The treatment was administered intravenously every three days for a total of five doses. Tumor-volume changes and body weight of each mouse were recorded every other day, and the mice survival rate was also tracked during the experimental period. Twenty-five days after the first dose, the major organs were harvested for H&E staining and tumors were stained with H&E, TUNEL, and PCNA for histopathology analysis.

Twelve of BALB/c mice were i.v. injected with NDX-CCS (DOX dose of 5 mg kg⁻¹) as the experimental group, and the control group was administrated saline. Fresh blood was collected from the treated mice at 1, 7, 14, and 28 days for routine blood examination and serum biochemical index analysis.

Statistical analysis

Data analysis and graphs were performed with Origin 8.0 software. The unpaired Student's t-test was used for two groups' comparison and one-way ANOVA test was used for comparisons between multiple groups. All of the data are expressed as a mean ± standard deviation (mean ± SD) unless otherwise indicated. Statistical significance was set as * $P < 0.05$, and extreme significance was set as ** $P < 0.01$. In vivo assays were performed with a minimum of $n = 6$ animals per group unless mentioned.

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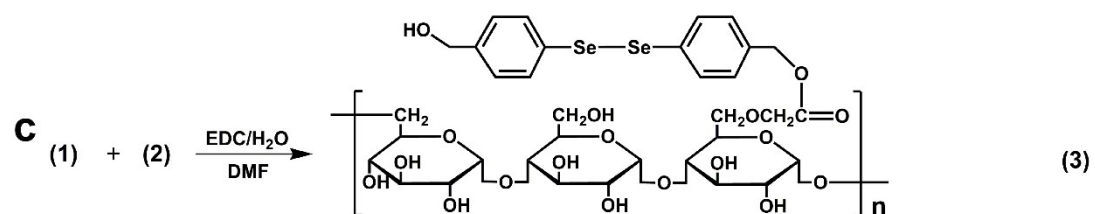
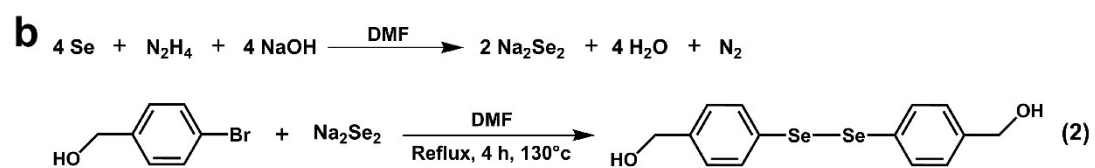
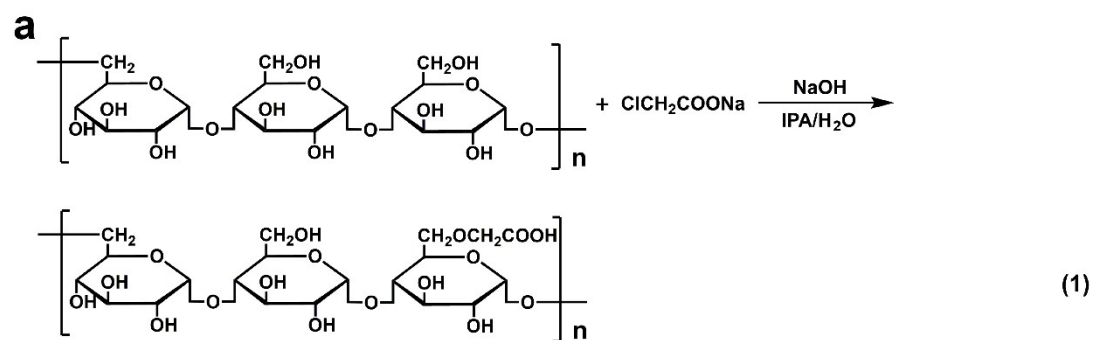


Figure S1. Synthesis of a) carboxymethyl pullulan (CMP), b) H₂Se and c) Pu-HBSe complex.

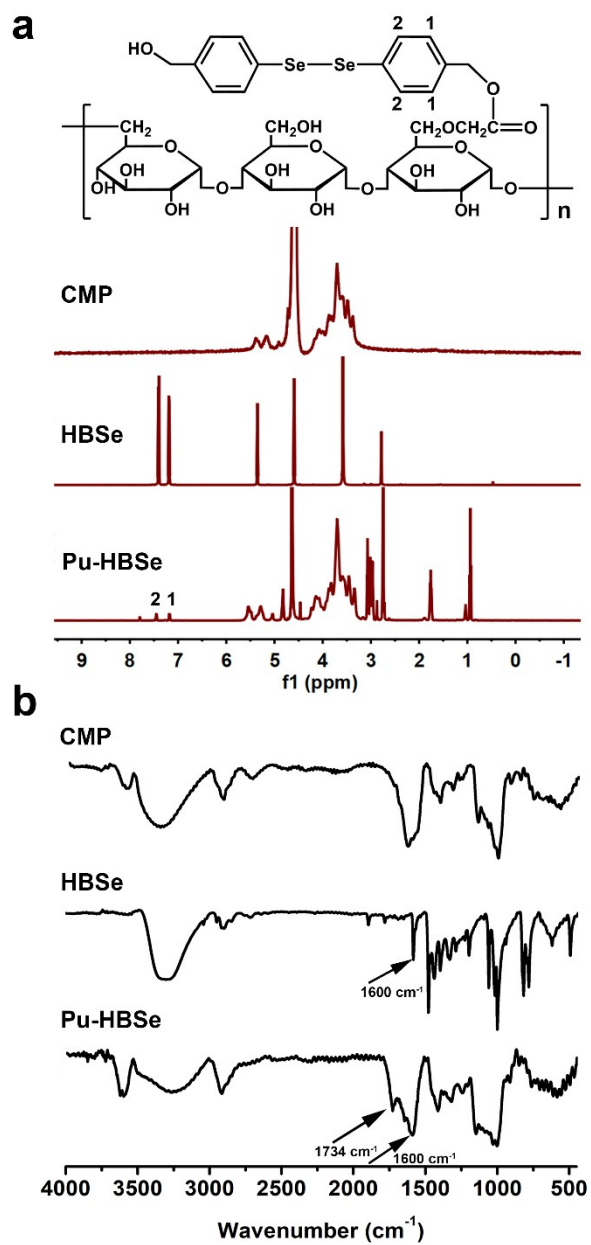


Figure S2. Characterization of NDX-CCS. a) $^1\text{H-NMR}$ and b) FTIR spectra of CMP, HBSe and Pu-HBSe.

Table S1. Calculated IC₅₀ values of different formulations in HepG2 cells after 24 h incubation.

	free DOX	NDX	NDX-NCS	NDX-CCS
IC ₅₀ (mg L ⁻¹) ^a	4.90±0.15	3.01±0.14** ^b	0.86±0.06**	0.41±0.02**

^a IC₅₀ value was calculated based on the concentration of DOX;

^b ** $P < 0.01$, compared to free DOX.

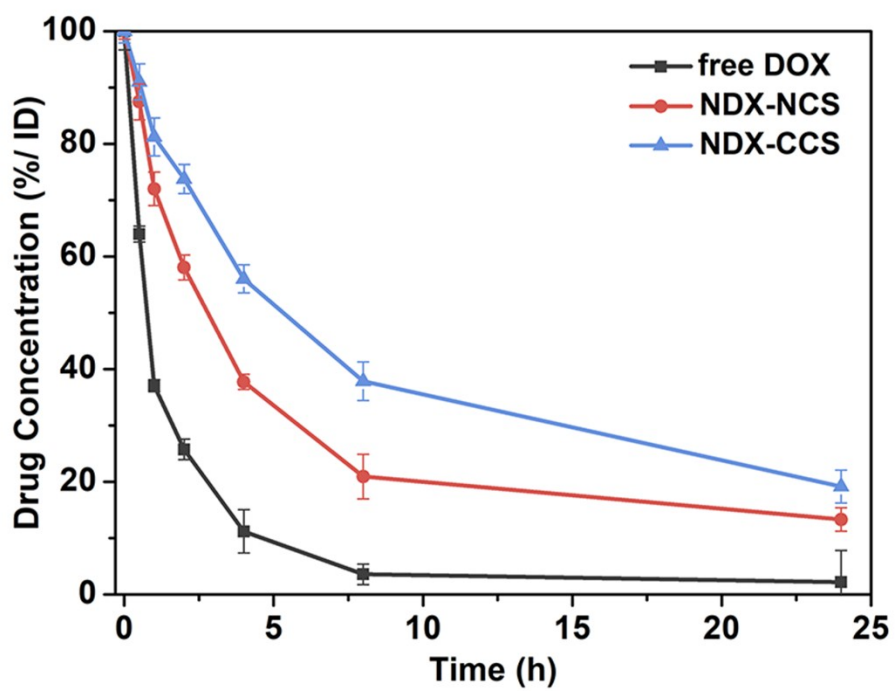


Figure S3. Pharmacokinetics profiles in rats after i.v. administration of free DOX, NDX-NCS and NDX-CCS at DOX dose of 5 mg kg^{-1} ($n = 6$).

Table S2. Pharmacokinetic parameters of DOX after i.v. administration to SD rats in free DOX, NDX-NCS and NDX-CCS ($n = 6$) in vivo.

Parameters	free DOX	NDX-NCS	NDX-CCS
$t_{1/2}$ (h) ^a	1.67±0.07	3.55±0.09** ^c	5.71±0.14**
AUC ₀₋₂₄ (µg mL ⁻¹ h ⁻¹) ^b	26.20±1.43	33.89±1.23**	45.57±2.02**

^a The elimination half-life;

^b Area under the plasma DOX concentration-time curve;

^c ** $P < 0.01$, compared to free DOX.

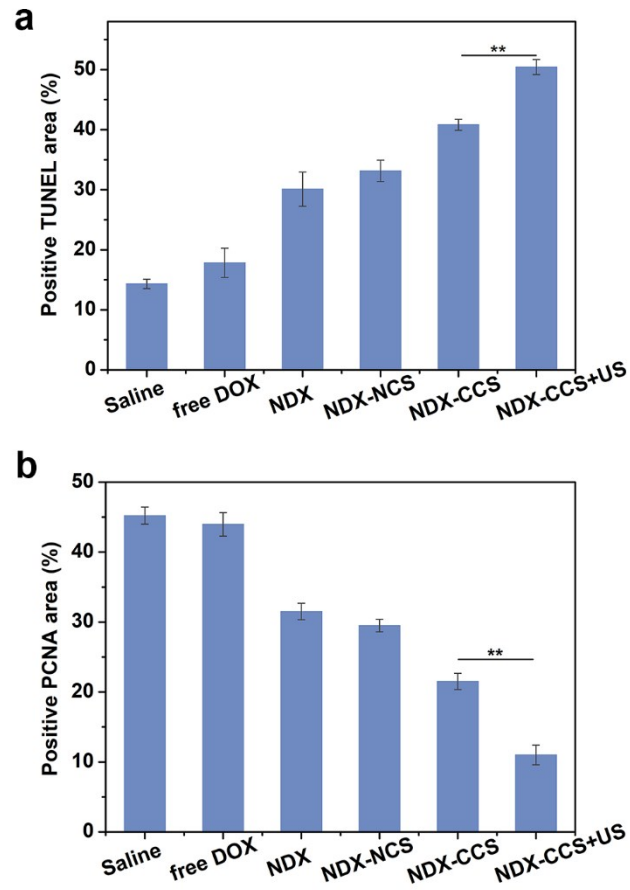


Figure S4. Quantitative analysis of a) TUNEL and b) PCNA staining of tumor section in different formulations-treated groups (Values are means \pm SD, $**P < 0.01$, $n = 6$).

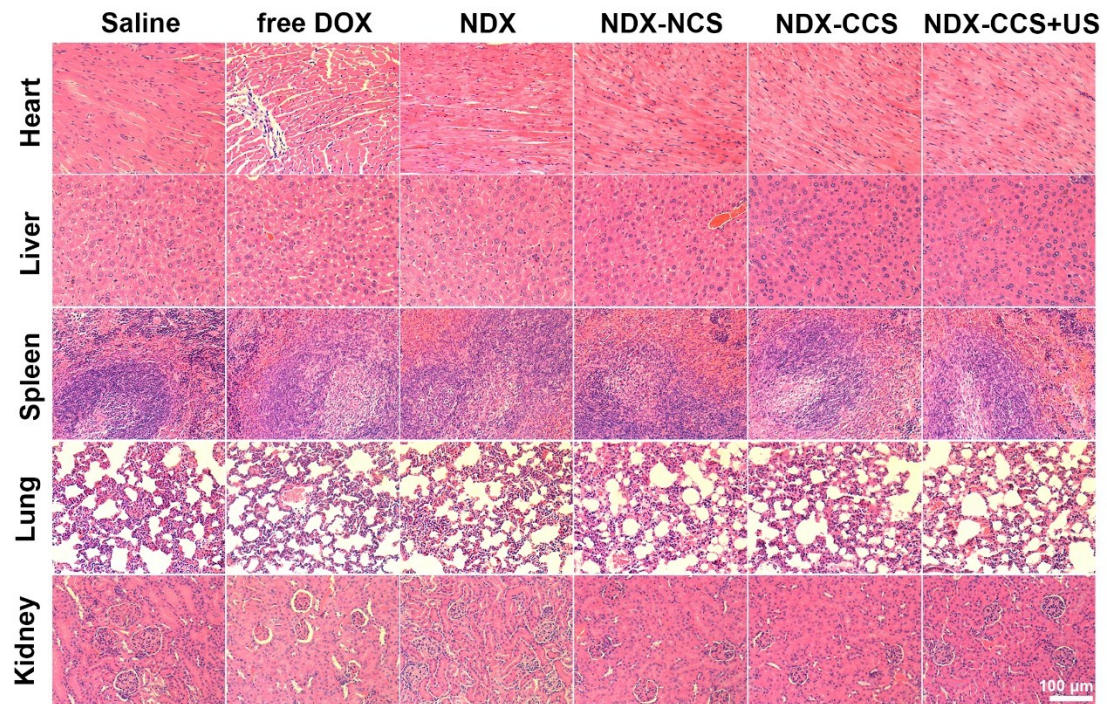


Figure S5. H&E staining of the major organs (heart, liver, spleen, lung and kidney) of HepG2 tumor-bearing nude mice after various treatments.

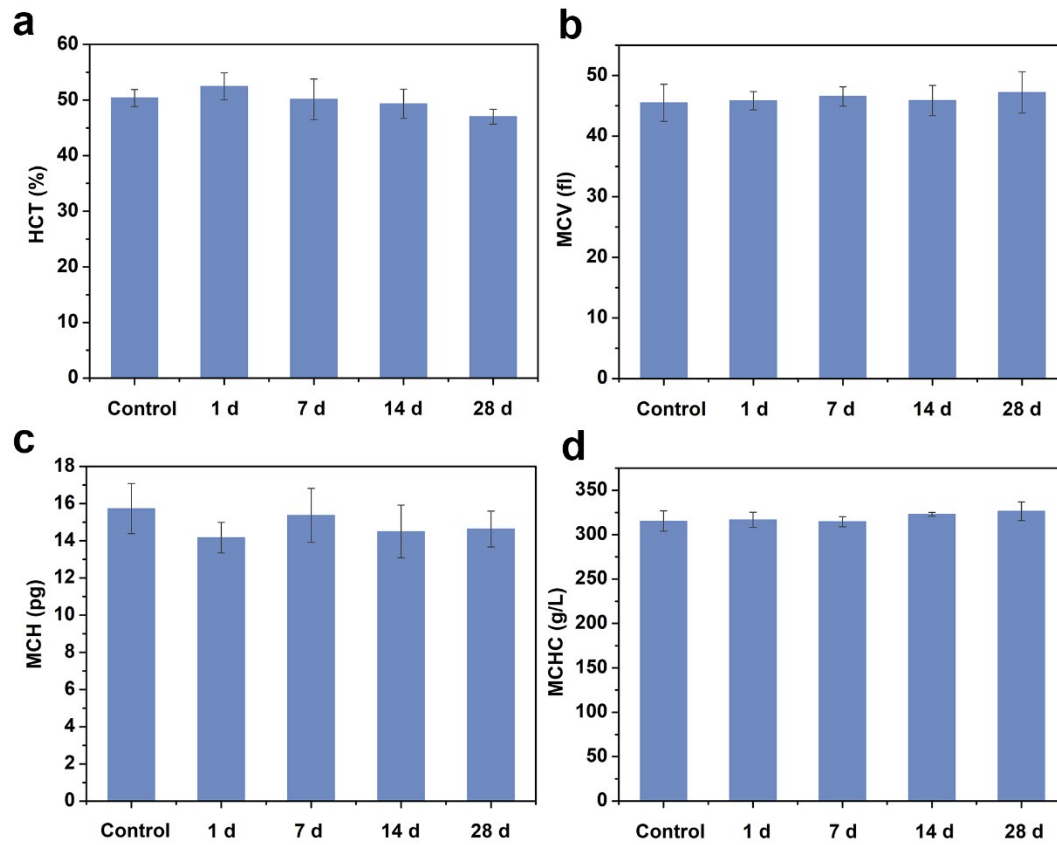


Figure S6. Blood routine indexes a) HCT, b) MCV, c) MCH and d) MCHC analysis of BALB/C mice in the control group and the experimental group at on days 1, 7, 14 and 28 post i.v. injection of NDX-CCS (DOX dose of 5 mg kg^{-1} , values are means \pm SD, $n = 3$).