Supplementary Information

Quadruple-responsive nanoparticle-mediated targeted combination chemotherapy for metastatic breast cancer

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1. Materials

1.1 RSF extraction

Silkworm cocoons were stripped to remove the silkworm chrysalis. The refined silkworm cocoons were boiled 3 times for 20 minutes each time in a Na_2CO_3 aqueous solution (0.5%, w/v), and rinsed thoroughly with deionized water to eliminate the outer glue-like sericin. Subsequently, the raw silk fibroin was fully dried at 40°C for 24 h, and the dried silk fibroin was dissolved in Ajisawa's reagent composed of $CaCl_2$:ethanol:H_2O (1:2:8 in molar ratio) for 2 h at 78°C. Insoluble impurities were removed by centrifugation for 10 min at 8,000 rpm and dialyzed against distilled water for at least 3 days (molecular weight cut-off = 1,400 Da). The RSF aqueous solution was filtered to remove the aggregates and freeze-dried. The dried RSF was stored at 4°C.

1.2 Physicochemical characterizations of NPs

The average particle size (nm), polydispersity index (PDI), and zeta potential (mV) of NPs were measured using a DLS (Malvern Zetasizer Nano S90, Worcestershire, UK). The average values of these parameters were based on the measurements of 3 batches of these NPs.

The morphologies of HA-CUR/5-FU-NPs (v) were observed using a SPA 400 AFM (Seiko instruments Inc., Japan) at tapping mode using high resonant frequency ($F0$ = 150 kHz) pyramidal cantilevers with silicon probes at a scan frequency of 1 Hz. The HA-CUR/5-FU-NP (v) suspension (0.02 mg/mL, 20 μL) was dropped onto a cleaned mica slice, followed by drying overnight at room temperature.

The drug loading amounts and encapsulation efficiencies of CUR and 5-FU in various NPs were determined by measuring their intrinsic fluorescence intensity and UV absorption, respectively. Briefly, NPs (5 mg) were fully dissolved in DMSO. The fluorescence intensities of CUR in the

obtained solution were determined using a fluorescence spectrophotometer (Shimadzu RF-5301 PC, Kyoto, Japan) at λ_{em}= 530 nm and λ_{ex}= 425 nm. The UV spectrum of 5-FU was measured at 270 nm on a Shimadzu UV-1700 UV/Vis spectrophotometer (Kyoto, Japan). The amounts of HA on the surface of NPs were quantified using alcian blue according to previous reports.[1, 2]

A dialysis method was used to investigate the release behaviors of CUR and 5-FU from NPs in buffers with different pH values. HA-CUR/5-FU-NPs (1:1.2) containing total drug amount of 200 μg were dispersed in various releasing buffers. The suspensions were introduced into dialysis bags with a molecular weight cut-off of 3,500 Da. Subsequently, these bags were sealed at both ends and put into centrifuge tubes (50 mL) supplemented with various releasing media (20 mL). Meanwhile, Tween-80 was supplemented into the releasing media to maintain the solubility of CUR in the aqueous phase, and the final concentration of Tween-80 was set as 0.1% (w/v). Tubes were placed in a thermostat shaker at 150 rpm and 37^{o}C. At pre-determined time intervals, the releasing media (2 mL) was withdrawn and replenished with the same volume of fresh releasing media. The amount of CUR and 5-FU in the outer solution was measured according to the method as described above.

1.3 In vitro cellular uptake efficiencies of NPs

4T1 cells were cultured in eight-chamber tissue culture glass slides (BD Falcon, Bedford, USA) at a final density of 5×10^4 cells per well and incubated overnight. After exposure to NP suspensions for 3 h, cells were rinsed with PBS to eliminate un-phagocytized NPs and fixed in 10% formalin solution for 30 min. AF633-labeled phalloidin was applied to stain cytoskeleton for 1 h, followed by staining the nuclei with DAPI. Samples were imaged using a confocal laser scanning microscope (Zeiss-800, Jena, Germany).

Cell internalization efficiencies of NPs were quantified using FCM. 4T1 cells and HUVECs were cultured in 12-well plates at a cell density of 3×10^5 and 5×10^5 cells/well, respectively. After overnight culture, the complete medium was replaced with a serum-free medium containing various NPs (equivalent to 16 μM of CUR). Cells without treatment were used as a negative control. After co-incubation for prescribed time intervals (1, 3, and 5 h), cells were thoroughly rinsed with cold PBS to eliminate the residual NPs which were not internalized. Subsequently, cells were harvested, transferred to polystyrene test tubes, and suspended in FCM buffer. The quantification of cellular uptake efficiencies of NPs were performed on a FCM (ACEA NovoCyte™, San Diego, USA) using a FITC channel. A total of 10,000 ungated cells were analyzed.

1.4 In vitro anti-migration properties of NPs

FCM was used to determine the pro-apoptotic properties of various NPs. 4T1 cells were grown in 12-well plates at a final density of 2×10^5 cells/well. After overnight culture, a sterilized toothpick was used to make a straight scratch in the center of wells. After rinsing the wells with PBS for 3 times, the initial images of 4T1 cells were captured using a CLSM (Zeiss-800, Jena, Germany). Subsequently, cells were incubated with 2 mL of various NP suspensions (total drug concentration: 16 μM). After incubation for 24 h, cells were rinsed with PBS for 3 times, and their images were captured using a CLSM.

1.5 Cell cycle distribution analysis of NPs

FCM was used to determine the cell cycle distributions of various NPs. 4T1 cells were grown in 12-well plates at a final density of 1×10^5 cells/well. After overnight culture, cells were incubated with various NPs (total drug concentration: 16 μM) for 24 h. Cells were thoroughly rinsed with PBS for 3 times, digested using trypsin, and collected by centrifugation at 2,000 g for 5 min.

Subsequently, cells were fixed in an ice-cold ethanol solution (70%, v/v) containing ribonuclease (RNase, 2 mg/mL) for 30 min. After co-incubation for 24 h, cells were harvested and stained with Annexin V-PE solution for 30 min in the dark. Finally, the obtained cell suspensions were analyzed using a PE channel on the ACEA Novo Cyte^{TM} FCM System (ACEA Biosciences, San Diego, USA). A total of 20 000 ungated 4T1 cells were analyzed.

1.6 Blood compatibility studies of NPs

Blood was collected from the eyelid posterior sinus vein of mice to evaluate the *in vitro* hemocompatibility of HA-CUR/5-FU-NPs (v). Subsequently, erythrocytes were harvested by centrifugation at 2,500 g for 10 min, washed 3 times and suspended in PBS (2%, v/v). These erythrocyte suspensions were mixed with HA-CUR/5-FU-NP (v) suspensions, and the total drug concentrations in the mixtures were from 0 to 128 μM. After incubation for 1 h at 37^{o}C, the mixtures were centrifuged at 3,000 g for 15 min. Finally, hemoglobin amounts in the supernatants were analyzed by spectrophotometric tests at 570 nm. The untreated erythrocyte suspension was utilized as a negative control, whereas erythrocyte suspension treated with Triton X-100 (1.0%, w/v) was used as a positive control.

In the context of the *in vivo* blood compatibility studies, female Kunming mice (8 weeks old; Chongqing Tengxin Biotechnologied Company, Chongqing, P. R. China) were intravenously injected with HA-CUR/5-FU-NP (v) suspensions at a total drug concentration of 10 mg drug/kg body weight. At the desired time point (6 h, 12 h, 1 d, 3 d, 5 d, and 7 d), mice blood was harvested and analyzed using a hematology analyzer (BC-3200, Mindray, Shenzhen, P. R. China).

1.7 In vivo bio-distribution of NPs

To detect the *in vivo* bio-distribution of NPs, we utilized Cy7 as a NIRF probe. CUL-Cy7-NPs and HA-Cy7-NPs were constructed following the same protocol used to generate HA-CUR-NPs, except that CUR was replaced with Cy7. Female BALB/c mice with breast tumors were intravenously injected with CUL-Cy7-NPs and HA-Cy7-NPs (5 mg Cy7/kg mouse). At the predetermined time points (12, 24, 48, and 72 h), mice were euthanized. The five main organs (heart, liver, spleen, lung, and kidney) and tumors were collected. The imaging was carried out using a living imaging system (FX Pro90200, Carestream, USA), and the regional fluorescence intensities were analyzed with the software of a living imaging system.

1.8 In vivo photoacoustic imaging of NPs

For photoacoustic imaging, female nude mice bearing breast tumor xenografts were intravenously injected with HA-Cy7-NPs (5 mg Cy7/kg mouse). Subsequently, at predetermined time points (12, 24, 48, and 72 h), tumor regions were imaged with an excitation wavelength at 770 nm using a VisualSonics (Vevo 2100; VisualSonics Inc., Ontario, Canada).

1.9 In vivo targeting capacities of NPs

To investigate the *in vivo* targeting capacities of HA-functionalized NPs, female BALB/c mice bearing breast tumor xenografts were intravenously injected with HA-CUR-NPs (5 mg CUR/kg mouse). Twenty-four hours after intravenous injection, mice were euthanized. Tumor tissues were collected, embedded in OCT, and sectioned into 5 μm slices. Subsequently, these slices were fixed in paraformaldehyde (4%, v/v) and stained with DAPI. Finally, the fluorescence images of these slices were acquired under a CLSM.

1.10 Statistical analysis

Statistical analysis was conducted using a student's *t*-test or one-way ANOVA test followed by SPSS statistical software 19.0 (SPSS Inc., Chicago, USA). All data were reported as mean ±

standard error of the mean (S.E.M.). Statistical significance was represented by *$P < 0.05$, **$P < 0.01$, or ***$P < 0.001$.

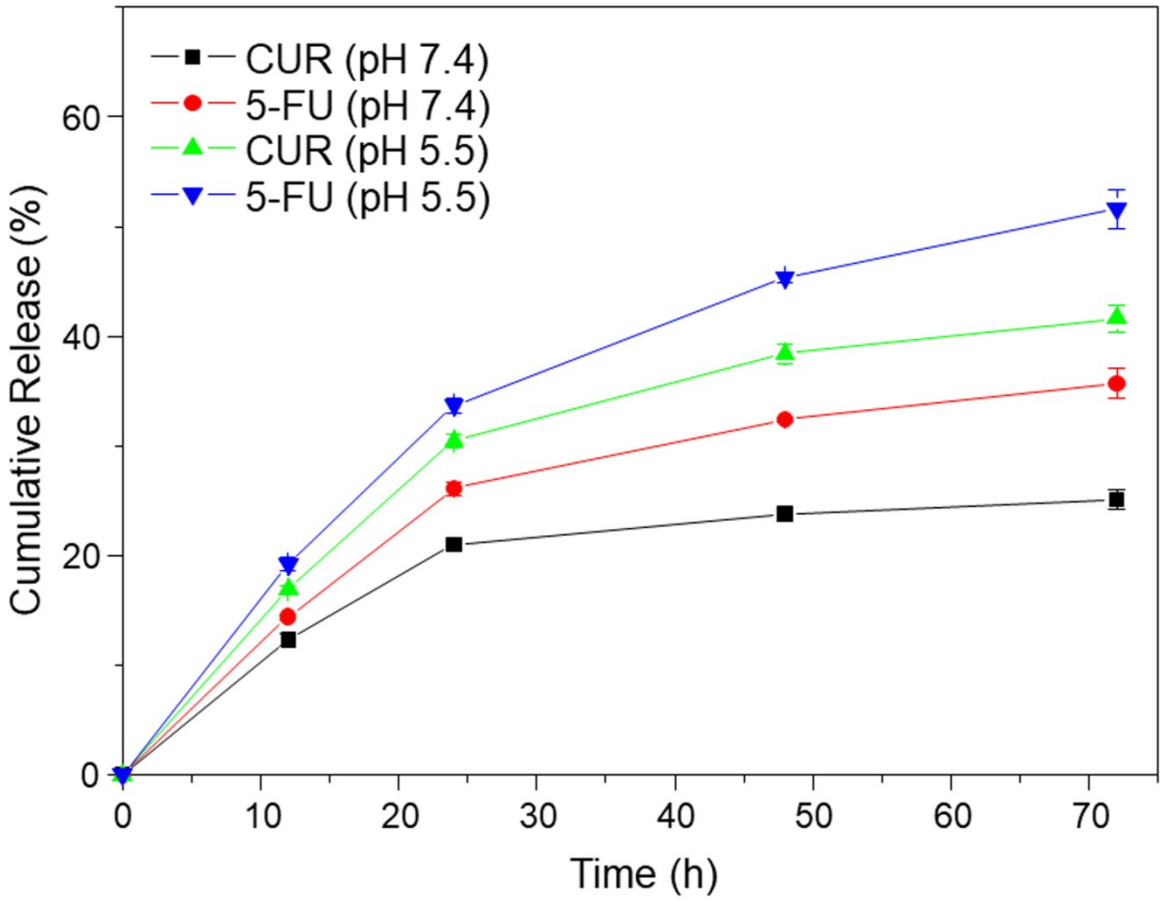

Fig. S1 *In vitro* cumulative release profiles of CUR and 5-FU from HA-CUR/5-FU-NPs (v) in buffers with different pH values (pH = 7.4 and 5.5).

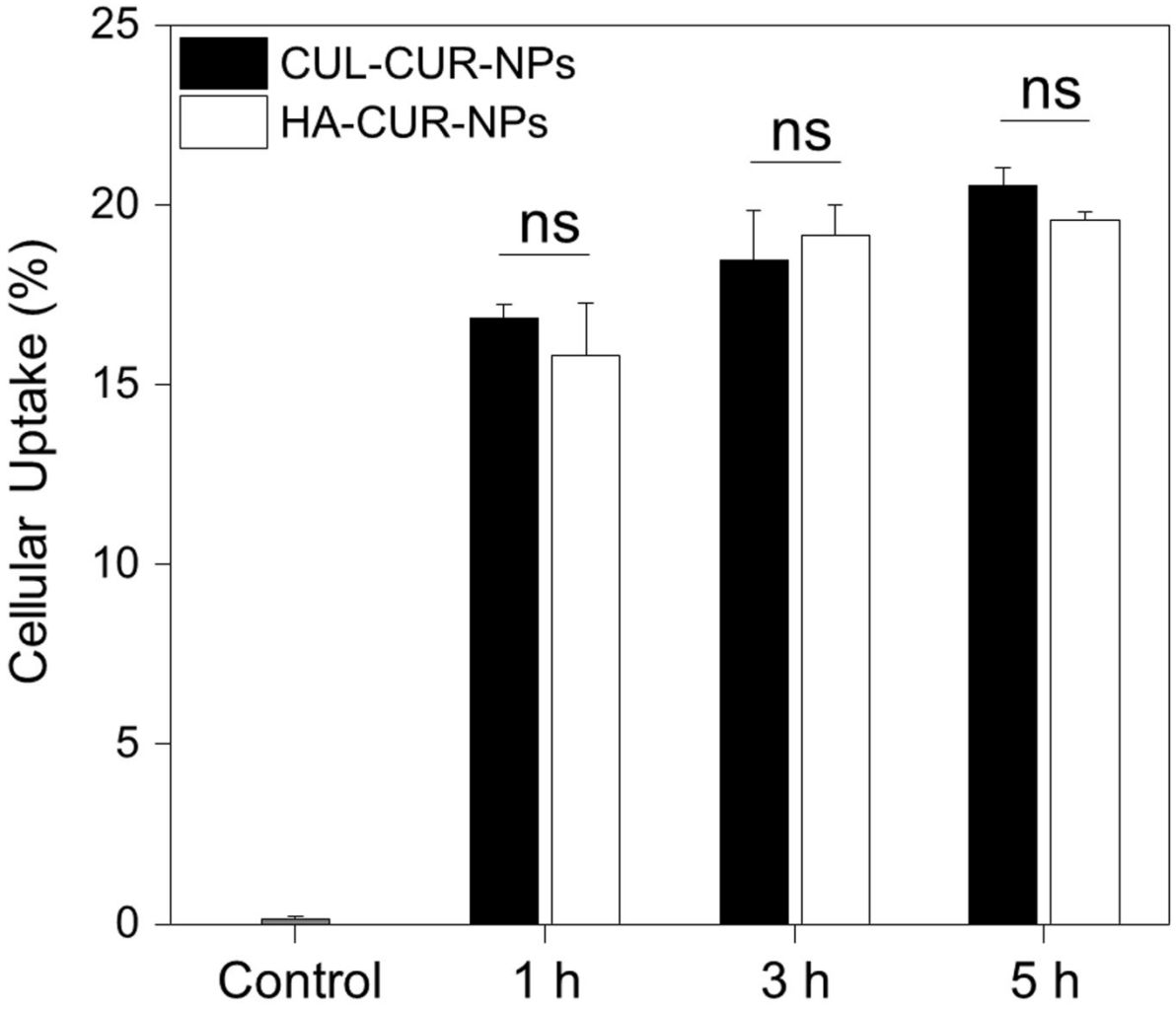

Fig. S2 Cellular uptake percentages of NPs in HUVECs after treatment with CUL-CUR-NPs and HA-CUR-NPs (CUR, 16 μM) for 1, 3, and 5 h, respectively. Data are expressed as means ± S.E.M. (n = 3; *$p < 0.05$, **$p < 0.01$, and ns = no significant).

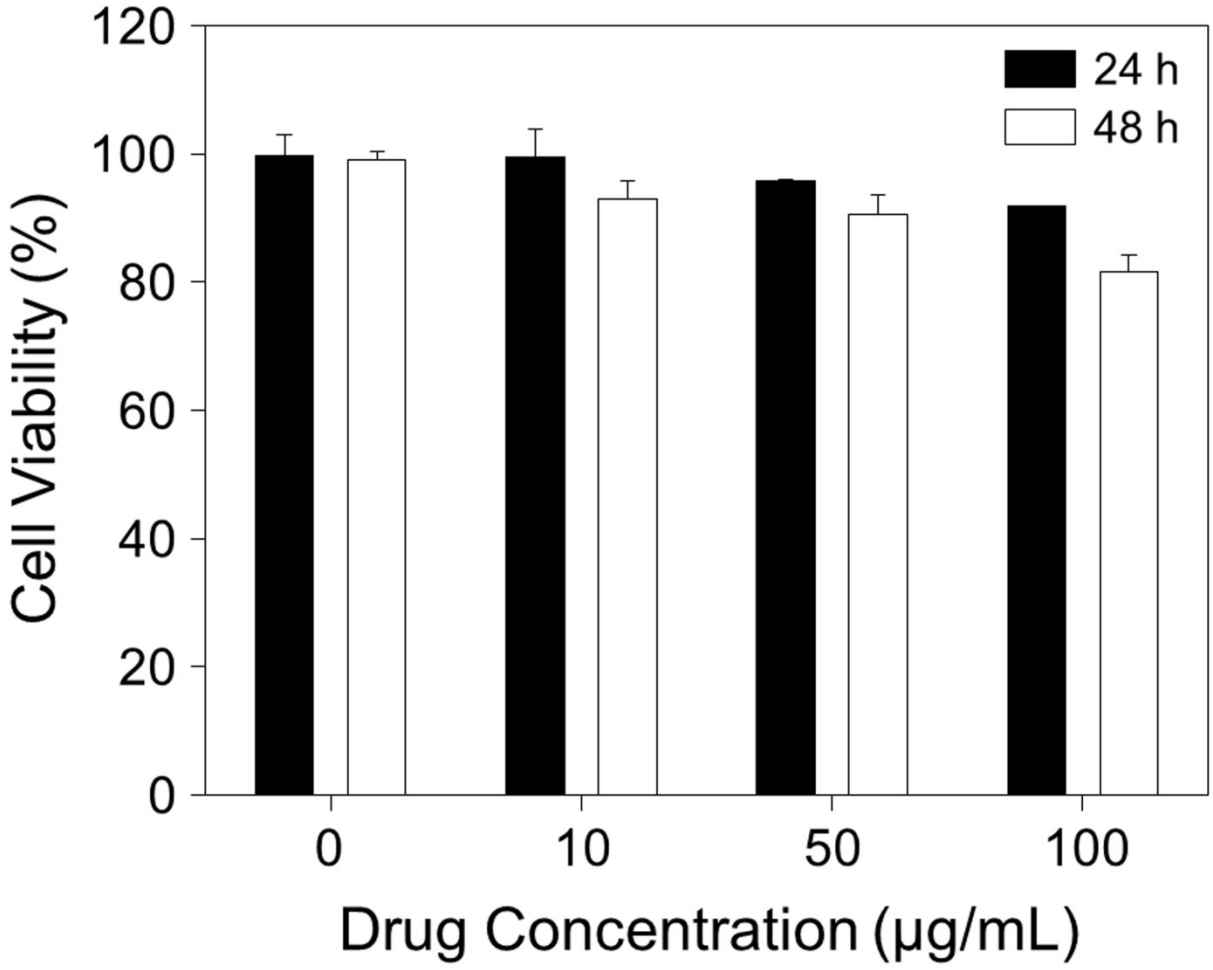

Fig. S3 *In vitro* cytotoxicities of blank HA-functionalized NPs against 4T1 cells after co-incubation for 24 and 48 h, respectively. Triton X-100 (0.5%, w/v) was used as a positive control to produce a maximum cell death rate (100%); cell culture medium was used as a negative control (death rate defined as 0%). Cytotoxicity is given as the percentage of viable cells remaining after treatment. Data are expressed as means ± S.E.M. (n = 5).

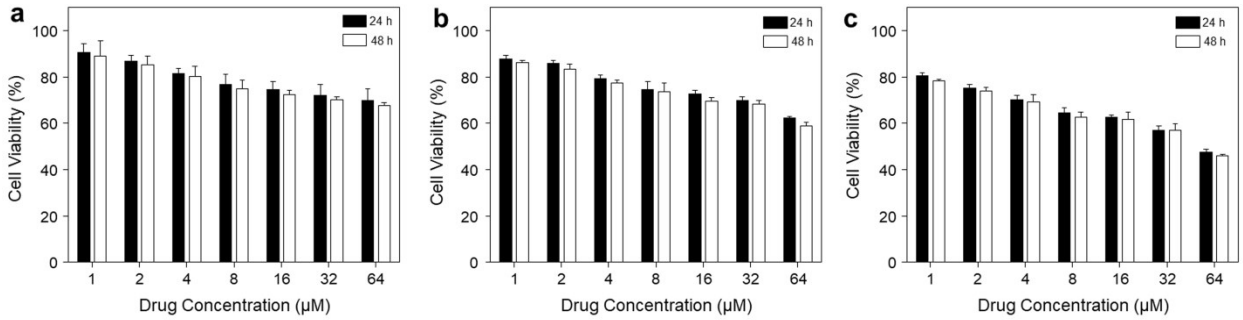

Fig. S4 *In vitro* cytotoxicities of (a) HA-CUR-NPs, (b) HA-5-FU-NPs, and (c) HA-CUR/5-FU-NPs (v) against HUVECs after co-incubation for 24 and 48 h, respectively. Triton X-100 (0.5%, w/v) was used as a positive control to produce a maximum cell death rate (100%); cell culture medium was used as a negative control (death rate defined as 0%). Cytotoxicity is given as the percentage of viable cells remaining after treatment. Data are expressed as means ± S.E.M. (n = 5).

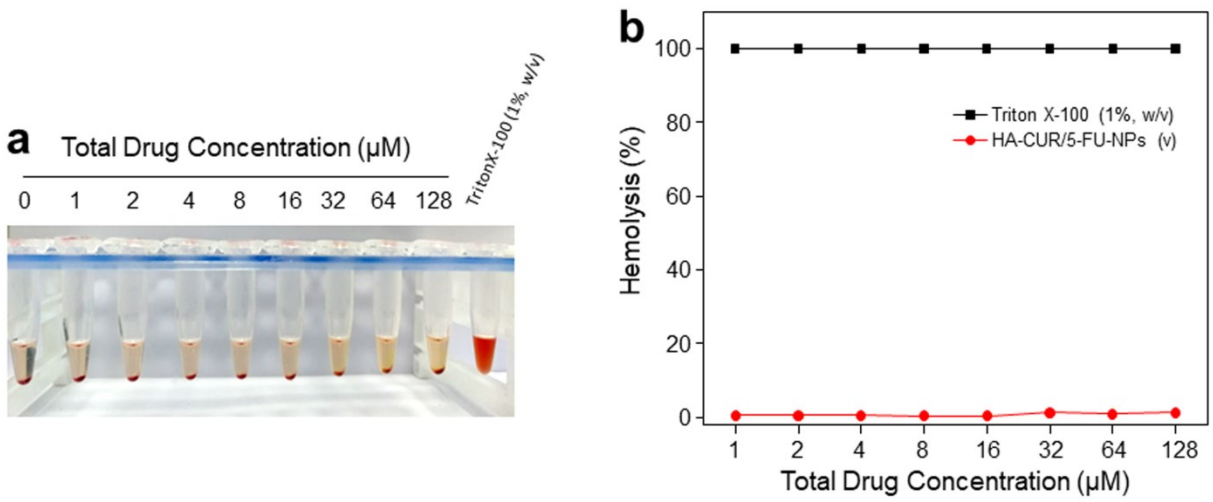

Fig. S5 *In vitro* hemocompatibility of NPs. (a) Photograph and (b) hemolytic analysis of erythrocyte suspensions with different treatments at various total drug concentrations. Triton X-100 (1.0%, w/v) was used as a positive control (100 %), whereas PBS was used as a negative control (0%). Data are expressed as means ± S.E.M. (n = 5).

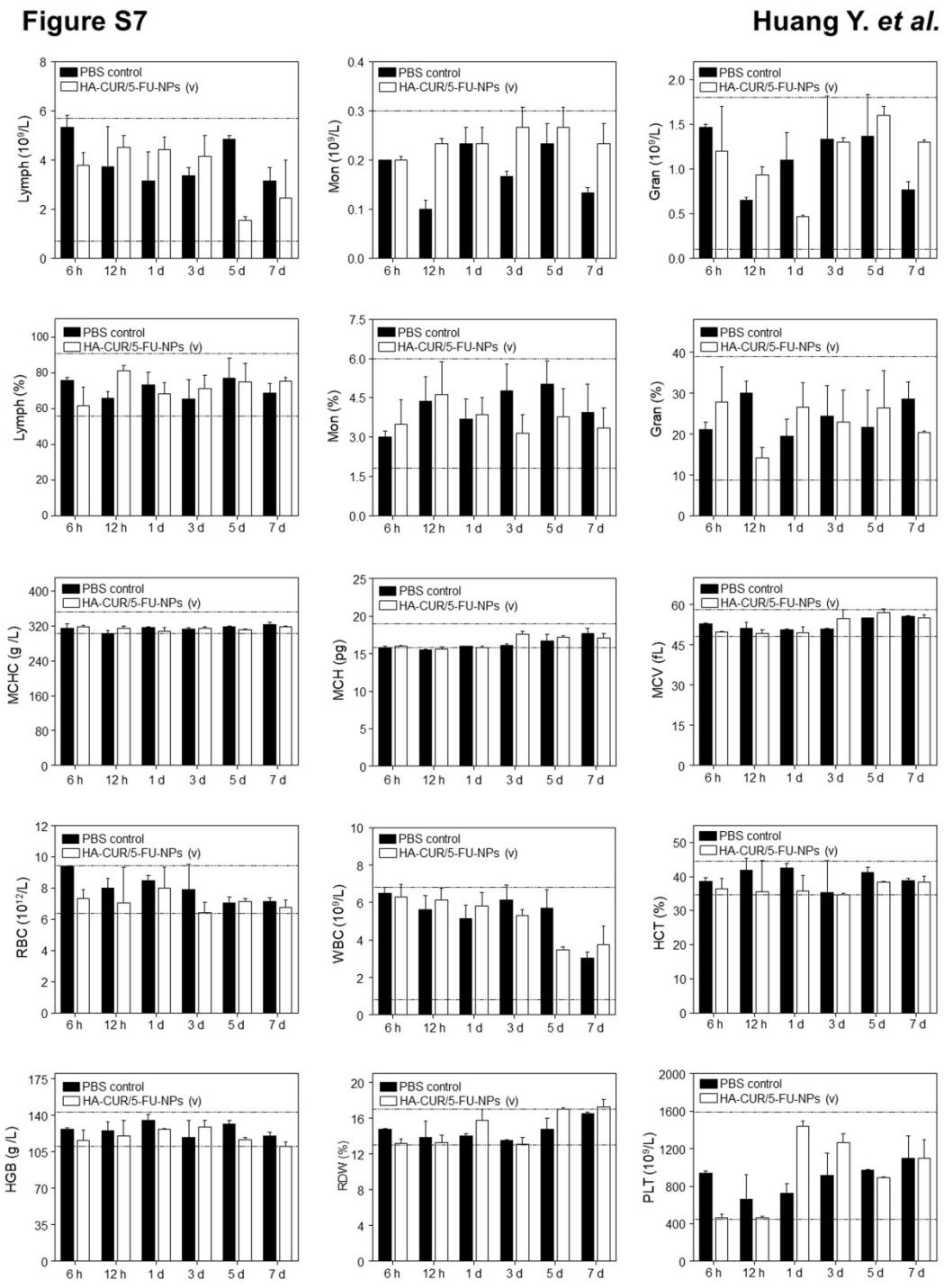

Fig. S6 Complete blood counts of typical parameters, including Lymph, Mon, Gran, MCHC, MCH, MCV, RBC, WBC, and HCT. Data are expressed as mean ± S.E.M. (n = 3).

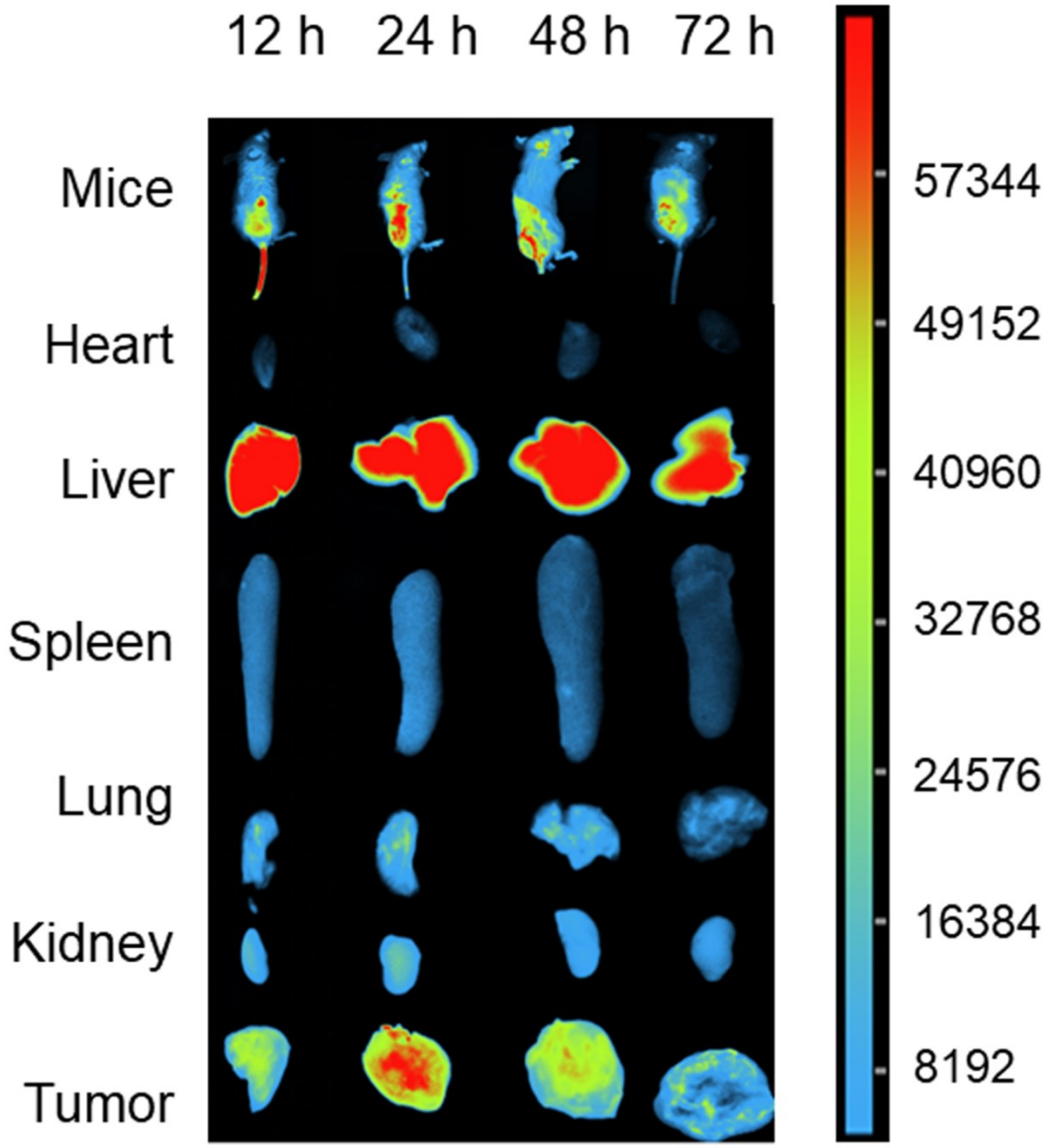

Fig. S7 Fluorescence images of mice bearing 4T1 carcinoma xenografts, five major organs, and tumors after intravenous injection of CUL-Cy7-NPs at different time points (12, 24, 48, and 72 h).

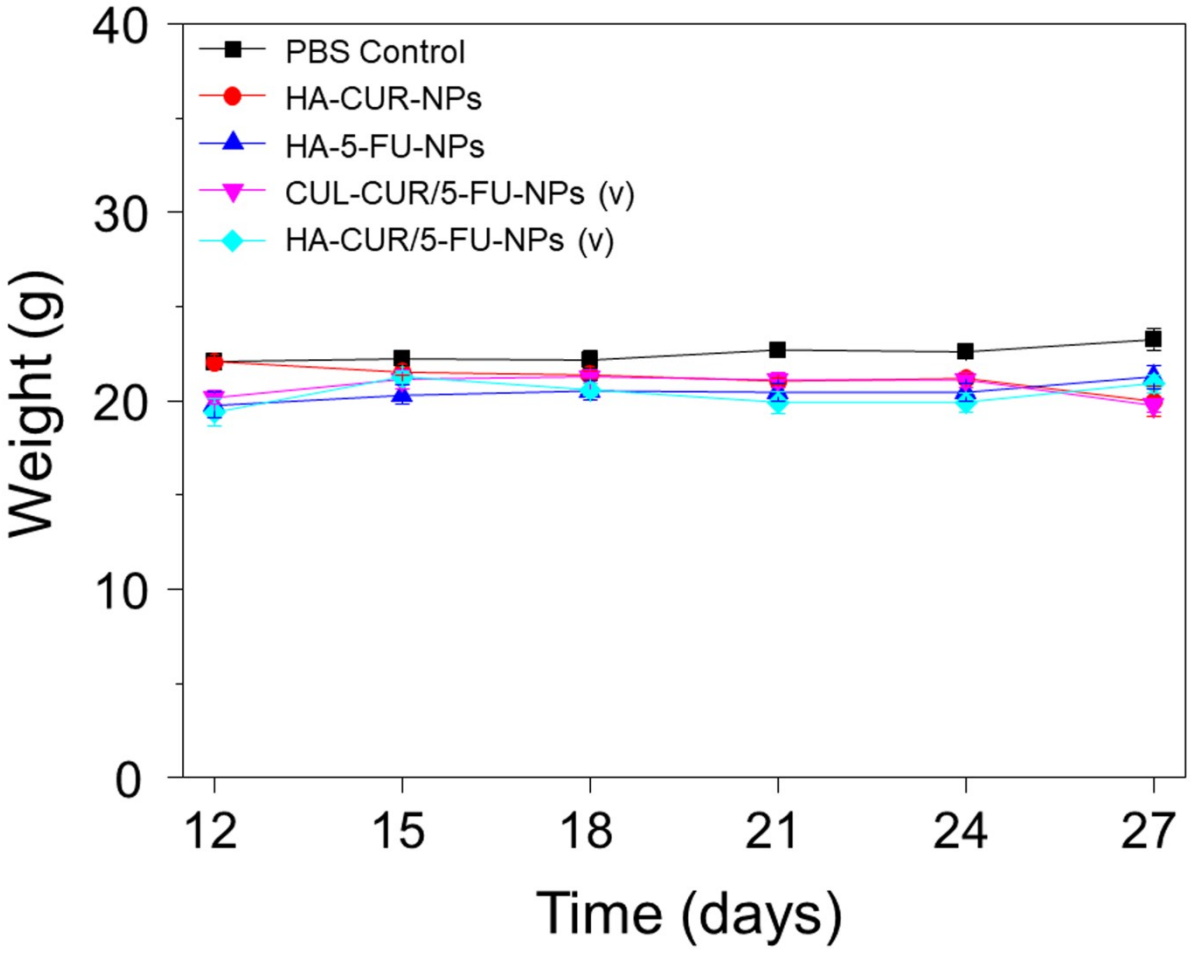

Fig. S8 Variations of body weights of various mouse groups. Data are expressed as means ± S.E.M. (n = 3).

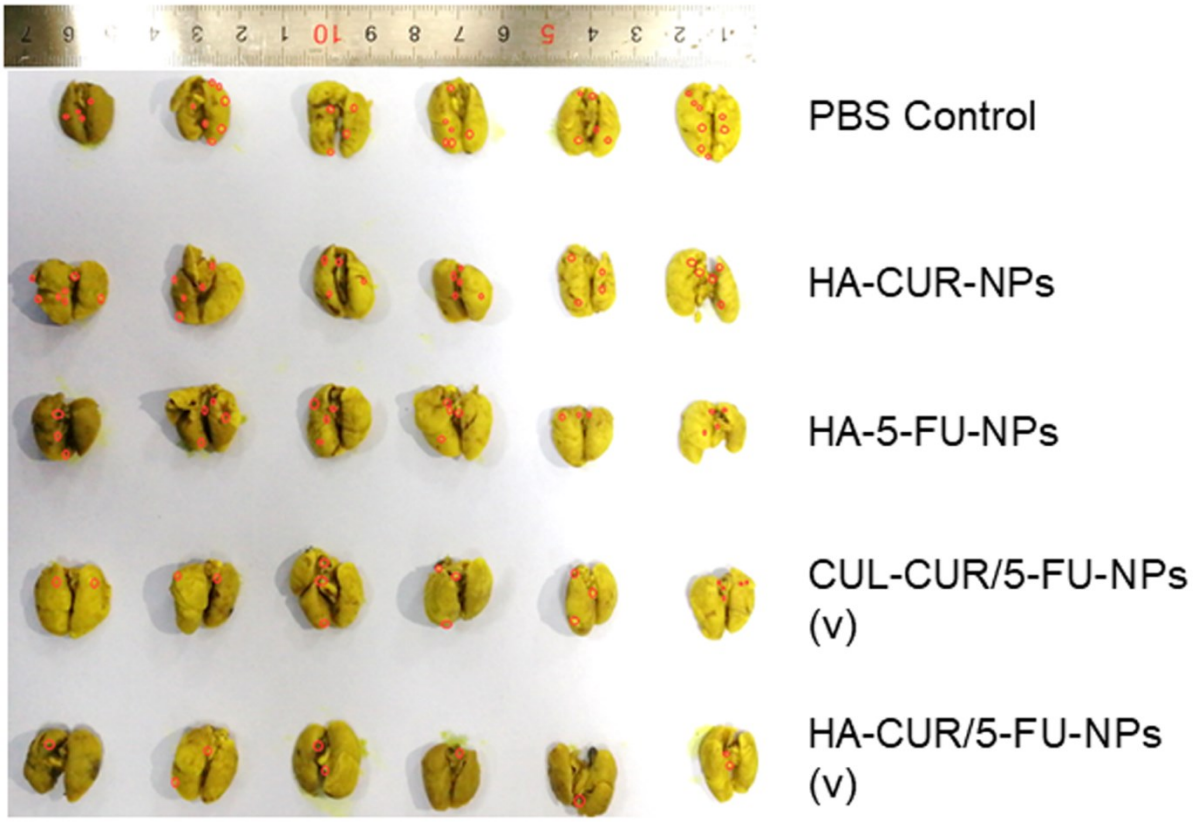

Fig. S9 Photographs of the lungs from various mouse groups.

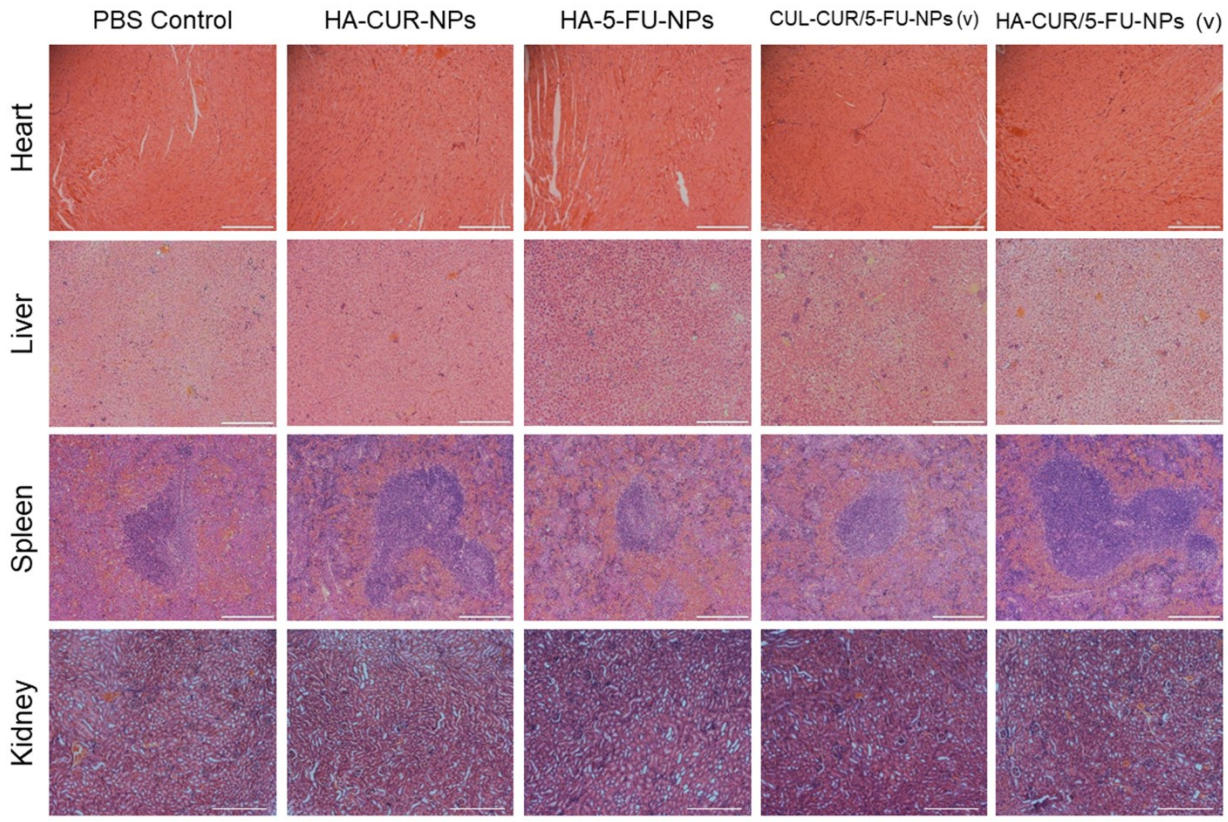

Fig. S10 H&E staining of the four major organs (heart, liver, spleen, and kidney) from various mouse groups. Scale bar is 100 μm.

Table S1

IC_{50} (μM, total drug concentration) of various nanotherapeutics against HUVEC cells.

NPs	24 h	48 h
HA-CUR-NPs	118.8	105.0
HA-5-FU-NPs	109.2	90.5
CUL-CUR/5-FU-NPs (v)	73.5	70.7
HA-CUR/5-FU-NPs (v)	40.0	37.5

1 S. Bjornsson, *Anal. Biochem.,* 1993, **210**, 282.

2 E. Gold, *Biochimica et Biophysica Acta*, 1981, **673**, 408.