Engineering of Yin Yang-like nanocarriers for varisized guest delivery and synergistic eradication of patient-derived hepatocellular carcinoma

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Experimental Section

Materials: Polyacrylic acid (PAA, *Mw* ≈ 1800) was purchased from Sigma (USA). Tetraethyl orthosilicate (TEOS, 28%), isopropyl alcohol (IPA), ethanol (EtOH), ammonia solution ($NH_3 \cdot H_2O$)(25-28%), oleic acid (OA)(90%) and sodium fluoride (NaF) were purchased from Sinopharm Chemical Reagent Beijing Co, Ltd. Yttrium (III) nitrate hexahydrate ($Y(NO_3)_3 \cdot 6H_2O$), ytterbium (III) nitrate hexahydrate ($Yb(NO_3)_3 \cdot 6H_2O$), erbium nitrate hexahydrate ($Er(NO_3)_3 \cdot 6H_2O$), doxorubicin hydrochloride (DOX) and 10-hydroxycamptothecin (HCPT) were obtained from Energy Chemical (Shanghai, China). Deionized (DI) water was used in all experiments.

Characterization: High-resolution transmission electron microscope (HR-TEM) characterizations were recorded by a JEM-2100 TEM under 200 kV accelerating voltage. Fourier transform infrared (FTIR) spectra were performed with a Magna 560 FTIR spectrometer (Nicolet, USA). UV-Vis absorption spectra were monitored by a U-3010 spectrophotometer (Hitachi, Japan). N_2 adsorption-desorption measurements were performed by an intelligent gravimetric analyzer Autosorb-iQ (Quantachrome). X-ray diffraction patterns (XRD) were obtained on a D8 Focus diffractometer (Bruker) with CuKα radiation (λ = 0.15405 nm). Scanning electron microscopy (SEM) images and the energy dispersive X-ray (EDX) spectrum were performed with an XL30 ESEM-FEG field-emission scanning electron microscope (FEI Co.). Confocal laser scanning microscopy (CLSM) was operated on an Olympus Fluoview FV1000. The upconversion luminescence microscopy (UCLM) instrument equipped with an inverted fluorescence microscope (Nikon Ti-S) and an external CW 980 nm laser diode was available for illuminating upon the samples.

Synthesis of PAA NPs: Firstly, PAA solution (100 μL, 0.2 g mL^{-1}) and $NH_3 \cdot H_2O$ (100 μL, 2 mol L^{-1}) were put into 10 mL DI water under ultrasonic sound for 15 min, afterwards, 40 mL IPA was dropwise added into the flask under continuously stirring.

Synthesis of $RE(OH)_3$/PAA NPs: In a 500 mL flask, $Y(NO_3)_3 \cdot 6H_2O$, $Yb(NO_3)_3 \cdot 6H_2O$, $Er(NO_3)_3 \cdot 6H_2O$ (Y:Yb:Er = 78:20:2) were added into 500 mL PAA NPs with magnetic stirring for 3 h. $RE(OH)_3$/PAA NPs were collected by centrifugation at a speed of 8000 rpm for 8 min and washed three times with DI water.

Synthesis of $RE(OH)_3$/PAA@SiO_2 NPs: $RE(OH)_3$/PAA NPs (0.1 g) were dispersed in deionized water (40 mL). Then, 200 mL of IPA was added into the mixture by ultrasonication to form a solution. Then we adjusted the pH value of 240 mL of as-obtained $RE(OH)_3$/PAA NPs solution to ≈ 8 with $NH_3 \cdot H_2O$ solution (2 mol L^{-1}). After that, the addition of 200 μL of TEOS was conducted four times for every 30 min intervals under rigorous stirring for 20 h, the resulting $RE(OH)_3$/PAA@SiO_2 NPs were collected by centrifugation and washed with IPA and DI water repeatedly to eliminate the excess precursors.

Synthesis of Yin Yang-like oleic acid-$NaYF_4$@hollow porous SiO_2 (Yin Yang-like OA-UCNPs@HPS NPs): The obtained $RE(OH)_3$/PAA@SiO_2 NPs (0.025 g) and 9 mL solvent (H_2O:EtOH:OA = 2:3:1) were brought to a 25 mL flask under magnetic stirring for 10 min. Afterwards, 0.013 g NaF was added to the above mixed suspension under magnetic stirring for 15 min. At last, the suspension was put into autoclave at 180 °C for 24 h, finally the OA-UCNPs@HPS NPs were obtained. The Yin Yang-like OA-UCNPs@HPS NPs were gathered by centrifugation and washed with EtOH and DI water several times.

Drug loading and release: To evaluate the drug loading capacity of Yin Yang-like OA-UCNPs@HPS NPs, the NPs after loading drugs were collected by centrifugation and washed with deionized water three times to remove the redundant drugs. The concentrations of HCPT and DOX in the collected supernatants were analyzed by UV-Vis absorption measurements. The drugs loaded in Yin Yang-like OA-

UCNPs@HPS NPs were calculated by the difference between the amount of the added drugs and the residual drugs in the supernatants. The drug loading efficiency (LE) was calculated according to Equation 1.

$$\mathrm{LE}(\%) = \frac{\text{weight of loaded drugs}}{\text{original weight of drugs}} \times 100\% \qquad (1)$$

The drug release experiment of the drug-loaded nanocarriers was carried out with a dynamic dialysis method. The UV-Vis absorption spectra were taken from the supernatant to measure the amount of HCPT and DOX released at certain time intervals. 3 mg drug-loaded nanocarrier was dispersed in 3 mL of PBS solutions (pH 7.4, pH 6.5 or pH 5.3) and sealed in a dialysis bag (MWCO 4000 Da), and then immersing the dialysis bag in 30 mL PBS solutions containing 5% dimethyl sulfoxide (DMSO) with pH 7.4, 6.5 or 5.3. At defined time periods, 200 μL of release media were collected to detect the content of drugs. All of the drug-releasing experiments were performed at 37 °C. The release of HCPT and DOX from NPs were measured by UV-Vis spectrum, and the release efficiency (RE) is calculated based on Equation 2.

$$\mathrm{RE}(\%) = \frac{\text{weight of released drugs}}{\text{weight of loaded drugs}} \times 100\% \qquad (2)$$

In vitro cytotoxicity assay: In vitro cytotoxicity of Yin Yang-like OA-UCNPs@HPS NPs was evaluated by MTT (3-(4, 5-cimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assays against human hepatocellular carcinoma (HepG2) cells, human pulmonary adenocarcinoma (A549) cells and human breast cancer (MCF-7) cells. Briefly, the cells were seeded in a 96-well plate in which cells density was 5000 cells per test well, and cultured overnight at 37 °C in a 5% CO_2 incubator. The Yin Yang-like OA-UCNPs@HPS NPs were added to respective test well and the cells were cultured for 24 h in 5% CO_2. The concentrations of the NPs were 6.25, 12.5, 25, 50, 100 and 200 μg mL^{-1}, respectively. One row of the 96-well plate was used as a blank control with culture medium only. After incubation for 24

and 48 h, the MTT assays were performed to quantitatively measure the relative cell viabilities. Cell viability was determined by Equation 3.

$$\text{Cell viability}(\%) = \frac{A_{(\text{test cells})}}{A_{(\text{control cells})}} \times 100\% \qquad (3)$$

Double-staining method to evaluate multi-drug chemotherapy in cell levels: Calcein acetoxymethyl ester (Calcein AM) stained the live cells with green emission where propidium iodide (PI) dye stained the dead cells with red emission. The samples (blank NPs, HCPT-loaded NPs, DOX-loaded NPs, cocktail and HCPT/DOX-loaded NPs) at a concentration of 5 μg mL^{-1} were incubated with cells. At the same time, a blank control group absent NPs was conducted as comparison, separately.

UC luminescent imaging and cellular uptake of the Yin Yang-like OA-UCNPs@HPS NPs: Cellular uptake was examined using UCLM. For UCLM, HepG2 cells were seeded in 48-well culture plates and were cultured overnight. After incubated with Yin Yang-like OA-UCNPs@HPS NPs at 37 °C for 2 h in the dark, the cells were washed with PBS several times for removing the residual NPs, and then fixed with 2.5% formaldehyde at 37 °C for 10 min before washing with PBS again. Hoechst 33342 solution was used for nucleus labeling of fixed cells for 10 min, and then the cells were washed with PBS several times. Lastly, the cells were visualized under UCLM after the cover slips were placed on a glass microscope slide. The samples were excited with 405 nm for nucleus and 980 nm for Yin Yang-like OA-UCNPs@HPS NPs. Upon verifying the excellent drug release efficiency in the present system, the time-dependent uptake and the intracellular release behaviours of the HCPT/DOX-loaded NPs were investigated by CLSM in HepG2 cells.

Combination index (CI): For combination drug therapy, it is expected that the net effect of two drugs is synergistic, or greater than the sum of effects of each drug alone.

One of the simplest formalisms to demonstrate synergistic interaction is CI, defined for the pair of drugs A and B as

$$CI = \frac{IC_X(A)_{pair}}{IC_X(A)} + \frac{IC_X(B)_{pair}}{IC_X(B)} \quad (4)$$

CI values are plotted against the drug effect level, where IC_{50} is the drug concentration that inhibits a cellular function or behavior (e.g., cell growth) by 50%, for each individual drug or for the drug given as an HCPT-DOX pair. Thus, if HCPT and DOX act synergistically, smaller doses of both HCPT and DOX are required to lead to the same cellular effect, so IC_{50} (HCPT or DOX)$_{pair}$ < IC_{50} (HCPT or DOX) and CI < 1. For drugs that act additively (independently), CI is near 1, and for those that act antagonistically, CI > 1.

Hemolysis assay of Yin Yang-like OA-UCNPs@HPS NPs: Hemolysis test was executed using human blood to evaluate the hematotoxicity of NPs *in vitro*. Red blood cells were obtained after washing with physiological saline, and centrifuged five times. After that, blood cells were diluted to 1:10 with PBS solution. Then, 0.3 mL of diluted cells suspensions was mixed with 1.2 mL of PBS (as a negative control), 1.2 mL of water (as a positive control), and 1.2 mL of product suspensions with varying concentrations of 15.63, 31.25, 62.5, 125, 250, 500 and 1000 μg mL^{-1}. The nine samples were shaken and kept steady for 2 h. Finally, the absorbance of the upper supernatants was measured by UV-Vis spectroscopy after the centrifugation of the the mixtures at 2500 rpm for 3 min. The percentage hemolysis of the red blood cells was calculated by the following Equation 5.

$$\text{Hemolysis}\,(\%) = \frac{A_{sample} - A_{control(-)}}{A_{sample(+)} - A_{control(-)}} \quad (5)$$

Immunofluorescence staining: For immunostaining of the established HepG2 cell lines for cleaved caspase 3 expression, the cells were fixed with 4% paraformaldehyde in 6-wells, blocked with 3% normal goat serum, and treated with

the cleaved caspase 3 (1:100; Cell Signaling, 9664). The cells were treated with NPs, HCPT-loaded NPs, DOX-loaded NPs, cocktail and HCPT/DOX-loaded NPs for 48 h. The nuclear staining was through the use of the mountant, ProLong Gold antifade reagent with DAPI (Life Technologies). The images were acquired using the Zeiss Axioplan 2 epifluorescence imaging system equipped with the Micro Manager software.

Cell apoptosis and cell cycle assay: HepG2 cells were seeded in 12-well plates (2.0×10^5 cells per well) in 2 mL of complete DMEM and cultured for 24 h. The cells were treated with NPs, HCPT-loaded NPs, DOX-loaded NPs, cocktail and HCPT/DOX-loaded NPs at the same concentration for 48 h. The treated cells were harvested and washed twice with ice-cold PBS, stained with Alexa Fluor® 488 annexin V and PI on the basis of the manufacturer's instructions for quantitative measurement of apoptosis. For cell cycle, treated cells were collected, washed for twice with ice-cold PBS, fixed with 70% ethanol at 4 °C overnight, then PI stained for 30 min in the dark. Both cell apoptosis and cycle were tested by flow cytometry (BD FACS Calibur, USA), and 1.0×10^5 events per sample were counted.

MRI/CT imaging in vitro and vivo： Firstly, different concentrations (0, 0.25, 0.5, 1.0, 2.0 and 4.0 mM) of Yb^{3+} in PBS were prepared to measure the ability of the MR imaging. To assess CT imaging efficacy, the Yin Yang-like OA-UCNPs@HPS NPs were dispersed in PBS with different Yb^{3+} concentrations over the range from 0 to 200 mM. T_2-weighted MR images of tumor-bearing mice were detected after administration of Yin Yang-like OA-UCNPs@HPS NPs with a dose of 200 μL (30 mg Yb^{3+} kg^{-1}) via intravenous injection. The Yin Yang-like OA-UCNPs@HPS NPs at a total dose of 200 μL (200 mg Yb^{3+} kg^{-1}) were injected into mice via intravenous injection to evaluate CT imaging. CT images were acquired by a Philips 256-slice CT Scanner (Philips Medical System). The MRI experiment was performed on a 3.0 T MRI instrument.

Cell line-derived xenograft (CDX) and patient-derived xenograft (PDX) models: CDX HCC model was established by subcutaneous injection of cancer cell lines (HepG2) at the flank of server combined immune-deficiency (SCID) mice. Apart from CDX model, we here established novel PDX mice models with high fidelity bearing different HCC xenografts (#199715-PDX and #200696-PDX) obtained by direct implantation of two patients resection HCC tissue fragments in immunocompromised nude mice. The study was approved by the Institutional Ethics Committee of the Eastern Hepatobiliary Surgery Hospital. Written informed consent was obtained from two patients for their HCC tissue to be used for research purposes. Fresh HCC samples were used for the establishment of our PDX HCC model in 5~6 week SCID mice, tumor passage was used in nude mice. The anticancer efficacy of drug-loaded NPs on the above CDX and PDX models was further evaluated.

Combination therapy *in vivo*: When the tumor size reached about 100 mm^3 (*Cell line-derived xenograft (CDX)* model) and approximately 3×3×3 mm^3 (*patient-derived xenograft (PDX)* models), the tumor-bearing mice were randomly divided into 9 groups (4 mice/group): PBS, NPs, HCPT, DOX, HCPT+DOX, HCPT-loaded NPs (in terms of 1.8 mg kg^{-1} HCPT), DOX-loaded NPs (in terms of 2.68 mg kg^{-1} DOX), cocktail, HCPT/DOX-loaded NPs. The day of the first intravenous injection counted as day 0 and the samples were injected through the tail vein every 3 days for CDX and 4 days for PDX models. Meanwhile, the body weight and tumor volume of each group of mice were measured at 3 days intervals for 15 days (CDX model). The body weight and tumor volume of each group of mice were monitored at 4 days intervals for 20 days (PDX models). The volume for each type of tumor was respectively calculated by the following Equation 6.

$$\text{Volume} = \left(\frac{\text{length} \times \text{width}^2}{2}\right) \qquad (6)$$

After that, the tumor-bearinng mice were sacrificed for the collection of the tumors. The full tumor from each mouse was removed and weighed. Tumor growth inhibition rate was determined using the following Equation 7.

$$\text{Inhibition (\%)} = \left(\frac{\text{C - T}}{\text{C}}\right) \times 100\% \qquad (7)$$

where C is the average tumor weight of the control group and T is the average tumor weight of each treated group.

Western blot assay: To detect the protein expression levels of GADPH, cleaved caspase 3, and caspase 3, western blot assay was used. Total cell lines protein and HCC tumor protein was extracted using RIPA Lysis Buffer and PMSF (Thermo Scientifc) in accordance with the manufacturers' instructions, after centrifuged at 13,000 g for 15 min, extracted the supernatant for further study. Western blots were carried out using specific cleaved caspase 3 cleaved caspase 3 (1:100; Cell Signaling, 9664), and caspase 3 antibody 1:100; Cell Signaling, 9662 and the GAPDH specific antibody (KC-5G4; Kangcheng Shanghai, China). Secondary antibodies and CA were purchased from Cell Signaling. The images were captured using the Gel Dox XR system (Bio-Rad, Philadelphia, PA).

Immunohistochemical assay and blood analysis: Paraffin-embedded tumor tissues from immunohistochemistry were performed on the tumor tissues from mice, which had antibodies against cleaved caspase 3 (1:100; Cell Signaling, 9664) and ki-67 (1:100; Cell Signaling, 11882). The tissue sections were also stained with hematoxylin and eosin (H&E). Finally, an optical microscope was used to detect the histological sections. The total area of positive staining was quantified by Image-Pro Plus software 3. For the reading of each antibody staining, a uniform setting for all the slides was applied.

In order to evaluate the toxicity and side effects of the NPs, healthy and tumor-free nude mice were used as the subjects. The mice were randomly divided into 3 groups (5 mice per group). The two groups of mice were treated with the NPs (20 mg kg^{-1}) and HCPT/DOX-loaded NPs via the tail vein. Other healthy mice were used as the untreated control. Approximately 0.8 mL of blood from each mouse was collected for

a blood chemistry test and complete blood panel analysis before the mouse was euthanatized.

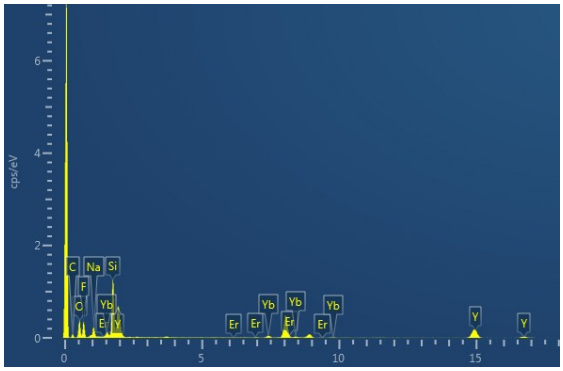

Figure S1. EDX analysis of Yin Yang-like OA-UCNPs@HPS NPs.

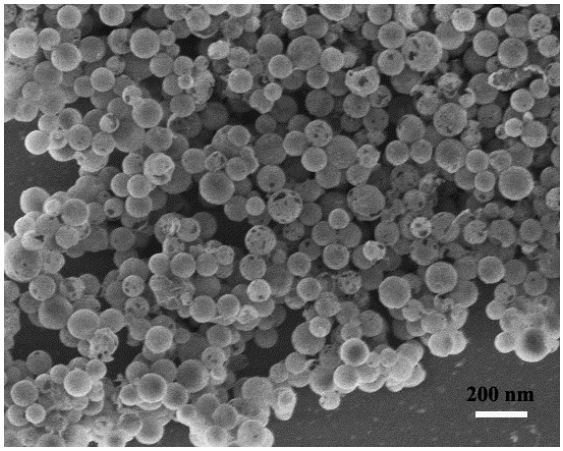

Figure S2. SEM image of Yin Yang-like OA-UCNPs@HPS NPs.

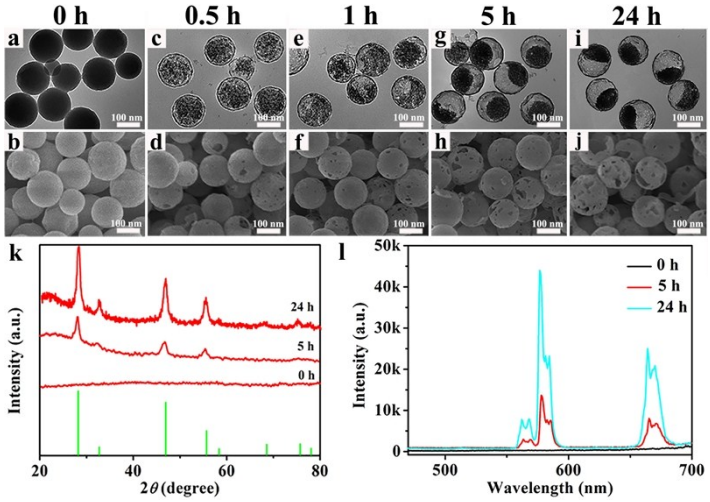

Figure S3. SEM, TEM images a-j) and the corresponding XRD k), UCL spectra l) of the samples at different reaction periods.

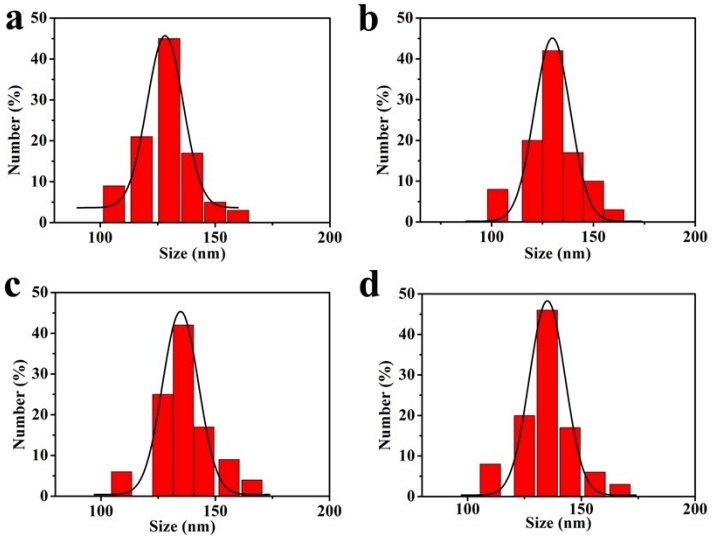

Figure S4. a) PAA NPs with diameter about 130 ± 15 nm, b) $RE(OH)_3$/PAA precursors with diameter about 130 ± 15 nm, c) $RE(OH)_3$/PAA@SiO_2 NPs with diameter about 136 ± 15 nm and d) Yin Yang-like OA-UCNPs@HPS NPs with diameter about 136 ± 15 nm were measured by dynamic light scattering.

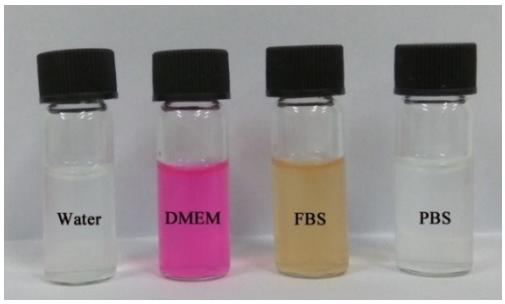

Figure S5. Photographs of the NPs in different solutions after 24 h. No precipitation of the NPs was observed in water, culture medium (DMEM), fetal bovine serum (FBS) or phosphate buffer saline (PBS).

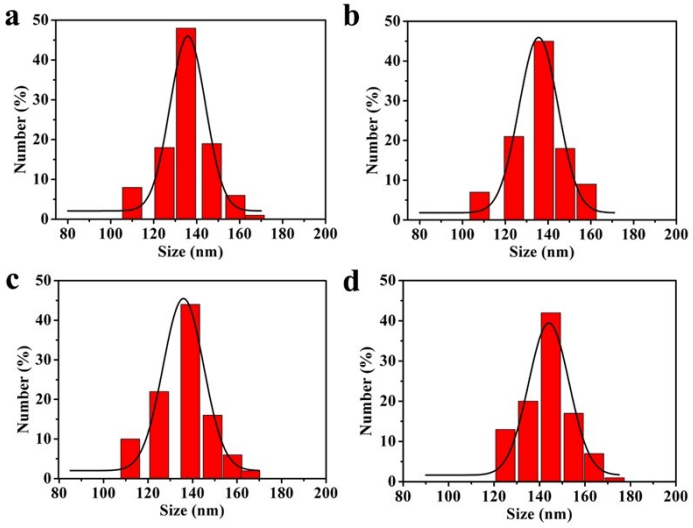

Figure S6. The hydrodynamic diameters of Yin Yang-like OA-UCNPs@HPS NPs dispersed in water (a), PBS (pH = 7.4) (b), DMEM (c) and FBS (d).

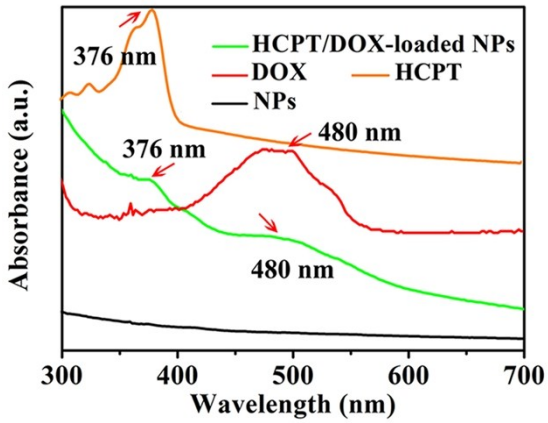

Figure S7. UV-Vis absorption curves of the Yin Yang-like NPs, HCPT, DOX, HCPT/DOX-loaded Yin Yang-like NPs.

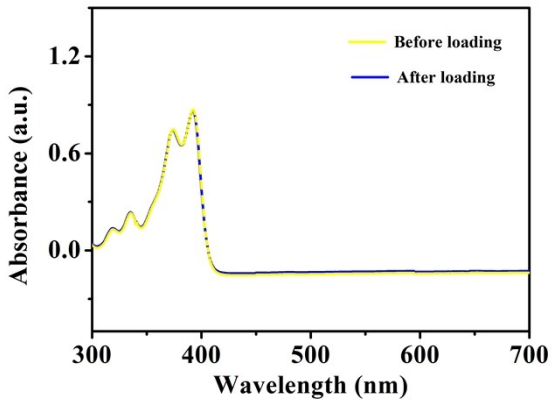

Figure S8. UV-Vis spectra of HCPT before and after loading by HPS.

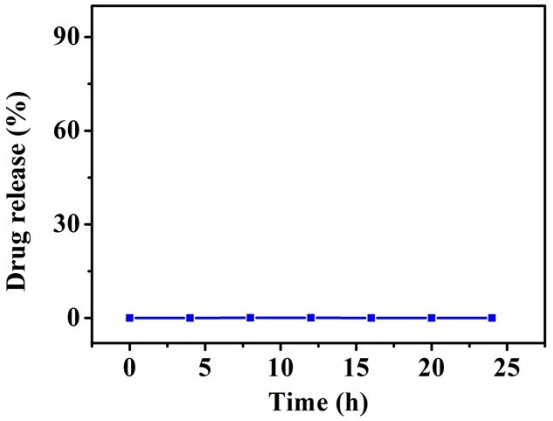

Figure S9. Release profile of HCPT from HPS in PBS (pH = 5.3) at different time points.

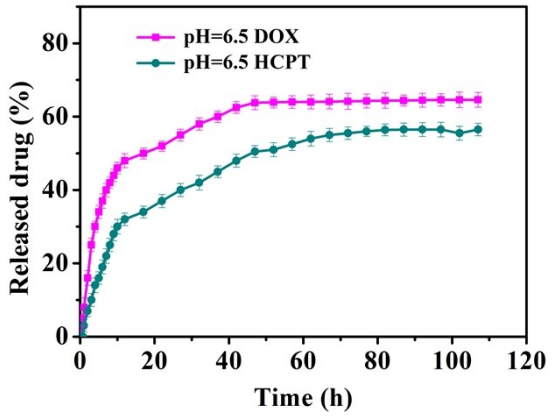

Figure S10. *In vitro* drug release curves of DOX and HCPT from drug-loaded Yin Yang-like NPs at pH 6.5.

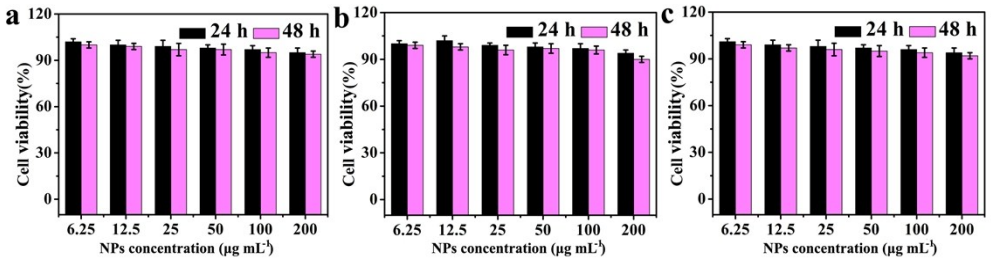

Figure S11. Cell viabilities of a) A549 cells, b) HepG2 cells and c) MCF-7 cells after treatment with Yin Yang-like NPs for 24 and 48 h.

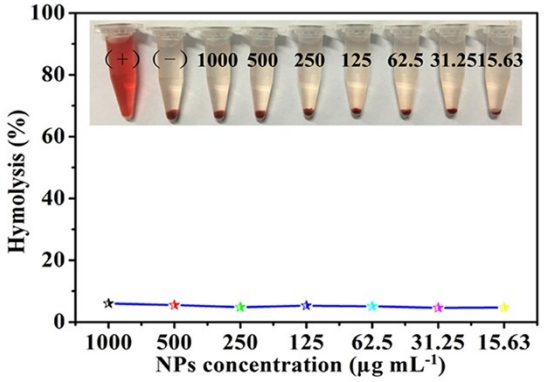

Figure S12. Hemolytic percent of red blood cells incubated with Yin Yang-like NPs at various concentrations for 2 h. Inset describes photo of Yin Yang-like NPs mixed with red blood cells after centrifugation. +: positive control. -: negative control.

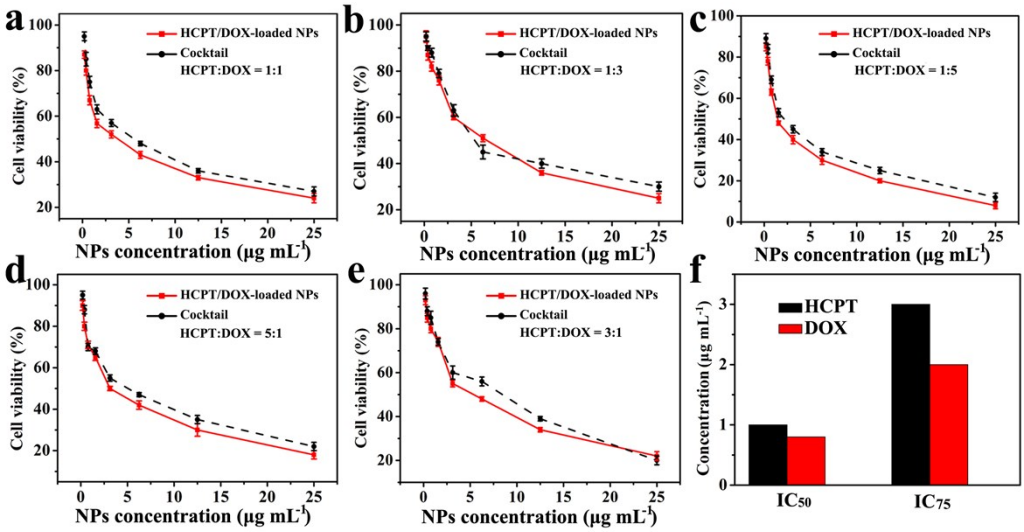

Figure S13. a-e) HCPT/DOX-loaded Yin Yang-like NPs and cocktail system exposed to cells for 48 h at indicated molar ratios. f) The IC_{50} and IC_{75} values of HCPT and DOX treated with HepG2 cells for 48 h.

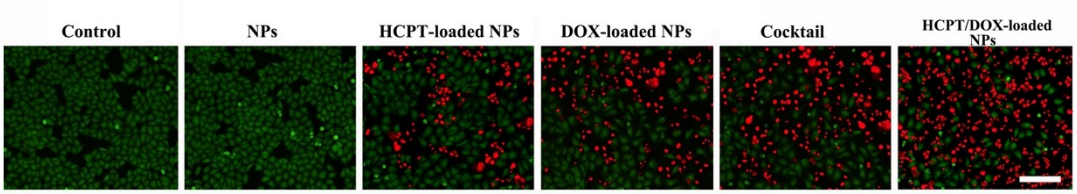

Figure S14. Fluorescence images of HepG2 cells with different treatments through staining with calcein AM/PI (scale bar: 200 μm).

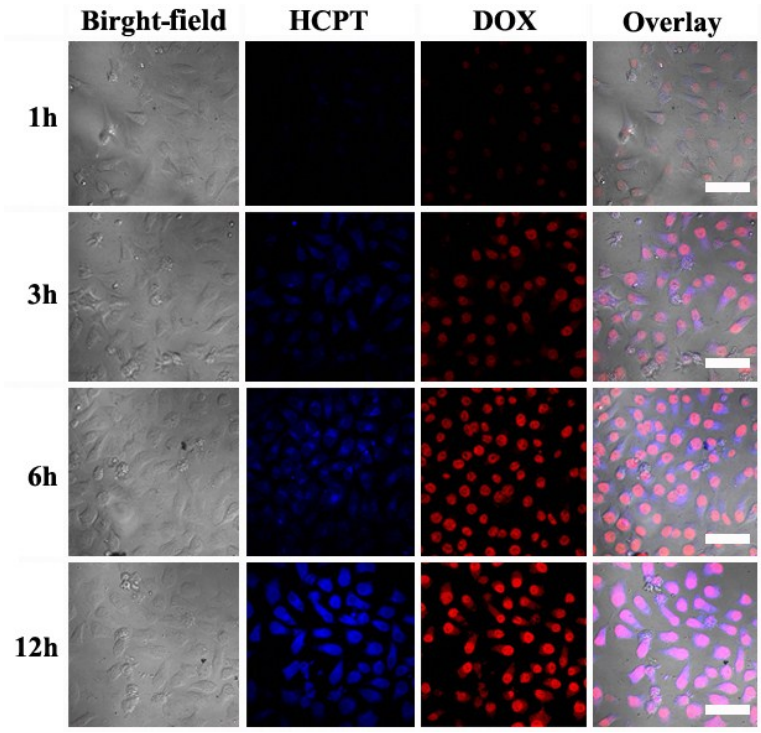

Figure S15. CLSM images of HepG2 cells treated with HCPT/DOX-loaded Yin Yang-like NPs. DOX is shown in red and the HCPT is shown in blue. The scale bars represent 50 μm.

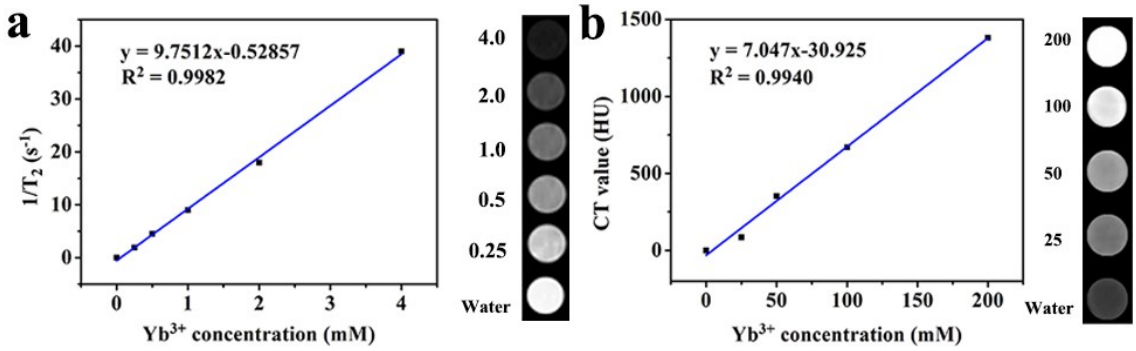

Figure S16. a) Relaxation rate ($1/T_2$, r_2) and *in vitro* T_2-weighted MR images of the Yin Yang-like NPs. b) CT values (HU) and *in vitro* CT images of the Yin Yang-like NPs.

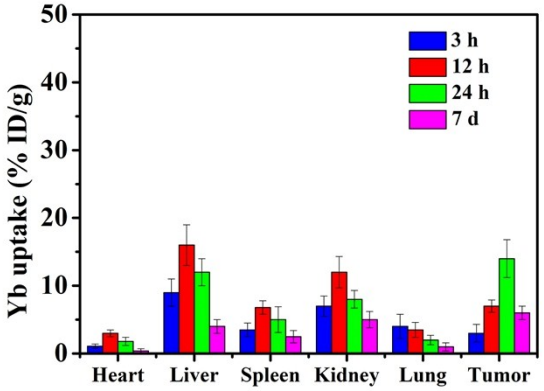

Figure S17. Biodistribution of Yb in HCC tumor-bearing mice.

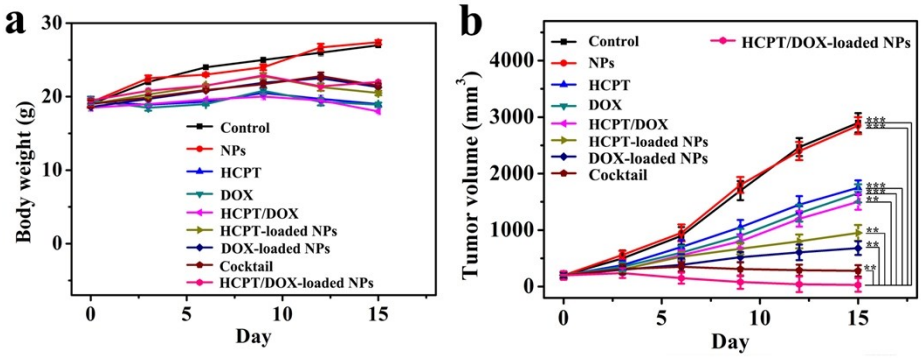

Figure S18. The body weight (a) and tumor volume (b) changes of mice in the various groups during the 15 day therapeutic period.

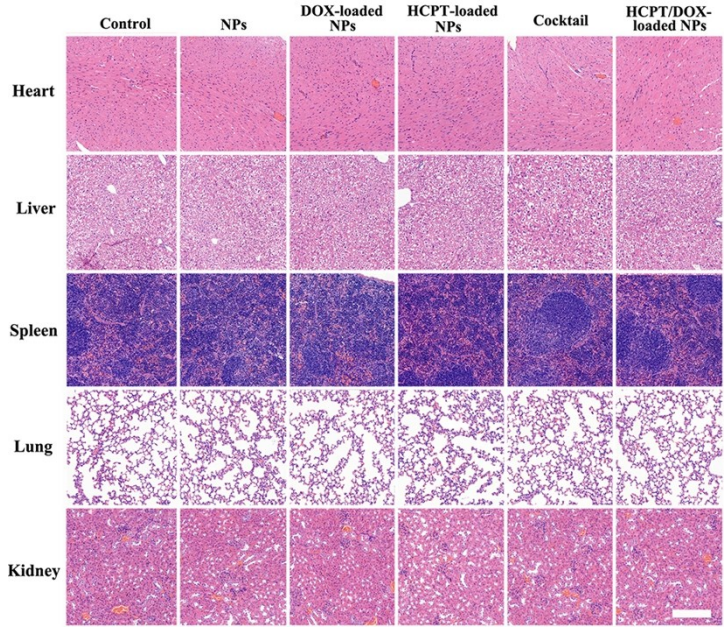

Figure S19. Histological section of major organ tissues stained with H&E after various treatments. Scale bar: 200 μm.

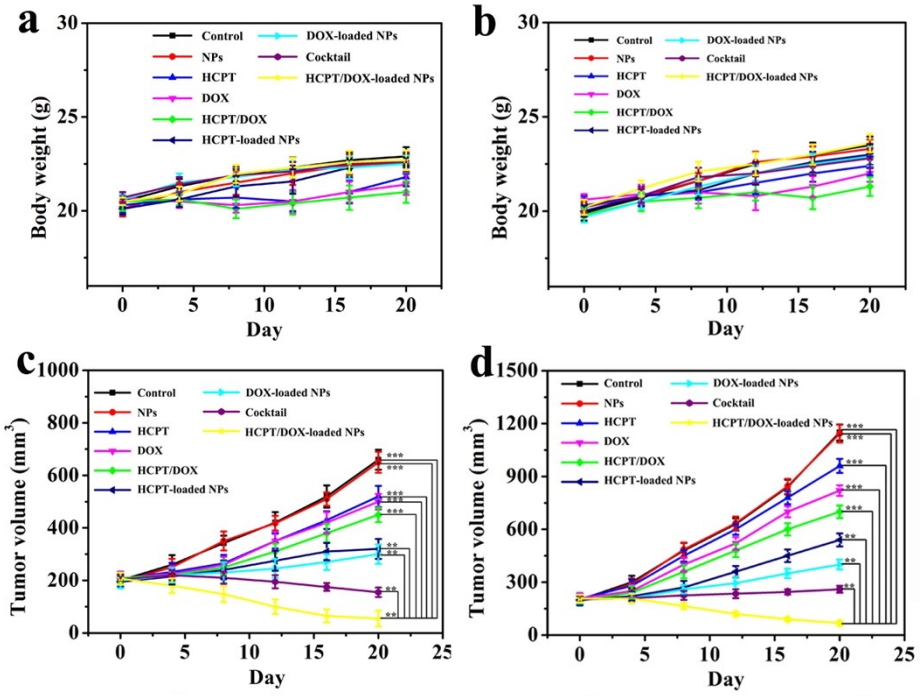

Figure S20. Body weight and tumor growth curves of the #208797-PDX (a, c) and #209390-PDX (b, d).

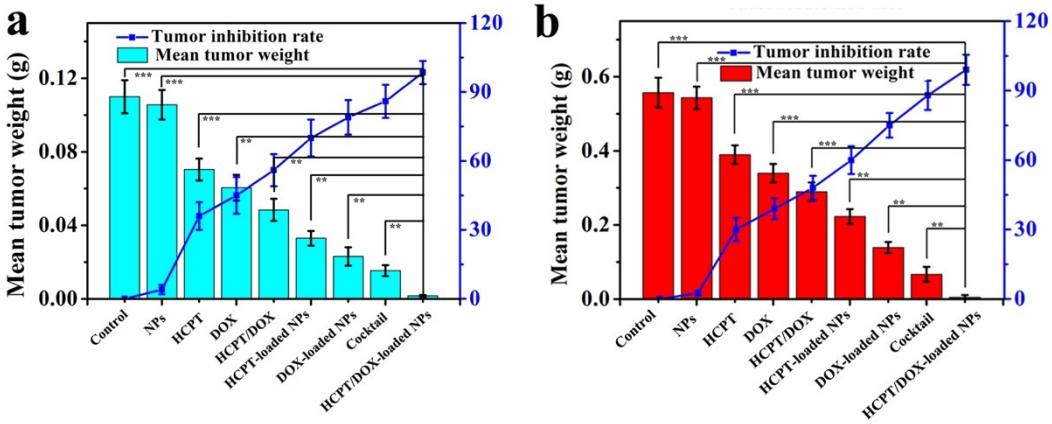

Figure S21. Mean tumor weight and tumor inhibition rate of each group of the #208797-PDX (a) and #209390-PDX (b).

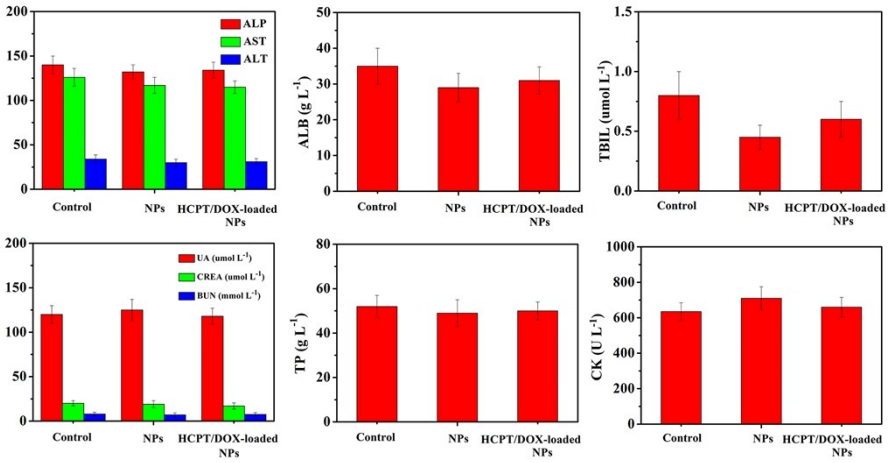

Figure S22. Blood test parameters for mice treated with saline, NPs and HCPT/DOX-loaded Yin Yang-like NPs.