Supporting Information

Amphiphilic Janus nanoparticles for imaging-guided synergistic chemo-photothermal hepatocellular carcinoma therapy in the second near-infrared window

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Experimental Section

Materials

Polyacrylic acid (PAA), dopamine hydrochloride (DA, 98%) and hydrogen tetrachloroaurate ($HAuCl_4 \cdot H_2O$) were purchased from Sigma (USA). Isopropyl alcohol (IPA), ethanol (EtOH), ammonia solution ($NH_3 \bullet H_2O$)(25~28%), oleic acid (OA)(90%) and sodium fluoride (NaF) were purchased from Sinopharm Chemical Reagent Beijing Co. Ltd. Yttrium (III) nitrate hexahydrate ($Y(NO_3)_3 \bullet 6H_2O$), ytterbium (III) nitrate hexahydrate ($Yb(NO_3)_3 \bullet 6H_2O$), erbium nitrate hexahydrate ($Er(NO_3)_3 \bullet 6H_2O$), 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-NIR absorption spectra were monitored by a U-3010 spectrophotometer (Hitachi, Japan). 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.

Preparation of PAA nanoparticles (NPs)

Firstly, 80 μL, 0.2 g mL^{-1} PAA solution and 80 μL, 2 mol L^{-1} $NH_3 \cdot H_2O$ were added to 10 mL DI in a round-bottom flask water with ultrasonic sound for 10 min, afterwards, 40 mL IPA was dropwise added into the flask under continuously stirring. When a color change from colorless PAA solution to a milk-white color, the PAA NPs were successful obtained.

Preparation of rare earth hydroxide/poly(acrylic acid) NPs ($RE(OH)_3$/PAA NPs)

In a 200 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 200 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.

Preparation of $RE(OH)_3$/PAA@polydopamine (PDA) asymmetric NPs

$RE(OH)_3$/PAA NPs were dispersed in mixture of DI water: IPA (40: 160 mL). Then we used the 2 mol L^{-1} $NH_3 \cdot H_2O$ solution to adjust the pH value of 200 mL of as-obtained solution to ~ 8.8. Then, DA was added to obtain $RE(OH)_3$/PAA@PDA asymmetric NPs with stirring for 16 h. The obtained $RE(OH)_3$/PAA@PDA asymmetric NPs were centrifuged for several times to dispose of unreacted reagents.

Preparation of oleic acid-$NaYF_4$ (Y:Yb:Er=78:20:2)/polydopamine double-layered nanobowls (OA-UCNPs/PDA double-layered NBs)

The prepared $RE(OH)_3$/PAA@PDA asymmetric NPs were added into 9 mL solvent (H_2O:EtOH:OA = 2:3:1) under magnetic stirring for 10 min. Afterwards, NaF was added to the above mixed suspensions under stirring for more than 15 min. Lastly, the mixture was put into autoclave at 180 °C for 24 h to obtain the oleic acid-$NaYF_4$ (Y:Yb:Er = 78:20:2)/PDA double-layered NBs. The resulted OA-UCNPs/PDA double-layered NBs were washed with EtOH and DI water several times.

Preparation of OA-UCNPs/PDA Au nanoflower Janus NPs (OA-UCNPs/PDA-AuF JNPs)

The prepared NBs were dispersed in 10 mL aqueous solution, firstly, we removed the 100 μL of NBs into the 29 mL aqueous solution under the constantly stirring. Then, 84 μL $HAuCl_4$ (30 mM) was added into the above mixture, with it, 10 μL HCl (1 M) and 170 μL $AgNO_3$ (3 mM) were introduced. Afterwards, the 50 μL LAA was added quickly. After 30 s of stirring reaction, the color of the solution was observed to turn gray blue, and then the OA-UCNPs/PDA-AuF JNPs was collected by centrifugation and washed three times. The centrifugation was performed at a speed of 8000 rpm for 8 min.

Drug loading and release

By adding HCPT (dissolved in DMSO) to the OA-UCNPs/PDA-AuF JNPs with stirring for 24 h to obtain the HCPT-loaded JNPs. Afterwards, HCPT-loaded JNPs were washed with IPA three times to get rid of the free HCPT adsorbed on the surface, the DOX aqueous was introduced into the HCPT-loaded JNPs with stirring for 24 h. Finally, the HCPT/DOX-loaded JNPs were acquired by centrifugation and washed three times with deionized water to remove the DOX adsorbed on the surface. We measured the concentrations of HCPT and DOX in the

collected supernatants by UV-Vis absorption measurements. The drugs loaded into the JNPs 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.

$$\mathrm{LE}\,(\%) = \frac{\mathrm{m}_{(\mathrm{initial})} - \mathrm{m}_{(\mathrm{insupernatant})}}{\mathrm{m}_{(\mathrm{initial})}} \times 100\,\% \qquad (1)$$

The release experiments of the drug-loaded JNPs were initiated by placing the end-sealed dialysis bag into 20 mL buffer solution (5% DMSO) at 37 °C. At given times, 200 μL of external release medium was taken out to detect the content of drugs. The amounts of released DOX and HCPT were tested by UV-Vis spectrophotometry at 480 and 376 nm, respectively, using the standard curve method. To check the laser irradiation could accelerate the drug release, the same amount of drug-loaded JNPs was added in PBS buffer (pH 7.4 or pH 5.3) with 1064 nm near infrared (NIR) laser irradiation (1 W cm^{-2}, 5 min) at the selected time interval. All of the drug release experiments were carried out at 37 °C. The release efficiency (RE) was evaluated based on Equation 2.

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

Photothermal effect

In order to research the photothermal effect of the JNPs, the JNPs with different concentrations (0, 50, 100, 200 and 400 μg mL^{-1}) in centrifuge tubes were exposed to 1064 nm NIR laser (1 W cm^{-2}) for 10 min, then the temperature of the solutions was recorded by a thermometer every 30 s. DI water was also treated with NIR laser light as a control. The photothermal conversion efficiency (η) was calculated according to the previous reported method

$$\eta = \frac{hS(T_{\max} - T_{surr}) - Q_{dis}}{I(1 - 10^{-A_{808}})} \qquad (3)$$

h is the heat-transfer coefficient, S is the surface area of the container, $T_{\max}$ is the equilibrium temperature, T_{surr} is the ambient temperature of the surroundings, Q_{dis} is the heat associated with the light absorbance by the solvent, I is the incident laser power (1 W cm^{-2}), and A_{808} is the absorbance of the JNPs at 1064 nm. The value of hS is derived according to Equation 4.

$$\tau_s = \frac{m_D C_D}{hS} \qquad (4)$$

Where τ_s is the sample system time constant, m_D and C_D are the mass and heat capacity of water used as the solvent, respectively.

Cell culture

Human hepatocellular carcinoma (HepG2) cells, human pulmonary adenocarcinoma (A549) cells, human breast cancer (MCF-7) cells and Vero cells were grown in a humidified incubator in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C in a 95% air 5% CO_2.

***In vitro* cytotoxicity assay and the synergic therapeutic efficacy**

The MTT (3-(4, 5-cimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assays were executed to evaluate the cytotoxicities of JNPs by MCF-7, A549, HepG2 and Vero cells. Briefly, the cells were seeded in a 96-well plate (1.0×10^4 cells) and cultured overnight at 37 °C in a 5% CO_2 incubator. The JNPs (6.25, 12.5, 25, 50, 100 and 200 μg mL^{-1}) were added to respective test well. After incubation for 24 and 48 h, the MTT assay was carried out to quantitatively test the relative cell viability.

The HCPT-loaded JNPs, DOX-loaded JNPs, Cocktail, HCPT/DOX-loaded JNPs and HCPT/DOX-loaded JNPs+laser was introduced into the HepG2 cells to test the synergic therapeutic efficacy.

The HepG2 cells incubated the JNPs (200, 100, 50, 25 and 12.5 μg mL^{-1}) with and without NIR laser irradiation (1 W cm^{-2}, 5 min) were used to assess the JNPs-induced photothermal therapy effect. Cell viability was determined by Equation 5.

$$\text{Cell viability} = \frac{\text{Abs}_{(\text{test cells})}}{\text{Abs}_{(\text{control cells})}} \times 100\ \% \quad (5)$$

Hemolysis assay of OA-UCNPs/PDA-AuF JNPs

Hemolysis test was carried out using human blood to sytudy the hematotoxicity of JNPs *in vitro*. After washing with physiological saline, the red blood cells were collected. Then, blood cells were diluted to 1:10 with PBS solution. Afterwards, 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 JNPs suspensions (15.625, 31.25, 62.5, 125, 250, 500 and 1000 μg mL^{-1}). The samples were shaken and kept steady for 2 h. Finally, the upper supernatants were obtained by centrifugation at 2500 rpm for 3 min and measured the absorbance by UV-Vis spectroscopy. The percentage hemolysis of the red blood cells was determined by the following Equation 6.

$$\text{Hemolysis}\,(\%) = \frac{\text{A}_{\text{sample}} - \text{A}_{\text{control(-)}}}{\text{A}_{\text{sample(+)}} - \text{A}_{\text{control(-)}}} \quad (6)$$

Combination index (CI)

One of the simplest formalisms to describe combination drug therapy is CI, defined for the pair of drugs A and B as

$$CI = \frac{IC_X(A)_{pair}}{IC_X(A)} + \frac{IC_X(B)_{pair}}{IC_X(B)} \quad (7)$$

CI values are plotted against the drug effect level, where IC_X is the drug concentration that inhibits a cellular function or behavior by X% (e.g., 50%), for each individual drug or for HCPT-DOX pair. CI < 1, = 1 or > 1 represent synergism, addition and antagonism respectively.

Cellular killing ability

The live staining method was used to evaluate photothermal effect in cell levels. Calcein acetoxymethyl ester (Calcein AM) stained the live cells with green emission. The samples (control, control+laser, JNPs, JNPs+laser) were treated with cells.

Confocal laser scanning microscopy (CLSM) and cellular uptake of the OA-UCNPs/PDA-AuF JNPs

HepG2 cells were seeded in 48-well culture plates and cultured overnight. After incubated with JNPs at 37 °C for 2 h, the cells were washed with PBS several times for disposing of the residual JNPs, and then fixed with 2.5% formaldehyde at 37 °C for 10 min before washing with PBS again. The nucleus was labeled by Hoechst 33342 solution. 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 JNPs were investigated by CLSM in HepG2 cells.

Cell apoptosis and cell cycle assays

HepG2 cells were seeded in 12-well plates (2.0×10^5 cells) in 2 mL of complete DMEM and cultured for 24 h. The JNPs, HCPT-loaded JNPs, DOX-loaded JNPs, cocktail and HCPT/DOX-loaded JNPs at the same concentration were added into the cells for 48 h. HepG2 cells without the treatment were used as a control. The treated cells were collected, washed for twice with ice-cold PBS, fixed with 70% ethanol at 4 °C overnight, followed by PI staining for 30 min in the dark. For quantitative measurement of apoptosis, the JNPs, HCPT-loaded JNPs, DOX-loaded JNPs, cocktail and HCPT/DOX-loaded JNPs were treated with cells, then the cells were harvested and washed twice with ice-cold PBS, stained with Alexa

Fluor® 488 annexin V and PI according to the manufacturer's instructions. Both cell apoptosis and cycle were analyzed by flow cytometry (BD FACS Calibur, USA)..

MR/CT imaging *in vitro* and *vivo*

The different concentrations (0, 0.625, 1.25, 2.5 and 5.0 mM) of Yb^{3+} in PBS were prepared and used to measure the corresponding values of transverse relaxation time T_2. To evaluate CT imaging efficacy of the JNPs, the different Yb^{3+} concentrations were dispersed in PBS over the range from 0 to 80 mM. As for *in vivo* MR/CT imaging, tumor-bearing nude mice were anesthetized by intraperitoneal injection of 10 wt% chloral hydrate. After administration of JNPs with a dose of 200 μL (30 mg Yb^{3+} kg^{-1}) via intravenous injection, the T_2-weighted MR images of tumor-bearing nude mice were obtained. The JNPs with a total dose of 200 μL (200 mg Yb^{3+} kg^{-1}) were injected into mice via intravenous injection to access CT imaging. The CT and MRI experiments were performed on a Philips 256-slice CT Scanner (Philips Medical System) and a 3.0 T MRI instrument.

Cell line-derived xenograft (CDX) and patient-derived xenograft (PDX) model

Fresh HCC samples were used for building up PDX HCC model in 5~6 week SCID mice. Specimens from clinical hepatectomies were orthotopically implanted into the subcutaneous of SCID mice and nude mice. Several samples were successfully grown and passes as transplantable tumor lines. CDX HCC model was established by subcutaneous injection of cancer cell lines (HepG2) at the flank of nude mice.

Combination therapy *in vivo*

The tumor-bearing nude mice were randomly divided into 12 groups (4 mice/group): PBS, PBS+laser, JNPs, JNPs+laser, HCPT, DOX, HCPT+DOX, HCPT-loaded JNPs (in terms of 1.8 mg kg^{-1} HCPT), DOX-loaded JNPs (in terms of 2.5 mg kg^{-1} DOX), cocktail, HCPT/DOX-loaded JNPs, HCPT/DOX-loaded JNPs+laser. The samples were injected through the tail vein every 3 days for CDX and 4 days for PDX models. In the meanwhile, the body weight and tumor volume of each group of mice were measured at 3 days and 4 days intervals for 15 days (CDX model) and 20 days (PDX model), respectively. The volume for each type of tumor was respectively calculated by the following Equation 8.

$$\text{Volume} = \left(\frac{\text{length} \times \text{width}^2}{2}\right) \qquad (8)$$

After that, the full tumor from each mouse was collected and weighed. Tumor growth inhibition rate was determined using the following Equation 9.

$$\text{Inhibition (\%)} = (\frac{\text{C - T}}{\text{C}}) \times 100\% \qquad (9)$$

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

The western blot assay was used to detect the protein expression levels of GADPH, cleaved caspase 3, and caspase 3. Total cell lines protein and HCC tumor protein were extracted using RIPA Lysis Buffer and PMSF (Thermo Scientifc) on the basis of the manufacturers' instructions, after centrifuged at 13,000 g for 15 min, extracted the supernatant for further study. Western blots were performed using specific 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

The immunohistochemical assay was performed to evaluate the therapy effect by paraffin-embedded tumor tissues, which had antibodies against cleaved caspase 3 (1:100; Cell Signaling, 9664) and caspase 3 antibody (1:100; Cell Signaling, 9662). The tissue sections were also stained with hematoxylin and eosin (H&E). Afterwards, an optical microscope was applied for detecting the histological sections.

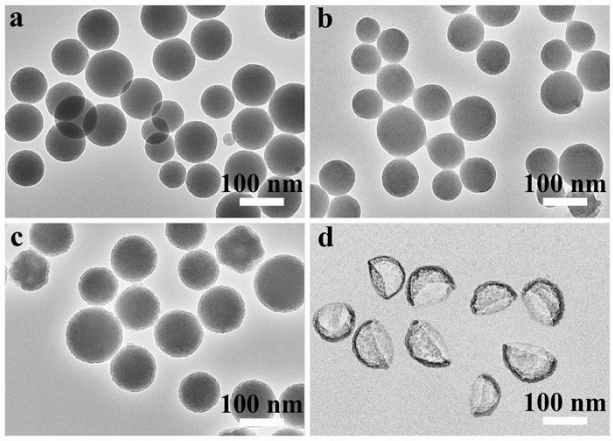

Figure S1. TEM images: a) PAA NPs. b) $RE(OH)_3$/PAA NPs. c) $RE(OH)_3$/PAA@PDA asymmetric NPs. In order to further confirm the structure of NBs, the as-prepared

$RE(OH)_3$/PAA@PDA asymmetric NPs were etched by HCl (0.1 M) to remove $RE(OH)_3$, resulting the bowl-like PDA NPs (d).

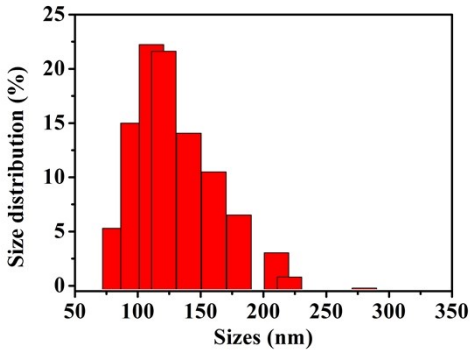

Figure S2. Dynamic light scattering size distributions of OA-UCNPs/PDA double-layered NBs in water.

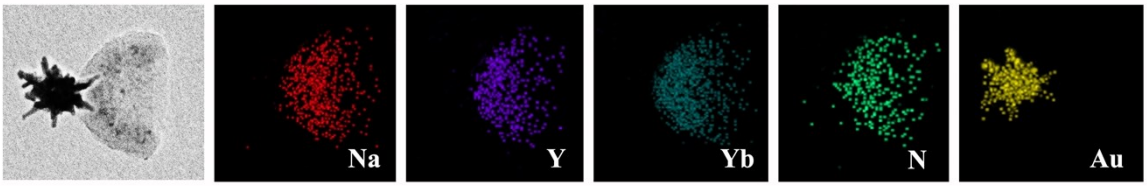

Figure S3. The corresponding TEM images and elemental mapping images of a single OA-UCNPs/PDA-AuF JNP.

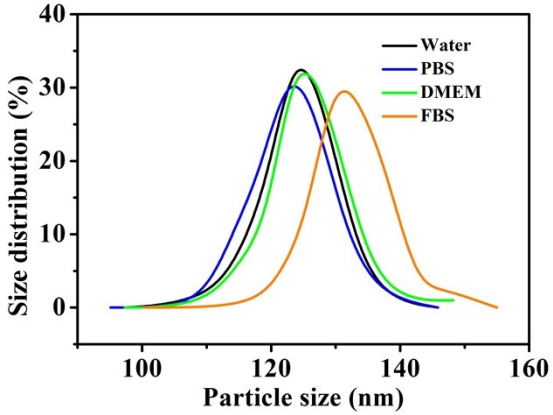

Figure S4. Dynamic light scattering size distributions of OA-UCNPs/PDA-AuF JNPs in different solvents: water, PBS, DMEM and FBS.

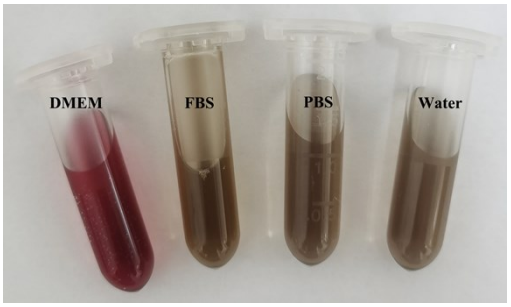

Figure S5. Photograph of OA-UCNPs/PDA-AuF JNPs in water, DMEM, FBS and PBS.

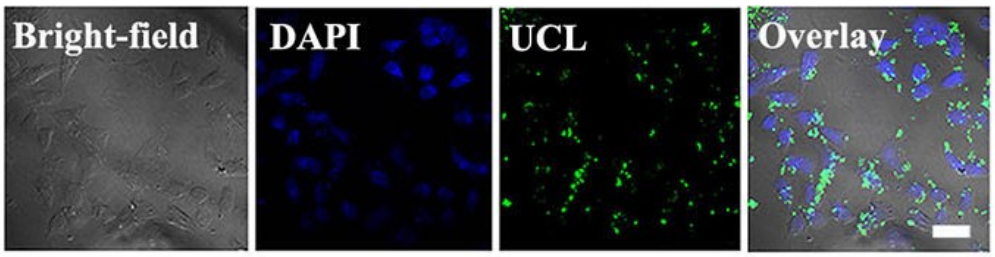

Figure S6. Inverted fluorescence microscope images of HepG2 cells. The bright-field image, nuclei of cells, UCL image and the overlay, respectively (Scale bar: 20 μm).

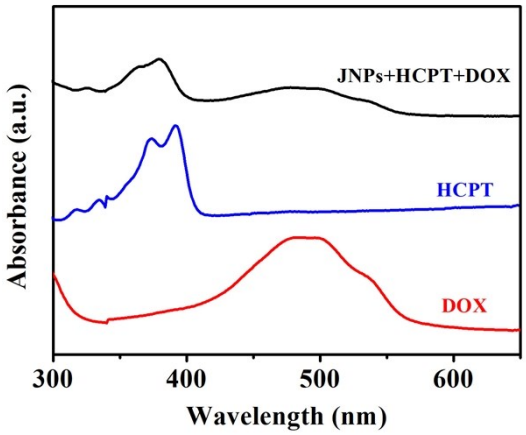

Figure S7. UV-Vis absorption curves of the HCPT, DOX, HCPT/DOX-loaded JNPs.

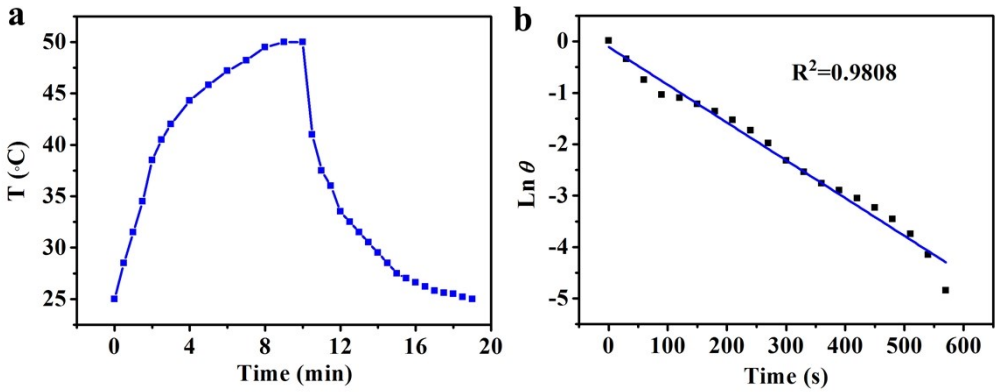

Figure S8. a) Photothermal effect of the aqueous dispersion of the JNPs under NIR laser irradiation for 600 s. b) Linear time date versus-lnθ obtained from the cooling period.

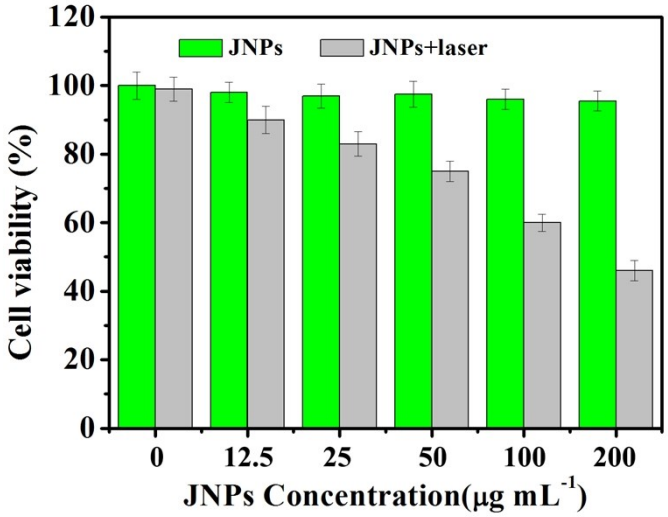

Figure S9. The photothermal effect of the JNPs treated with HepG2 cells (1064 nm, 10 min, 1 W cm^{-2}).

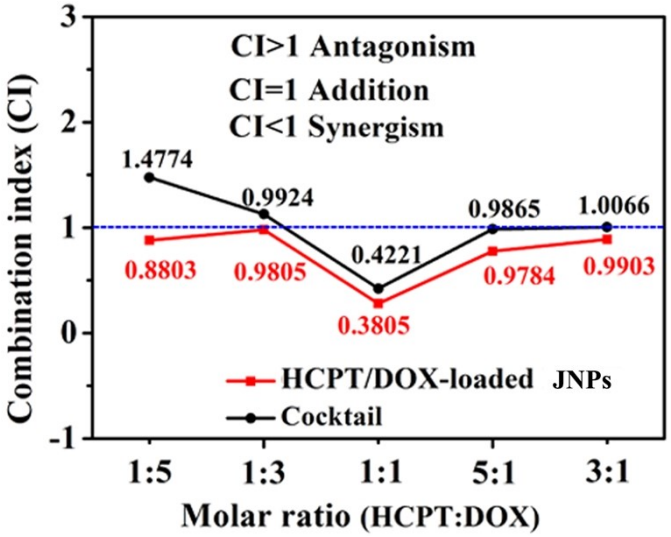

Figure S10. CI values of HCPT/DOX-loaded JNPs with various ratios against HepG2 cells at IC_{50}.

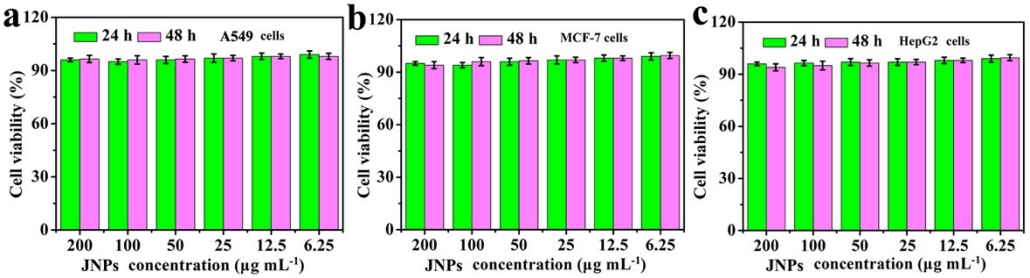

Figure S11. Cell viabilities of a) A549 cells, b) MCF-7 cells and c) HepG2 cells after treatment with JNPs for 24 and 48 h.

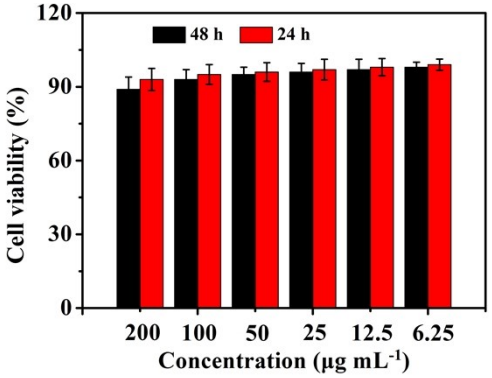

Figure S12. Cell viabilities of Vero cells after treatment with JNPs for 24 and 48 h.

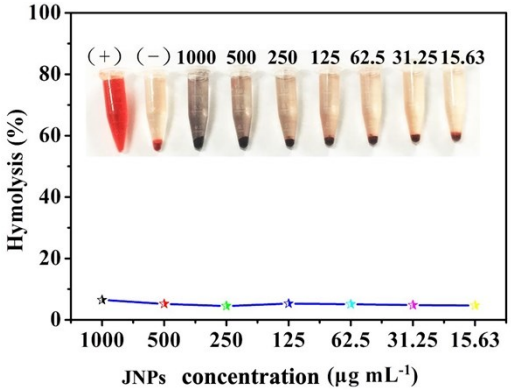

Figure S13. Hemolytic percent of red blood cells incubated with JNPs at various concentrations for 2 h. Inset describes photo of JNPs mixed with red blood cells after centrifugation. +: positive control. -: negative control.

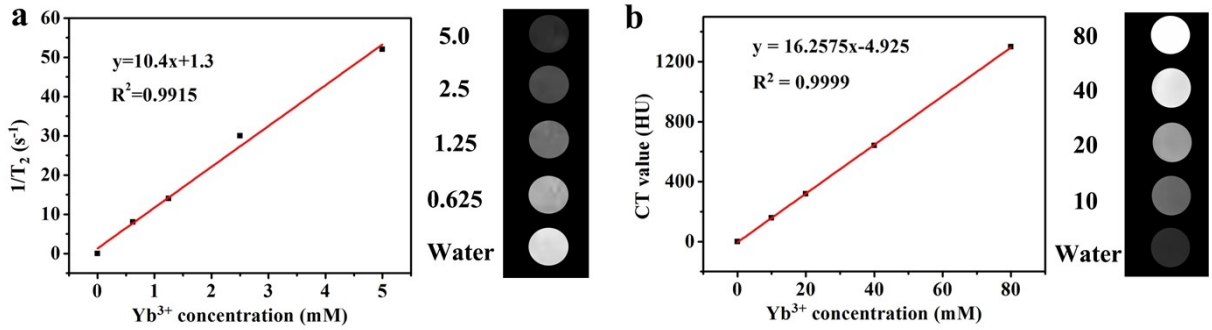

Figure S14. a) Relaxation rate ($1/T_2$, r_2) and *in vitro* T_2-weighted MR images of the JNPs. b) CT values (HU) and *in vitro* CT images of the JNPs.

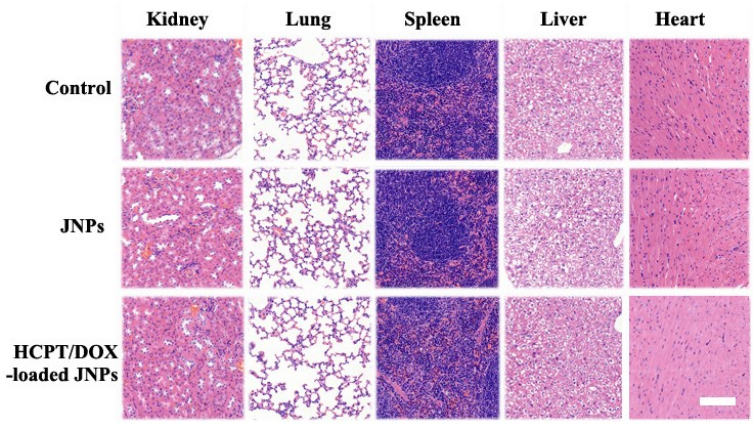

Figure S15. Histological section of major organ tissues stained with H&E after various treatments. Scale bar: 200 μm.