Electronic Supplementary Information (ESI)

Hyaluronic acid functionalized gold nanorods combined with copper-based therapeutic agents for chemo-photothermal cancer therapy

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Contents

1. Experimental section 2
1.1 Materials 2
1.2 Cells and animals 2
1.3 Synthesis of AuNRs-CTN@THA 3
1.4 Characterization of AuNRs and AuNRs-CTN@THA 4
1.5 Photothermal evaluation 4
1.6 Stability with respect to laser irradiation 4
1.7 Stimuli-responsive release of AuNRs-CTN@THA 4
1.8 Coordination interaction between CTN and THA 5
1.9 In vitro cytotoxicity 5
1.10 DNA cleavage activity 5
1.11 In vivo antitumor efficacy 6
1.12 Tumor deep penetration 6
1.13 Statistical analysis 6
2. Figures 7
Fig. S1. Preparation of CTN and THA 7
Fig. S2. Characterization of AuNRs 7
Fig. S3. UV−vis absorbance and photothermal stability. 8
Fig. S4. pH response of hybrid nanocomposites 8
Fig. S5. DNA cleavage ability of AuNRs-CTN@THA combined with laser 8
Fig. S6. Pharmacodynamics and safety evaluation 9
Fig. S7. Hematoxylin-eosin staining (H&E) images 9
3. References 10

1.Experimental section

1.1 Materials

Hydrogen tetrachloroaurate (III) tetrahydrate ($HAuCl_4 \cdot 4H_2O$), trisodium citrate, sodium borohydride ($NaBH_4$), cetyltrimethylammonium bromide (CTAB), hyaluronic acid (HA, WM 10000), ascorbic acid, cysteamine, 4-mercaptobenzoic acid (4-MBA), $Cu(NO_3)_2 \cdot 3H_2O$ and poly (hydroxyethyl methacrylate) (pHEMA) were obtained from Sigma-Aldrich (Shanghai, China). The sodium chloride (NaCl), sodium bicarbonate, sodium hydroxide (NaOH), hydrochloric acid (HCl), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl), D-glucose, dimethyl sulfoxide (DMSO), 4-morpholineethanesulfonic acid hydrate (MES) buffer, and DL-dithiothreitol (DTT) were obtained from Shanghai Sino pharm Chemical Reagent Co., Ltd (China). Isoflurane, 5, 5-Dithiobis-(2-nitrobenzoic acid) (DTNB, 99%) and tris (hydroxymethyl) aminomethane hydrochloride were purchased from Aladdin (Shanghai, China). Loading buffer (0.05% bromophenol blue, 0.035% xylene cyanol FF, 36% glycerol and 30 mM EDTA) and pBR322 DNA were purchased from TaKaRa Biotechnology (Dalian, China). Calf thymus DNA (CT-DNA), Tris, agarose gel electrophoresis and ethidium bromide (EB) were purchased from Sunshine Bio (Nanjing, China). MTT, FITC, PE-CD31 antibody, dialysis bag (MW 3500D), 4% paraformaldehyde (PFA), 4´,6-diamidino-2-phenylindole (DAPI), trypsin-EDTA solution and DMEM were supplied by KeyGEN BioTECH Co. Ltd (Nanjing, China). All the reagents were used without further purification. The ultra-pure water was obtained from Milli-Q deionized water (Millipore, 18.2 MΩ cm^{-1}). All the glassware was cleaned by aqua regia and rinsed with deionized water prior to the experiments.

1.2 Cells and animals

Mouse melanoma (B16F10) cell line was purchased from American Type Culture Collection (ATCC), cultured in DMEM with 10% (v/v) FBS, 100 U/mL penicillin and 100 mg/mL streptomycin in an incubator (Thermo Scientific, Waltham, MA) at 37 °C under an atmosphere of 5% CO_2 and 90% relative humidity. Cells were sub-cultivated approximately every four days at 80% confluence.

For the culture of 3D tumor spheroids, 450 mg pHEMA was dissolved completely in 30 mL ethanol-H_2O (28.5:1.5, v/v) solvent, and then 8 mL of them was added into the culture flask, dried in 60 °C oven and exposed to UV irradiation for 2 h. Afterwards, B16F10 cells suspended at a density of 5×10^5 cells/mL in DMEM culture medium were introduced into the prepared cell culture flask above, cultured as above. 3D tumor spheroids were used for the following experiments when the diameter reached around 100 μm.

The animal experiments were performed according to the guidelines for the care and use of laboratory animals and approved by the Animal Ethics Committee of Nanjing University of Chinese Medicine. Male C57BL/6 mice (18–22 g) were purchased from Shanghai Jie Esprit Company

(Nanjing China). To establish the tumor model, 200 μL B16F10 cells suspended in PBS (2×10^7 cells/mL) were implanted subcutaneously into the right lateral inguen of each mouse and treatments began when the tumor volume reached about 100 mm^3.

1.3 Synthesis of AuNRs-CTN@THA

Preparation of gold nanorod (AuNRs) AuNRs were prepared using classic seed mediated growth method previously reported [1]. Briefly, seed solution was synthesized by adding $HAuCl_4$ (1 mM, 1 mL), CTAB (0.2 M, 1.88 mL) and ice-cold $NaBH_4$ (0.01 M, 0.5 mL) to 5.35 mL deionized water (DI water) in order, and stirred vigorously to obtain a brownish-yellow solution. After that, the resultant solution was kept undisturbed at room temperature for 2 h and ready for the next step. In parallel, growth solution was prepared by adding $HAuCl_4$ (1 mM, 1.65 mL) and CTAB (0.2 M, 2.365 mL) to 890 μL DI water subsequently. Then, 0.1 M ascorbic acid was added dropwise to the above mixture as a mild reducing agent till the reaction solution turned colorless. Thereafter, the pH was adjusted by adding 2 μL 37% HCl, and then the prepared solution was left still at room temperature. By adding 40 μL seed solution to the growth solution with gentle stirring, the mixture was left undisturbed overnight. Then the AuNRs were obtained through centrifuged, washed twice and dispersed with DI water. The gold concentrations were quantified using inductively coupled plasma-mass spectrometry (ICP-MS, Perkin-Elmer Corporation, USA).

Preparation of thiolated hyaluronic acid (THA) THA was synthesized as followed [2]: 400 mg HA was dissolved in 80 mL MES buffer, followed by introducing 384.4 mg EDC·HCl into the mixture dropwise. After 30 min stirring, the solution containing 61.72 mg cysteamine and 1 M sodium bicarbonate were added dropwise to the above reactants in sequence, and then the mixture was stirred overnight under the protection of nitrogen. The resultant suspension was dialyzed (molecular weight cutoff: 3500) against DI water for 24 h, and then DTT (mole ratio, cysteamine: DTT=1:10) was introduced into the collected dialysate with moderate stirring and nitrogen protection for 5 h. The resultant solution was respectively dialyzed against 1 L HCl solution (pH 3.5, 0.1 M NaCl) and DI water for 1 h. Following this step, the dialysate was lyophilized to obtain the THA, characterized by ^1H NMR spectroscopy (400 MHz, Bruker AMX-400, USA). The degree of thiol substitution was measured by a modified Ellman method.[3] Following this method, DTNB reacted with the free thiol group and the product had characteristic absorption peak at 412 nm with UV test, which could be used for the quantitation of the free thiol group.

Preparation of AuNRs-CTN@THA To form the THA corona, AuNRs was centrifuged at 12 000 rpm for 30 min and washed twice to remove CTAB. 500 μL AuNRs suspensions with different Au concentrations were added into 1 mL of 2 mg/mL THA solutions (the final concentration of Au was 0.1, 0.5, 1, 2 and 3 nM) for 2 h incubation at 37 °C. The collected AuNRs@THA were centrifuged at 12 000 rpm for 15 min and washed once followed by the addition of CTN to obtain AuNRs-

CTN@THA. CTN loading content was measured by HPLC.

1.4 Characterization of AuNRs and AuNRs-CTN@THA

The morphology, particle distribution and structure of AuNRs and AuNRs-CTN@THA were determined by the transmission electron microscopy (TEM, Hitachi H-8000, Japan) with an accelerating voltage of 200 kV. The zeta potential, hydrodynamic diameters and polydispersity index (PDI) were determined by a dynamic light scattering (DLS) method using Malvern Zetasizer (Zetasizer Nano-ZS90, Malvern, England) at 25 °C with a detection angle of 90°. The UV-Vis absorption spectra of nanorods and nanocomposites were investigated by a Shimadzu UV-3600 spectrophotometer (UV-3600, Japan). All experiments were performed at least in triplicate.

1.5 Photothermal evaluation

The *in vitro* photothermal effects of AuNRs, AuNRs@THA and AuNRs-CTN@THA (Au concentration of 0.5 nM) were evaluated with PBS solution as a control. The irradiation under an 808 nm laser with power density of 1 W/cm^2 was employed. With the addition of samples in a quartz cell, the real-time temperature of samples was monitored by a FLIR Thermal Imaging System for 150 s.

For *in vivo* photothermal efficacy evaluation, the xenograft tumor mice were injected intravenously with PBS (100 μL) or AuNRs-CTN@THA (100 μL, 5 mg/kg). The tumor regions of all the mice were irradiated by an 808 nm laser (120 s, 1 W/cm^2) 12 h post injection. The thermal imaging of tumor sites was captured at a fixed time interval. Finally, Fotric AnalyzIR software was utilized to analyze temperature changes of these thermographs.

1.6 Stability with respect to laser irradiation

A laser irradiation (808 nm, 1W/cm^2) was used to excite the AuNRs or AuNRs@THA with a spot size of 6 mm. In brief, 100 μL of AuNRs or AuNRs@THA in 3×3 mm quartz cuvette was exposed to the laser irradiation for 5, 10 and 20 min and absorption spectra were captured after laser irradiation. A quantitative measure of the stability was developed as an aggregation index (AI).[4] The AI is a measure of the longitudinal surface plasmon resonance (LSPR) peak broadening derived from the total area under the absorption spectrum of the LSPR from 450 to 900 nm, divided by LSPR intensity. The AI gives the equivalent bandwidth of the longitudinal peak (with units of nm) for a spectrum normalized to the LSPR peak intensity. A higher degree of aggregation corresponds to a higher AI value.

1.7 Stimuli-responsive release of AuNRs-CTN@THA

In order to validate the effects of pH and laser irradiation on CTN release, an 808 nm laser (1 W/cm^2) was employed to heat AuNRs-CTN@THA dispersed in different pH (4.0, 6.5, 7.4) PBS for 0,

60 and 120 s, respectively. After that, AuNRs-CTN@THA were centrifuged and the supernatants were collected for quantification of the CTN by HPLC. The release was also monitored by dialysis method in PBS with different pH (4.0, 6.5, 7.4) values. An air bath table concentrator was used to sustain the temperature at 37 °C or 50 °C, respectively simulating the common condition or hyperpyrexia. AuNRs-CTN@THA solution containing 0.2 mg CTN was loaded in the pretreated dialysis bags (MW cutoff: 3500D) and immersed in 50 mL release medium. The dialysate samples were withdrawn at predetermined time intervals for determining CTN concentration by HPLC. All experiments were carried out in triplicates.

1.8 Coordination interaction between CTN and THA

The formation, cleavage, and pH dependence of coordination interactions between CTN and THA were studied by UV-vis spectra. Briefly, 2 mM CTN in ethanol was added dropwise into 2mL of 1mg/mL THA solution under agitation until equilibrium. Real-time UV-vis spectra of resultant mixture were recorded under successive pH values adjusted with dilute HNO_3 or NaOH.

1.9 *In vitro* cytotoxicity

The cytotoxicity of AuNRs-CTN@THA was evaluated by MTT assay. Briefly, B16F10 cells were seeded at a density of 5×10^4 cells per well in 96-well plates in 37 °C, 5% CO_2 incubator overnight. Then the cells were incubated with AuNRs-CTN@THA (2 μg/mL CTN) or AuNRs@THA for 4 h and treated with/without a laser irradiation (808 nm 1 W/m^2, 120 s). After another 20 h incubation, MTT assay was carried out at 490 nm using a microplate reader (Infinite® 200 Pro NanoQuant, Tecan).

Chemo-photothermal combination effect was further assessed on 3D multicellular tumor spheroids. The tumor spheroids were treated by AuNRs-CTN@THA (containing CTN of 1.0 or 2.0 μg/mL, respectively) with/without laser irradiation (808 nm, 1 W/m^2, 120 s) once a day for 5 days. The diameters of spheroid were monitored as an evidence of tumor growth inhibition.

1.10 DNA cleavage activity

The cleavage activity of the AuNRs-CTN@THA on Supercoiled pBR322 DNA was investigated by agarose gel electrophoresis. In brief, Supercoiled pBR322 DNA (200 ng) was treated with AuNRs-CTN@THA (alone or with laser irradiation, 1 W/m^2, 120 s) and ascorbic acid (1 μL) at a 100-fold molar excess relative to the AuNRs-CTN@THA in the PBS buffer (0.1 M, pH 5.0). The mixtures (10 μL) were incubated for 90 min at 37 °C in the dark, quenched with 2 μL 6 × DNA loading buffer. The product was loaded onto the agarose gel (1%) and subjected to electrophoresis in a TAE buffer (40 mM Tris acetate/1 mM EDTA). DNA bands were stained by EB, visualized and photographed

by a Bio-Rad Gel-Doc XR imaging system.

1.11 *In vivo* antitumor efficacy

The melanoma tumor-bearing mice were randomly divided into five groups (n=7): AuNRs-CTN@THA+L, AuNRs@THA+L, AuNRs-CTN@THA, CTN, and saline as a control group. The mice were intravenously injected with the corresponding formulations at a dose of 2 mg/kg CTN every other day for 12 days. Laser irradiation (808 nm, 1 W/cm^2, 3 min) was applied on the tumor sites 24 h post injection. The tumor size and body weight were regularly monitored. On day 14, one animal from each group was randomly selected and euthanized. The tumor and main organs were collected, washed with saline twice, and fixed in 4% PFA for hematoxylin & eosin (H&E) staining or TUNEL assay.

1.12 Tumor deep penetration

3D tumor spheroids were employed to evaluate the photothermally induced tumor deep penetration. FITC-labeled AuNRs@THA (FITC-AuNRs@THA) was prepared by incubating FITC with AuNRs@THA as previously reported.[5] About 10 spheroids were transferred into a 5 mL EP tube and exposed to FITC- AuNRs@THA with an appropriate concentration at 37 °C for 2 h. After an 808 nm irradiation at 0.5 W/cm^2 for 150 s, tumor spheroids were cultured for another 6 h. As a control, tumor spheroids were incubated with FITC-AuNRs@THA without laser irradition for 8 h. After incubation, the medium was removed and tumor spheroids were washed with PBS (pH 7.4) before observed with CLSM.

In order to further explore the tumor deep permeability of AuNRs-CTN@THA *in vivo*, melanoma tumor-bearing mice were divided into two groups randomly (3 mice per group) and injected with FITC-AuNRs@THA intravenously. For the photothermal intervention, the tumors were exposed to an 808 nm laser irradiation (0.5 W/cm^2, 150 s) 24 h post injection. Such a laser exposure would induce only mild heating without causing significant cell death. Then the mice were sacrificed and tumors were harvested and fixed with 4% PFA. The blood vessels and nuclei were stained with PE-CD31 antibody and DAPI, respectively. Finally, the slices were sealed with anti-fluorescent quencher and observed using a fluorescence microscope (Olympus X51, Tokyo, Japan). The Image J software was used to semi-quantitative fluorescence, and the penetration ability was expressed by the ratio of mean fluorescence intensity in the tumor parenchyma and blood vessels.

1.13 Statistical analysis

Data were expressed as mean ± standard deviation (SD) and analyzed using Student's test. A statistically significant difference was shown as $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

2. Figures

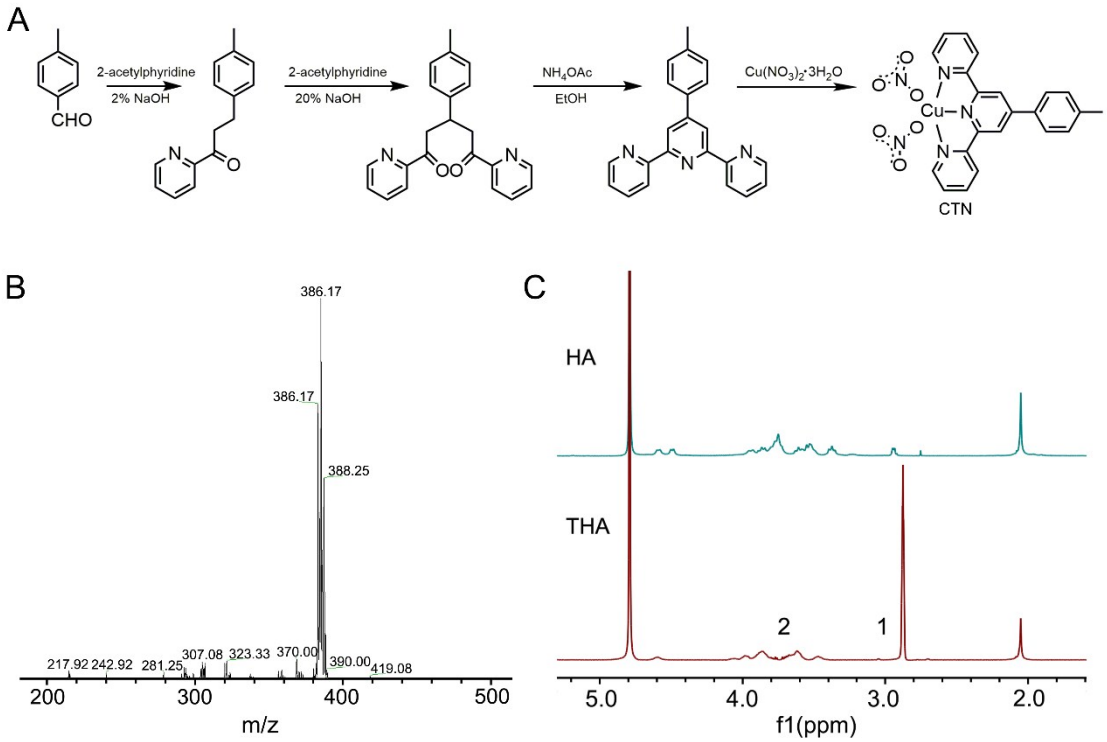

Fig. S1. Preparation of CTN and THA. (A) Synthesis of CTN. (B) ESI-MS spectrum (positive mode) of CTN. (C) ^{1}H NMR (D_2O) spectra of HA and THA.

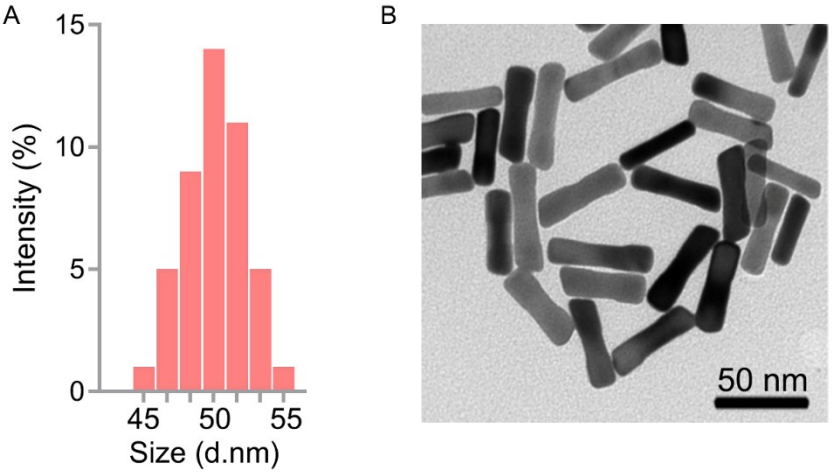

Fig. S2. Characterization of AuNRs. The size of AuNRs measured by DLS (A) and TEM (B). Scale bar, 50 nm.

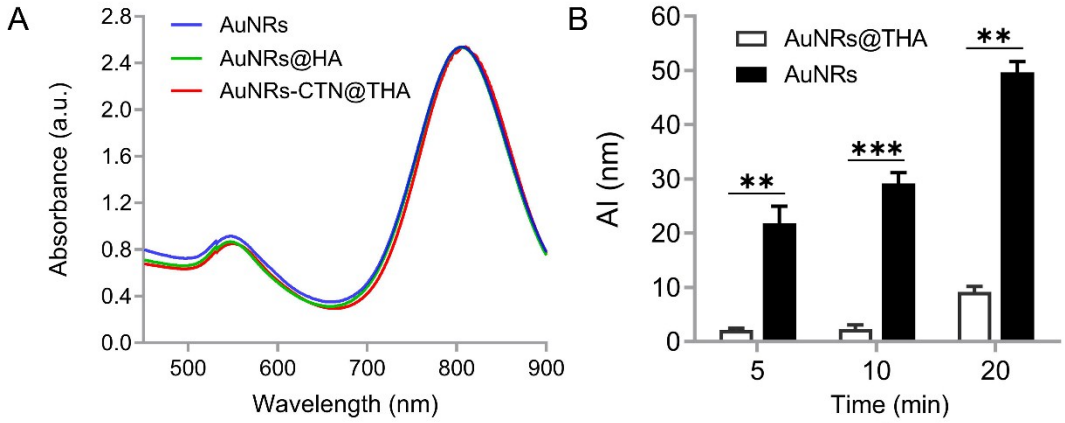

Fig. S3. UV−vis absorbance and photothermal stability. (A) UV−vis absorbance of AuNRs, AuNRs@THA and AuNRs-CTN@THA at 808 nm. (B) Increased aggregation index (AI) based on absorption spectra. Data were expressed as mean ± SD, n=3, $^{**}P < 0.01$, $^{***}P < 0.001$.

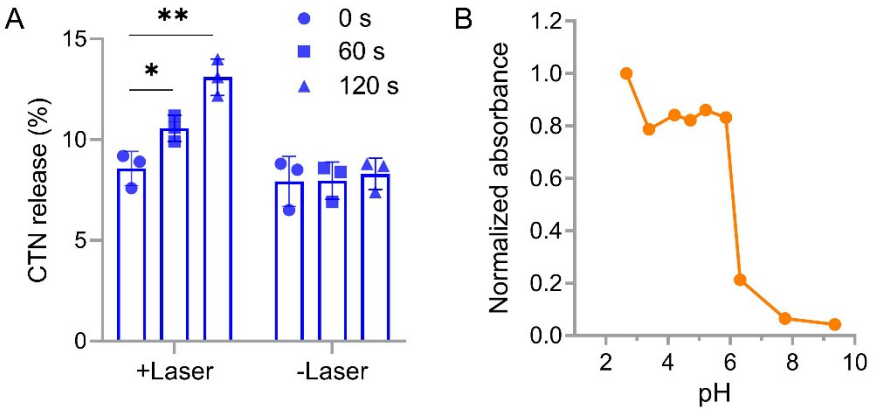

Fig. S4. pH response of hybrid nanocomposites. (A) CTN release from AuNRs-CTN@THA at pH 4.0 PBS with the increasing exposure to laser irradiation (0, 60 and 120 s). (B) pH-responsive formation and breakage of coordination bonds between CTN and THA. Data were expressed as mean ± SD, n=3, $^{*}P < 0.05$, $^{**}P < 0.01$.

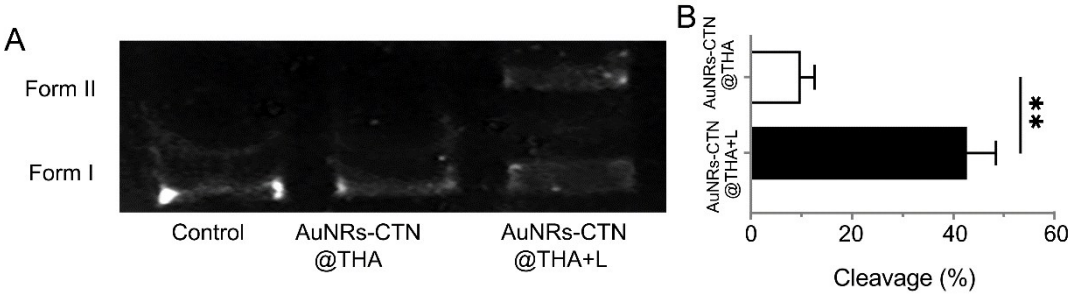

Fig. S5. DNA cleavage ability of AuNRs-CTN@THA combined with laser. (A) The cleavage patterns of the agarose gel electrophoresis and (B) the corresponding cleavage extent (%) for pBR322

plasmid DNA by AuNRs-CTN@THA or/and irradiation. Data were presented as mean ± SD, n=3, $^{**}P < 0.01$.

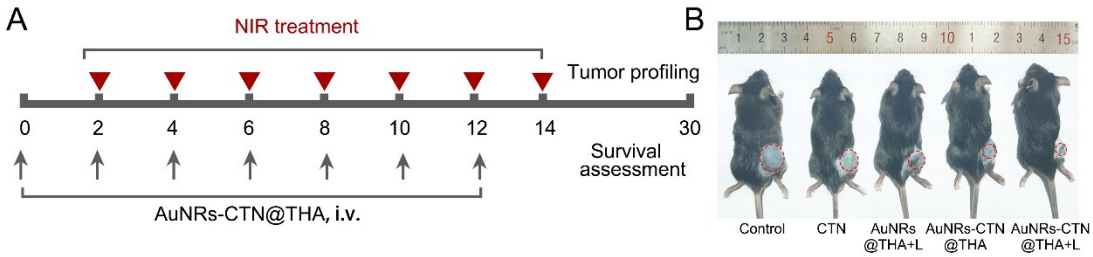

Fig. S6. Pharmacodynamics and safety evaluation. (A) Experiment flow: on day 0, AuNRs-CTN@THA were injected into melanoma tumor-bearing mice once every two days (*n*=7 per group). NIR treatment was applied once every two days from day 2 to day 14 using an 808 nm laser at 1 W for 3 min. (B) Representative images of B16F10 xenograft tumors in mice after different treatments on day 15, with tumor cycled in red dashed line.

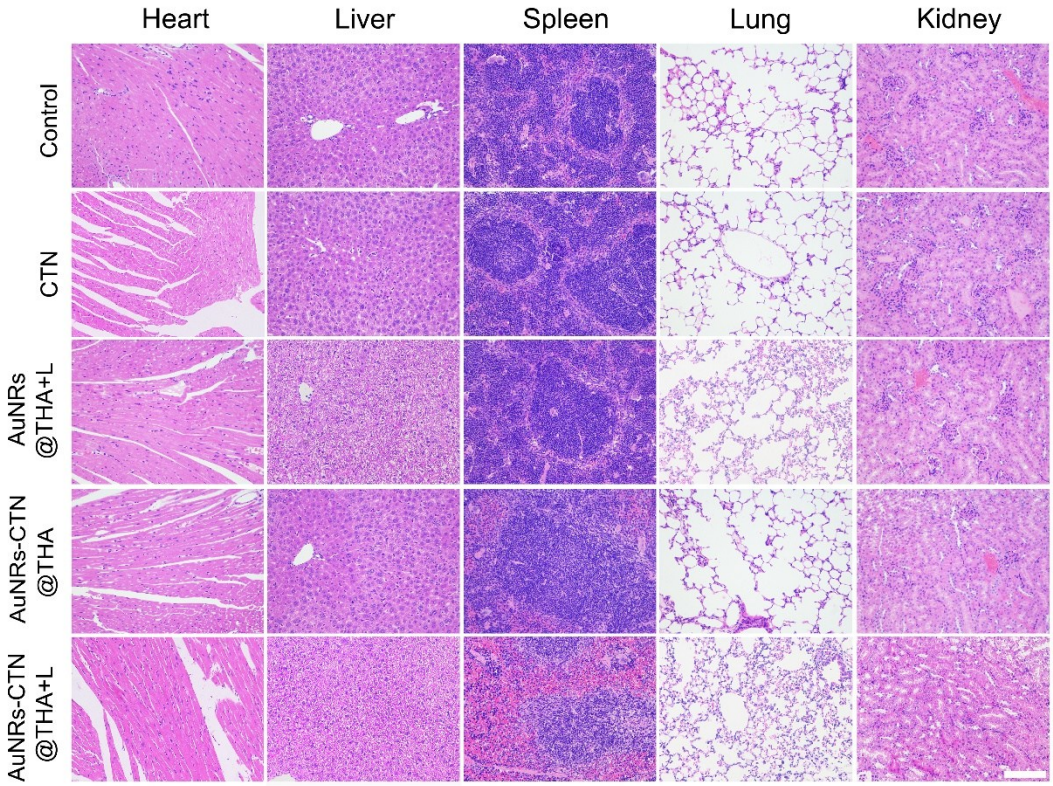

Fig. S7. Hematoxylin-eosin staining (H&E) images of the heart, liver, spleen, lung, kidney tissues from mice treated with different agents, scale bar, 50 μm.

References

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