Supporting Information

Enhancing the photothermal stability and photothermal efficacy of gold nanorods and nanostars by grafted with ruthenium(II) complexes

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Experimental Section

Materials

The materials and reagents used in this work are as follows: hexadecyltrimethyl ammonium bromide (CTAB), L-ascorbic acid, trisodium citrate ($Na_3C_6H_5O_7$), 2-[4-(2-hydroxyethyl) piperazine-1-yl]ethanesulfonic (HEPES) and sodium borohydride ($NaBH_4$) were purchased from Sigma Aldrich (Shanghai, China). $AgNO_3$, $RuCl_3 \cdot xH_2O$ and Au(III) chlorate ($HAuCl_4 \cdot xH_2O$) were purchased from Alfa Aesar (Shanghai, China). The ruthenium complex was synthesized as previously described by our group.[1] The water used in the research was Milli-Q deionized water (18 $M\Omega \cdot cm^{-1}$). We washed the glassware with aqua regia before experiments (HNO_3:HCl, 1:3) (Be careful! Aqua regia is a strong oxidizing agent).

Instruments

The morphology of the gold nanoparticles was recorded by transmission electron microscopy (TEM) (JEM2010-HR, JEOL Model, 200 kV). The X-ray photoelectron spectroscopy (XPS) (Thermo VG, ESCALab250) used 200 W Al K_R radiation with a twin anode. The UV-Vis-NIR spectra were measured on a UV-3150 spectrophotometer (Shimadzu, Model UV-3150). The emission spectra were tested on a Perkin-Elmer LS55 spectrofluorophotometer. A diode 808 nm laser was purchased from Hi-Tech Optoelectronics Co., Ltd. (Beijing, China).

Synthesis of AuNRs@Ru

7.5 mL CTAB (0.2 M) in water and 0.25 mL $HAuCl_4$ solution (30 mM) was mixed rapidly. Then 0.6 mL $NaBH_4$ (10 mM) under 0 °C was added drop by drop. The mixed solution was stirred for 2 min and left it for 1 h at 37 °C as growth solution. The color was brown-yellow. 40 mL CTAB (0.2 M) in water and 3 mL $HAuCl_4$ solution (30 mM) were mixed in a separate container, 0.5 mL $AgNO_3$ (10 mM) was then added and stirred slowly, and the color change to white at right when 0.55 mL L-ascorbic acid solution (0.1 M) was added finally. The above growth solution was added drop by drop and then left overnight at RT without stirring. The reaction mixture became purple-red in color. Upon cooling at 4 °C, the redundant CTAB was filtered. The filtrate was monodisperse Au nanorods. 5 mL Ru(II) complex (2 mM) dissolved in water were added into the gold nanorods solution. The mixture was stirring for 24 h at 37 °C. The as-prepared AuNRs@Ru were centrifuged at 20000 rpm for 30 min, washed with water three times, and discarded the supernatant to remove unbound ruthenium complex. The final AuNRs@Ru was resuspended in H_2O.[2]

Synthesis of AuNTs@Ru

100 mM HEPES solution was prepared with H_2O and pH was 7.4±0.1 at room temperature using 1 mol NaOH. 60 mL of H_2O was mixed with 40 mL of 100 mM HEPES, then added into 1 mL $HAuCl_4$ solution (30 mM). The color was from yellow to purple without shaking, and finally to greenish blue for 0.5 h. The product was collected by centrifugation after 1 h. 5 mL ruthenium complex (2 mM in H_2O) was added into the above AuNTs, which were left overnight to form the Ru@AuNT nanoconstructs. To increase the surface concentration of ruthenium, we salted the mixture solution with 2 mL of 100 mM NaCl solution. At last, the samples were washed and separated by centrifugation at 15000 rpm for 1 h.[3]

Size Distribution Measurements

We used JEM2010-HR (200 kV) to record TEM images of the nanoparticles and then analyzed the

distribution of the size. Two hundred particles were recorded to get the average size.

Determination of the Amount of Ru(II) Complex by ICP-MS Analysis

The AuNRs@Ru/AuNTs@Ru (50 μg/mL) solution was digested at 50 °C for 4 h using 5 mL of aqua regia. Then the samples were diluted with Milli-Q H_2O to 2% HNO_3 and measured by ICP-MS (Thermo Elemental Co., Ltd.).

Photothermal Effect Measurements

100 μL of 50 μg/mL of the nanoparticle solutions was irradiated by the 808 nm laser (0.25 W/cm^2, 5 min). The temperature increases were collected every 30 seconds by a thermal camera (MAG30, Magnity Electronics, Thermal Imaging Expert). The control groups of AuNRs and AuNTs were measured with 3 replicates. The photothermal conversion efficiency of the AuNRs@Ru and AuNTs@Ru was analyzed according to a reported method.[4]

Cytotoxicity Test

HeLa cells (Human cervical carcinoma cancer) were obtained from the Center for Experimental Animals, Sun Yat-sen University (Guangzhou, China). 5×10^3 cells were seeded in each well in a 96-well white plate and then incubated for 24 h in the incubator. After adding different concentration of AuNRs@Ru/AuNTs@Ru solutions, the cells were incubated for 12 h. Then the cells were irradiated for 5 min by 808 nm laser (0.25 W/cm^2) and the 96-well plates were put in the incubator for another 12 h. The survival rate was tested using MTT assay by a microplate spectrophotometer (590 nm).

Cellular Uptake Assay

HeLa cells were incubated with AuNRs@Ru/AuNTs@Ru (50 μg/mL) for 1 h. After that the cells were fixed with 2% glutaraldehyde for 2 h, and dehydrated in CH_3CH_2OH and then embedded in Spurr's resin. The obtained ultrathin slices were loaded on copper grids, stained with uranyl acetate and lead citrate, then imaged on an TEM (JWOL, Model TEM 100 CX, Japan).

3D Multicellular Tumor Spheroids Culture

Multicellular spheroids (MCTSs) were cultured by the liquid overlay method.[5] The single HeLa cell suspension was seeded in a standard flat-bottom 96-well plate, which was previously loaded with 60 μL 1.0% (wt/vol) agarose solution in the PBS to get a non-adherent surface. To generate multicellular spheroids, 150 μL solution with 5×10^3 cells was added into the prepared plate, the plate was incubated for 72 h in the incubator until the spheroids produced. The average diameter of spheroids was ~400–500 μm after 3 days growth. The generated spheroids were used for the following research.

Cell Viability Assay on MCTSs

The viability of the nanoparticles to the 3D MCTSs was tested by the Cell Titer Glo kit (from Promega).[6] The above MCTSs were carefully treated by replacing half of the medium with drug-supplemented standard medium. The treated MCTSs were incubated for 24 h, then irradiated for 5 min upon an 808 nm laser (0.25 W/cm^2). In the control, we treated with the solvent-containing or solvent-free medium with/without irradiation. The viability of the MCTSs was measured on a microplate reader (Promega).

Live/Dead Viability Assay

This assay was performed using the LIVE/DEAD Viability/Cytotoxicity Kit (Life Technologies). Live cells were determined by the enzymatic conversion of the virtually non-fluorescent calcein AM to the

strong fluorescent calcein (λ_{ex} = 488 nm, λ_{em} = 521±25 nm). After MCTSs treatment with AuNRs@Ru/AuNTs@Ru and 808 nm laser irradiation and the control cell treatment in the absence of AuNRs@Ru/AuNTs@Ru with irradiation, the MCTSs were stained with calcein AM (4 μM) for 1 h and imaged using a fluorescence microscope (Zeiss, Model Axio Observer D1, Germany).

Photothermal Therapy In Vivo

4–5 weeks old BALB/c-(nu/nu) female nude mice were given and bred in the Center of Experimental Animals at Sun Yat-sen University. Our experimental protocol was approved by the Sun Yat-sen University Animal Care and Use Committee. 5 × 10^6 HeLa cells were injected into BALB/c-(nu/nu) female nude mice *via* subcutaneous injection (s.c.). When the size of tumor was ~120 mm^3, the mice were divided into 5 groups (8 mice each group) for the following experiments.

Group 1 (physiological saline+laser): mice were injected with physiological saline as a control; Group 2 (AuNRs+laser): mice were injected with the AuNRs (100 μL/20 g body weight of 1 mg/mL solution, 100 μg AuNRs/20 g body weight); Group 3 (AuNTs+laser): mice were intratumorally injected with the AuNTs (100 μL/20 g body weight of 1 mg/mL solution, 100 μg AuNTs/20 g body weight); Group 4 (AuNRs@Ru+laser): mice were injected with the AuNRs@Ru (100 μL/20 g body weight of 1 mg/ mL solution, 100 μg AuNRs@Ru /20 g body weight); Group 5 (AuNTs@Ru +laser): mice were injected with the AuNTs@Ru (100 μL/20 g body weight of 1 mg/ mL solution, 100 μg AuNTs@Ru /20 g body weight). Then each mouse was irradiated with an 808 nm laser (Hi-Tech Optoelectronics Co., Ltd. Beijing, China). Thermal images were measured by the thermal camera when the tumors were irradiated for 5 min by the 808 nm laser (0.25 W/cm^2). After the irradiation (on day 0), we used a caliper to measure the size of the tumor sizes every 3 days and a digital color camera to photograph pictures on day 0, day 5, and day 15. The tumor volumes were calculated based on the formula tumor volume (V) = (tumor length) × (tumor width)^2/2. The relative tumor volume was named as V/V_0, where V_0 is the initial tumor volume on day 0.

Histological Examination

After the PTT *in vivo*, the organs (including kidney, liver, heart, spleen, brain, lung, ovary and intestine) and tumor tissues of the mice were collected. Then we used 4% paraformaldehyde to immerse these fresh tissues at 4 °C. 6 μm sections were cut from paraffin-embedded samples for the morphological studies. These sections were rehydrated (xylene, alcohol, water) and stained by hematoxylin-eosin (H&E). In general, nuclei are stained blue by hematoxylin, whereas the cytoplasm and extracellular matrix are stained pink by eosin. The sections were imaged on an Olympus microscope.

Statistical Analysis

Data were presented in the form of mean ± standard deviation, and significance was assessed using a student's test. Differences were considered to be significant at $P < 0.05$.

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4 Q. Tian, F. Jiang, R. Zou, Q. Liu, Z. Chen, M. Zhu, S. Yang, J. Wang, J. Wang and J. Hu, *ACS Nano,* 2011, **5**, 9761–9771.

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6 H. Y. Huang, P. Y. Zhang, H. M. Chen, L. N. Ji and H. Chao, *Chem. Eur. J.,* 2014, **21**, 715−725.

Table S1. The binding energy and elements analyzed of the nanoparticles by XPS.

Name	AuNRs@Ru		AuNTs@Ru	
	Peak BE/ev	At. %	Peak BE/ev	At. %
C_{1s}	284.75	61.21	284.66	61.09
O_{1s}	532.43	13.49	531.91	13.11
N_{1s}	400.22	12.59	400.51	13.27
Cl_{2p}	198.40	2.91	198.53	2.98
Ag_{3d}	367.84	1.15	NO	NO
Au_{4f}	84.46	8.32	84.49	8.99
Ru_{3d}	281.10	0.33	281.13	0.56

Table S2. The concentrations of Ru and Au/Ag atoms in the 50 μg/mL AuNRs@Ru/AuNTs@Ru determined by ICP-MS.

Nanoparticles (μg/mL)	Au atoms	Ag atoms	Ru complex	m(Au+Ag)/m(Ru complex)
AuNRs@Ru	40.8±1.3	3.1±0.1	6.0±0.2	7.3
AuNTs@Ru	40.6±0.8	NO	9.4±0.1	4.3

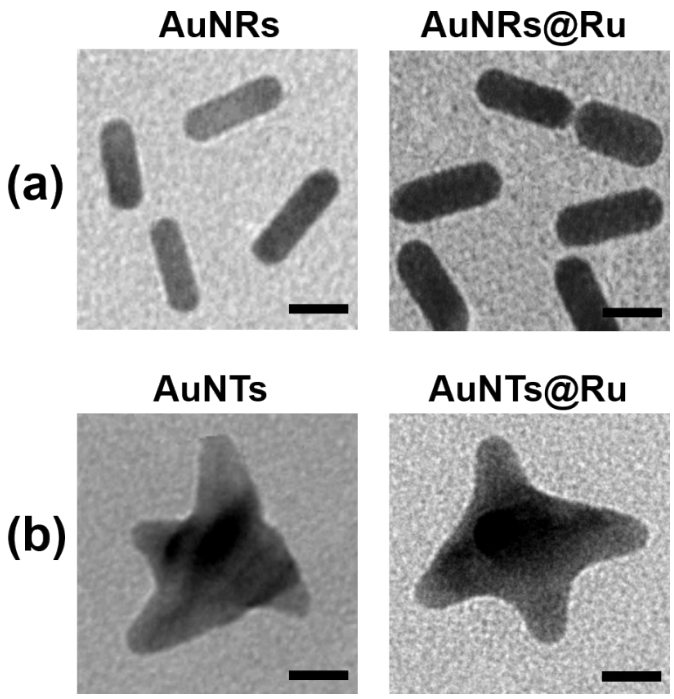

Figure S1. The high resolution TEM (HR-TEM) images of the (a) AuNRs and (b) AuNTs before and after modification with the Ru complex.

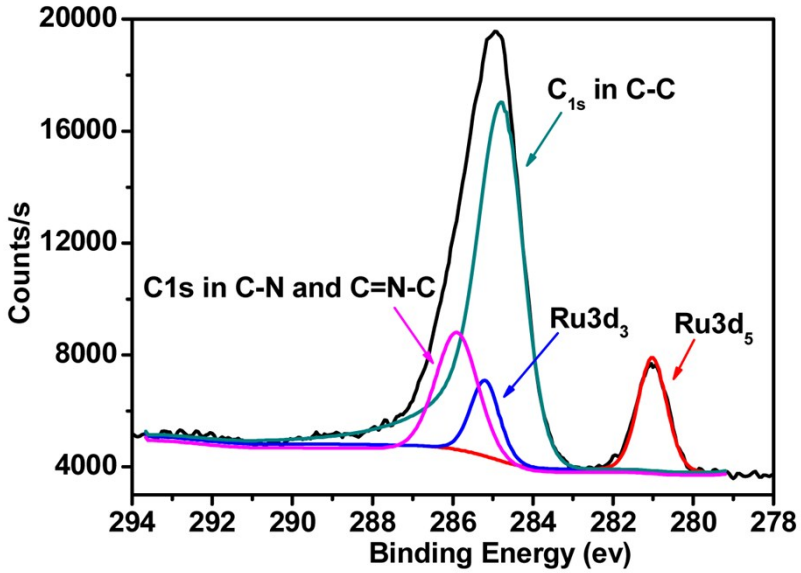

Figure S2. Energy distributions of the elements Ru_{3d} and C_{1s} in the AuNRs@Ru as determined by XPS measurements.

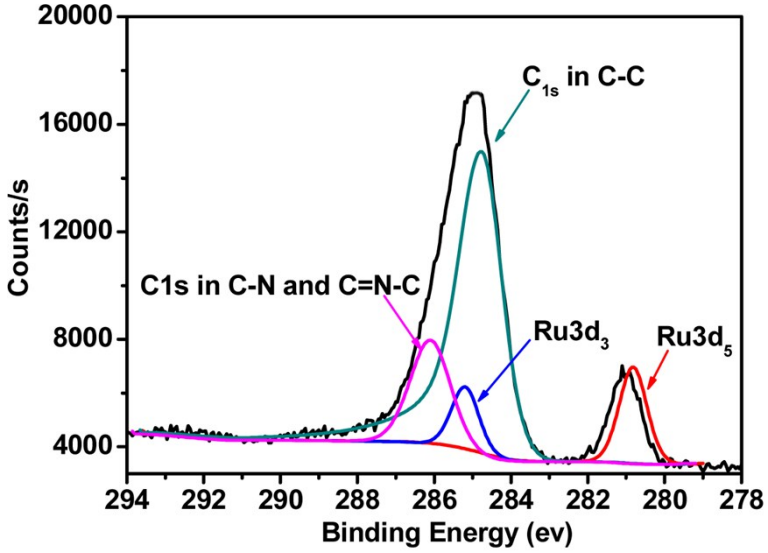

Figure S3. Energy distributions of the elements Ru_{3d} and C_{1s} in the AuNTs@Ru as determined by XPS measurements.

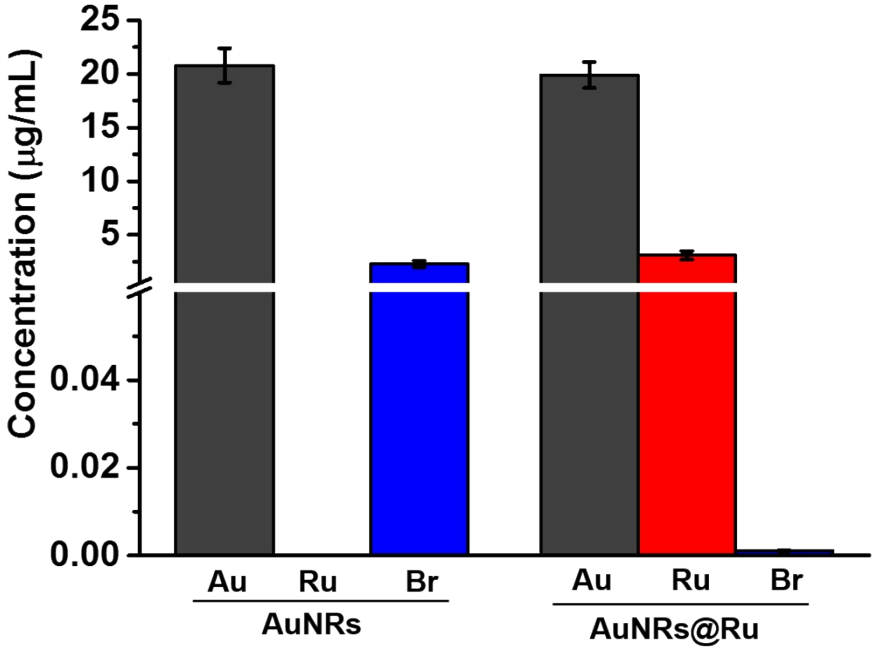

Figure S4. ICP-MS measurement of the elements of AuNRs before and after Ru complex modification.

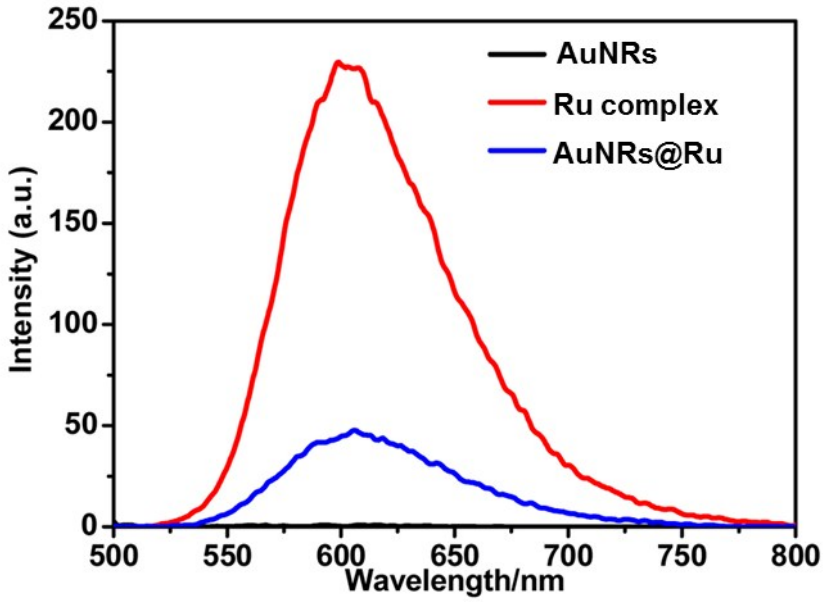

Figure S5. Luminescence spectra of the Ru(II) complex, AuNRs and AuNRs@Ru at 460 nm excitation.

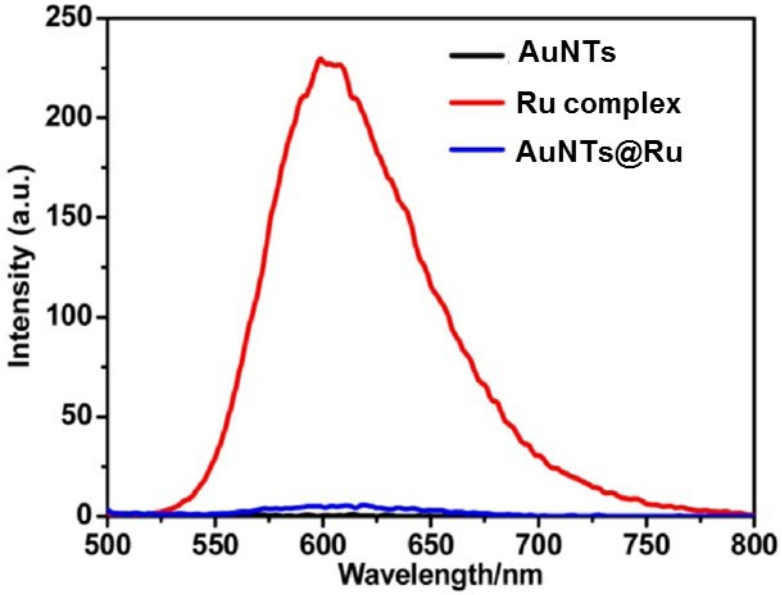

Figure S6. Luminescence spectra of the Ru(II) complex, AuNTs and AuNTs@Ru at 460 nm excitation.

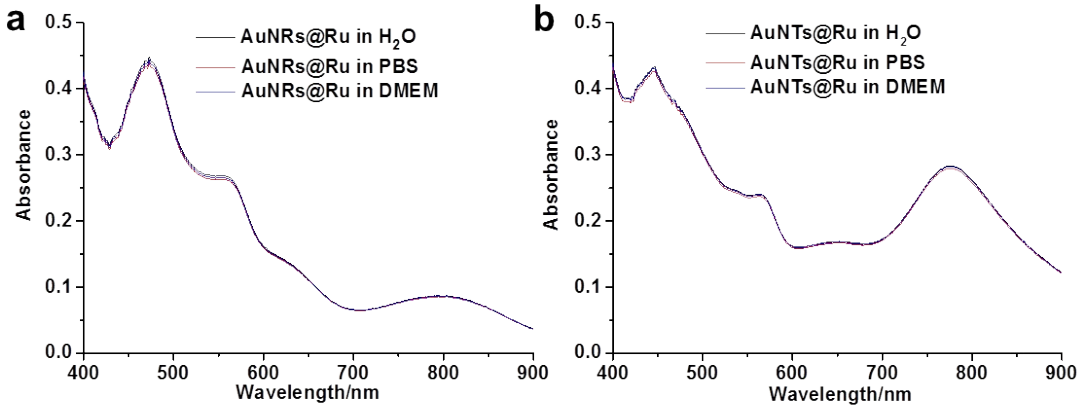

Figure S7. UV-vis-NIR spectra of the AuNTs@Ru/AuNTs@Ru in H_2O, PBS and DMEM medium for 72 h.

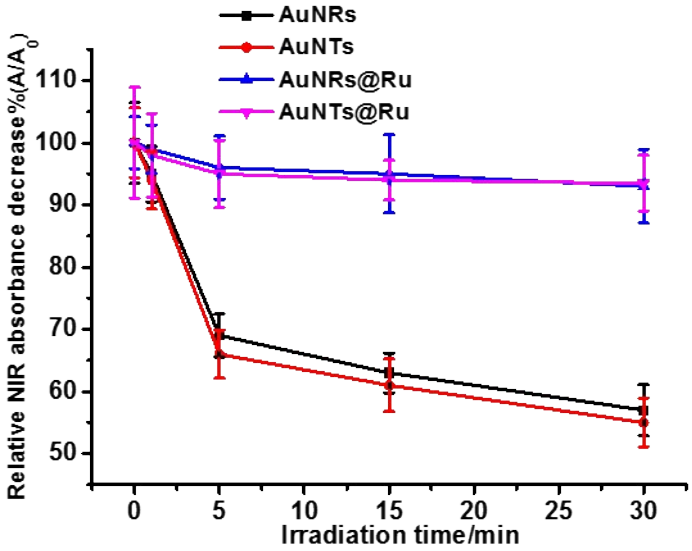

Figure S8. The photothermal stability of AuNRs@Ru and AuNTs@Ru in comparison with AuNRs and AuNTs; extinction peak shifts as a function of irradiation time (0-30 min, 0.25 W/cm^2) were recorded.

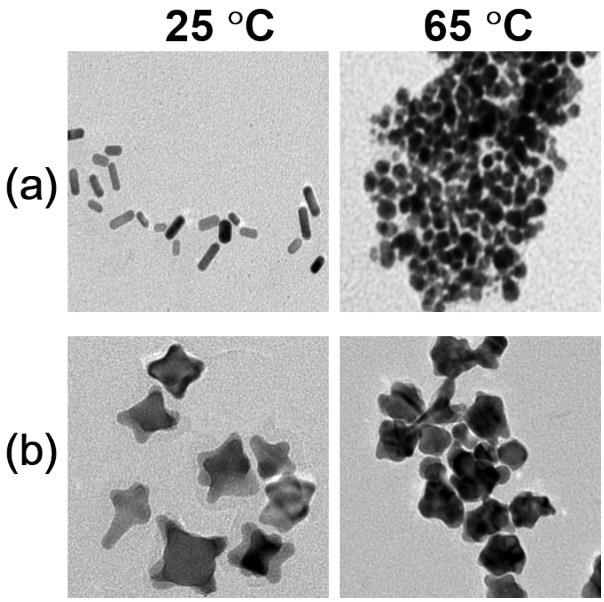

Figure S9. The stability of AuNRs (a) and AuNTs (b) at 25 °C and 65 °C incubation for 30 min, respectively.

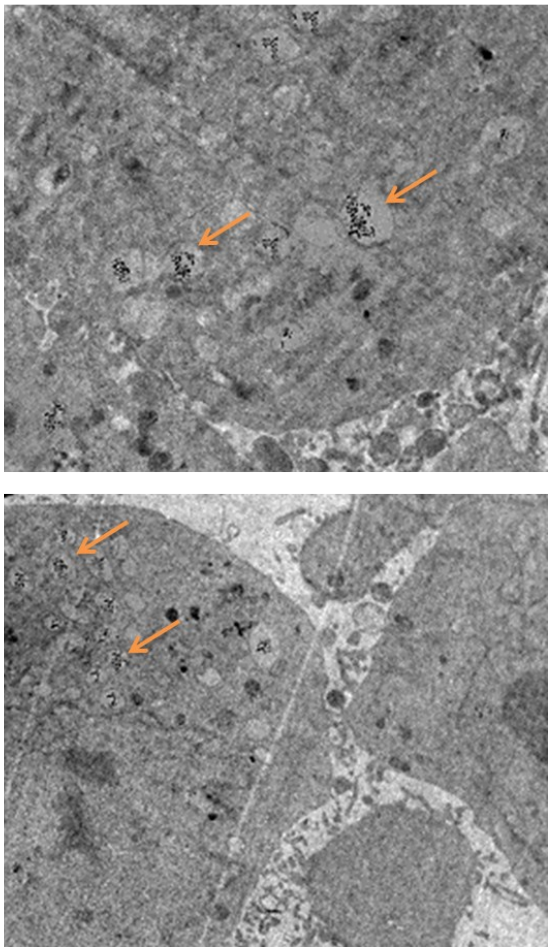

Figure S10. TEM images showed cellular uptake of the AuNRs@Ru (upper) and AuNTs@Ru (below) in the cells and an endosome (yellow arrows) after entering into cells.

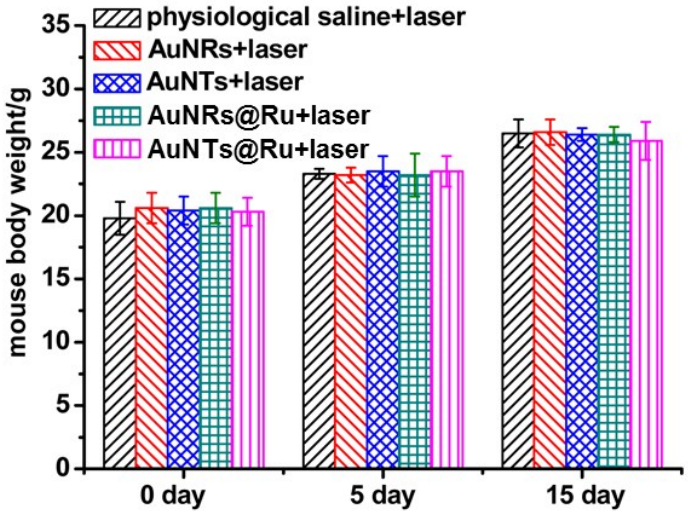

Figure S11. The body weights of mice after various treatments. Error bars represent the standard deviation of 8 mice per group (Irradiation at 808 nm, 0.25 W/cm^2).

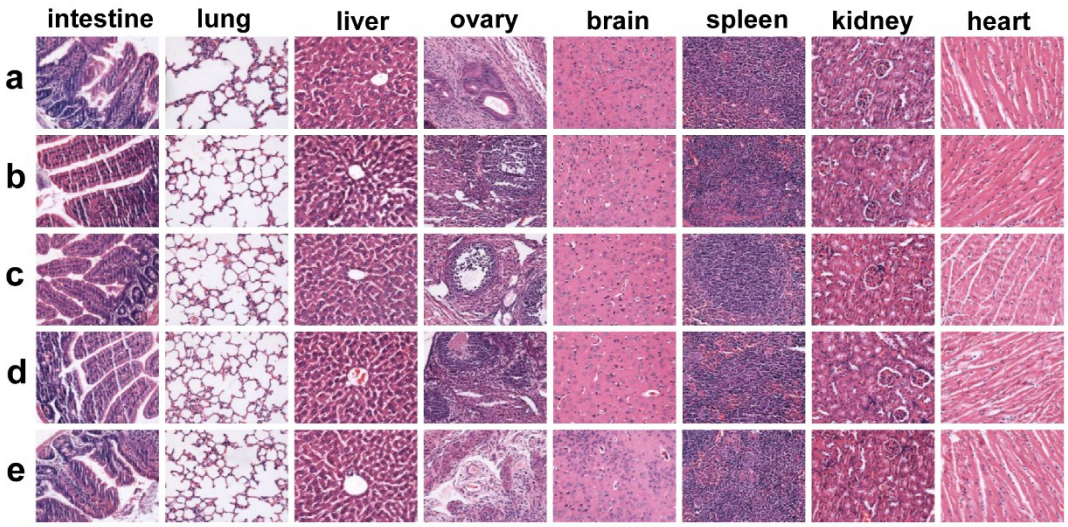

Figure S12. Histological examination of primary organs (a) physiological saline+Laser; (b) AuNRs+Laser; (c) AuNTs+Laser; (d) AuNRs@Ru +Laser; (e) AuNTs@Ru +Laser (Including intestine, lung, liver, ovary, brain, spleen, kidney and heart). Sections for light microscopy were stained with hematoxylin-eosin (H&E). Samples were from three different mice of the experiment. Magnification was 200×.