Electronic Supplementary Material (ESI) for Chemical Communications.

Supporting Information

Nucleus-targeting ultrasmall ruthenium(IV) oxide nanoparticles for photoacoustic imaging and low temperature photothermal therapy in the NIR-II window

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Experimental Section

Materials

Ruthenium(III) chloride trihydrate ($RuCl_3 \cdot 3H_2O$), and Ru standard solution were purchased from Aladdin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), polyvinylpyrrolidone (**PVP**, 600), and Hochest 33342, were purchased from Sigma. Calcein-AM, and propidium iodide (PI) were purchased from Thermo Fisher Scientific. HSP90 was purchased from Abcom. Chitosan (**CS**) and bull serum albumin (**BSA**) were purchased from Energy Chemical.

Characterization

TEM (Transmission Electron Microscopy) and the corresponding EDS (Energy Dispersive Spectrometry) mapping were performed on a JEOL JEM-ARM200P microscope. The elemental concentration of Ru was measured by inductively coupled plasma mass spectrometry (ICP-MS) on a Thermo Fisher Scientific iCAP RQ series spectrometer. A Malvern zetasizer Nanoseries was used to record the zeta potential and hydrodynamic particle size. Infrared spectrometer experiments were performed on a Thermo-Nicolet Nexus 670. X-ray photoelectron spectra of the samples were acquired on a Thermo Fisher Scientific ESCALab250. Confocal laser scanning microscope images were acquired on a LSM 810 NLO, Carl Zeiss AG. Photothermal effects were measured using a 1064 nm laser (Wave Particle Technology). And the thermal imaging and temperature detection were recorded using a FLIR A325SC infrared thermal imaging camera. Photoacoustic (PA) signal collection *in vitro* and *in vivo* PA imaging of tumors were acquired from TomoWave Laboratories (LOIS-3D, USA). Positive fluorescence microscope were acquired from Carl Zeiss AG (AX10). Milli-Q water was obtained from a Milli-Q system of Millipore Company.

Synthesis of RuO_2NPs

The ultrasmall CS-RuO_2NPs were synthesized by the solvothermal method. Chitosan (**CS**) (30 mg) was dissolved in 3% acetic acid (10 mL). $RuCl_3 \cdot 3H_2O$ (5 mL, 2.5 mg/mL) was then added to the **CS** solution, before stirring for 30 min. The mixture was placed in a round-bottomed flask and heated to 120 °C for 6 h. The dark green solution were obtained and dialyzed for 48 hours after collection.

2 nm-**PVP-RuO_2NPs** were prepared using the same method, with **PVP**(4 mg/mL) instead of **CS**.

The 2 nm-**BSA-RuO_2NPs** were synthesized by the reported method[1] with little modification. $RuCl_3 \cdot 3H_2O$ solution (5 mL of 2.5 mg/mL) was added to a 50 mL beaker, along with **BSA** (15 mL of 2 mg/mL), then fresh $NaBH_4$ (3 mL, 0.01 mol/L) was added dropwise with stirring until the solution turned dark brown. The ~25 nm **CS-RuO_2NPs** were prepared by the hydrothermal method. $RuCl_3 \cdot 3H_2O$ solution (5 mL of 2.5 mg/mL) was added to a 50 mL Teflon reactor, **CS** (10 mL of 4 mg/mL) was added, and then the mixture was heated at a temperature of 80 °C for 12 h.

Photothermal Performance of the Ultrasmall CS-RuO_2NPs

To assess the photothermal performance of the ultrasmall CS-RuO_2NPs, various concentrations of ultrasmall CS-RuO_2NPs (from 10 to 50 μg/mL) aqueous solution (200 μL) were placed in a 1.5 ml centrifuge tube and irradiated with a 1064 nm laser with power density of 1.0 W/cm^2. Various power densities were also assessed with 30 μg/mL ultrasmall **CS-RuO_2NPs** (200 μL). The temperature and the thermal images at different times were recorded and obtained by an infrared camera. The photothermal conversion efficiency of ultrasmall **CS-RuO_2NPs** was measured according to the literature method.[2]

The absorbance of ultrasmall **CS-RuO_2NPs** at 1064 nm was measured. 1 mL ultrasmall **CS-RuO_2NPs** solution (30 μg/mL) was prepared added to a cuvette and irradiated with a 1064 nm laser, followed by natural cooling after the laser was turned off. In Equation 1., m is the solution mass and equal to 1.0 g, c stands for the heat capacity of water which equal to 4.2 J·g^{-1}, T_{max} and T_{sur}, are the maximum temperatures of ultrasmall **CS-RuO_2NPs** and water, respectively. I represents the power density of the laser, and τ_s is the system time constant which

calculated according to the linear regression of the cooling profile. In order to obtain τ_s, a dimensionless driving force temperature, ϑ, was introduced, which was calculated using Equation 2. τ_s was then obtained with Equation 3.

Equation 1.

$$\eta = \frac{mc \cdot (T_{max} - T_{sur})}{I \cdot (1 - 10^{-A}) \cdot \tau s}$$

Equation 2.

$$\theta = \frac{T - T_{sur}}{T_{max} - T_{sur}}$$

Equation 3.

$$T = -\tau_s \cdot ln\theta$$

MCF-7 Cells Culture Conditions

MCF-7 cells were maintained as monolayer cultures in high-glucose Dulbecco's modified eagle medium (DMEM, Gibco, USA) supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin streptyomycin at 37 °C in a humidified atmosphere under 5% CO_2.

Cell Uptake and Nucleus Targeting

To analyze the cell uptake and distributions of the **RuO_2NPs** with different charges and sizes. MCF-7 cells were incubated with 80 μg/mL **RuO_2NPs** at various time, and then the cells were collected and the concentration of Ru measured by ICP-MS. In order to observe the process of cell uptake and determine the cell nucleus targeting ability, MCF-7 cells were incubated in 10 cm dishes with ultrasmall **CS-RuO_2NPs** (60 μg/mL) for 4 h, then the cells were collected, fixed and sliced before measurement by bio-TEM.

Cell Viability

Cell viability analysis was carried out on MCF-7 cells incubated in 96-well plates and cultured for 24 h. The culture media was refreshed with media containing ultrasmall **CS-RuO_2NPs** at various concentrations (0, 20, 40, 60, 80, 100, 120 μg/mL) and the cells incubated for a further 4 h. The cells were then washed with phosphate buffered saline (PBS) 3 times, and the cells cultured in fresh media for 12 h. The 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) method was used to measure the cell viabilities.[3]

Live/Dead Cell Imaging

MCF-7 cells were incubated with ultrasmall **CS-RuO_2NPs** (60 μg/mL) for 4 h, before being washed three times and the culture medium refreshed. The MCF-7 cells were then irradiated using a 1064 nm laser at different power densities (0.8, 0.9, 1.0 W/cm^2) for 10 min, and then co-stained with calcein-AM (live cells, green) and PI (dead cells, red) for 30 min. The cells were then washed before imaging using an inverted fluorescence microscope.

The Morphology of MCF-7 Nuclei Before and After Irradiation

MCF-7 cells were seeded in 2 cm dishes and cultured for 24 h. The culture medium was exchanged with fresh DMEM containing ultrasmall **CS-RuO_2NPs** (60 μg/mL), and the cells incubated for 4 h. The culture medium was then removed and fresh medium was added, the MCF-7 cells were then irradiated using a 1064 nm laser at

power density of 1.0 W/cm^2 for 10 min. And then the cells washed three times with PBS before fresh culture medium containing Hoechst dye was added. After 30 mins incubation confocal laser scanning microscopy was used to observe the morphology of the nuclei.

Western Blot

To analyze the expression level of HSP 90, MCF-7 cells were seeded into 10 cm dishes. After incubation for 24 h, the cells culture medium was refreshed and given different treatments: none (control), hyperthermia at 45 °C, ultrasmall **CS-RuO2NPs**, and ultrasmall **CS-RuO$_2$NPs** with NIR irradiation. The cells were then collected and Laemmle Sample Buffer was applied to lyse the cells. A BCA protein assay Kit (manufacturer) was used to quantify the total protein concentration. And then the experimental procedures of western blotting were performed according to the literature.[4]

In vivo PAI

PA imaging was performed using TomoWave Laboratories (LOIS-3D, USA). A tumor-bearing mouse was anaesthetized and after intratumoral injection of ultrasmall **CS-RuO$_2$NPs** with a volume of 20 μL at a dose of 2 mg/kg. At 1064 nm laser irradiation, images were recorded at various time.

In vivo PTT

When the volume of tumor reached about 150 mm^3 after ten days of MCF-7 cells implant, the female nude mice (4 weeks old, Beijing Vital River Laboratory Animal Technology Co., Ltd.) were divided into 4 groups (n = 5), and the tumor bearing mice were intratumorally injected with PBS only (20μL) , PBS (20μL) + laser only, ultrasmall **CS-RuO$_2$NPs** (20μL, 2 mg/mL) only and ultrasmall **CS-RuO$_2$NPs** (20μL, 2 mg/mL) combined with 1064 nm laser irradiation . After 4 h, the tumor location was irradiated with a 1064 nm laser (1.0 W/cm^2) for 10 min and maintained the temperature at the tumor site at approximately 45-46 ℃. The body weights and the tumor size of mice were recorded every three day for 18 days.

Histological Examination

At the end of experiments, mice from four groups were sacrificed, and tissues (heart, liver, spleen, lung, kidney and tumor) of mice were collected, fixed with paraformaldehyde solution (4%, PBS), embedded by paraffin, and finally treated with hematoxylin and eosin (H&E) staining. The stained tissues were observed by a positive fluorescence microscope.

Statistical Analysis

Data were presented as mean result ± standard deviation, and significance was assessed with each experiment was subjected to statistical analysis by the Student–Newmann–Keuls analysis of variance and with the *t*-test for grouped data. Differences were considered significant at *P* less than 0.05.

Ethics Statement

This study was performed with the approval of the Experimental Animal Manage Committee (EAMC) of Sun Yat-Sen University. Animals were treated as the guidelines of EAMC.

Supporting Figures and Tables

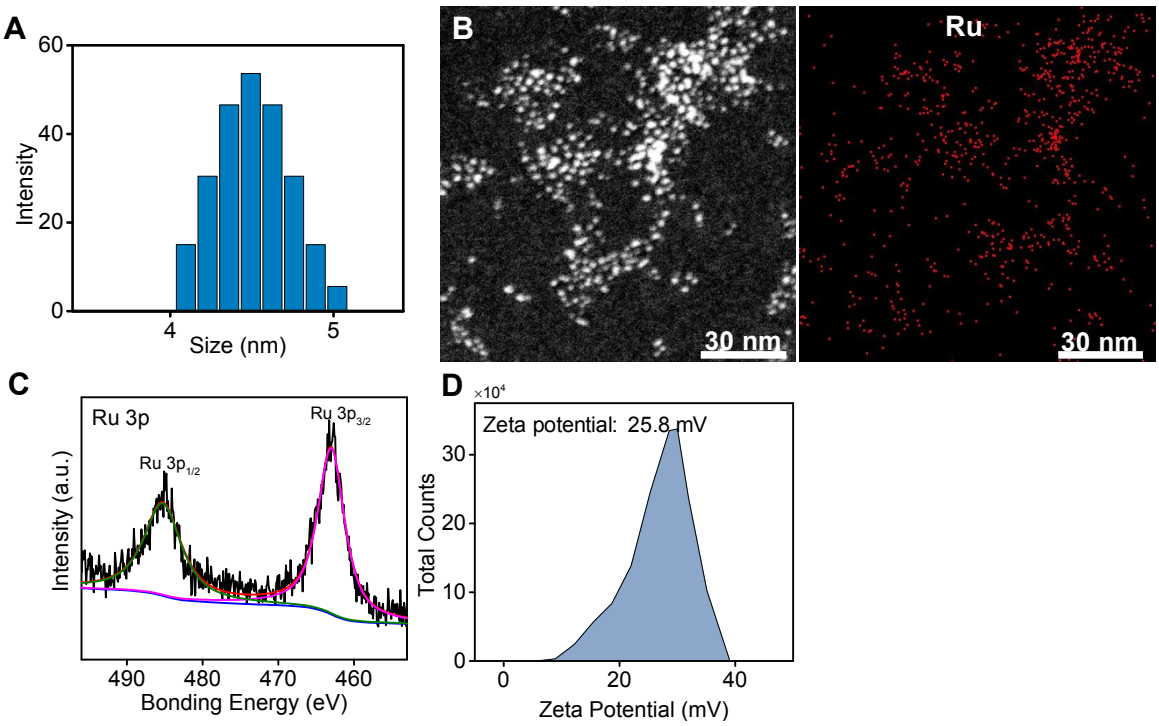

Figure S1. A) Size distribution of ultrasmall **CS-RuO$_2$NPs**. B) EDS mapping of ultrasmall **CS-RuO$_2$NPs**. C) XPS spectra of Ru 3p peak of ultrasmall **CS-RuO$_2$NPs**. D) The zeta potential of ultrasmall **CS-RuO$_2$NPs**.

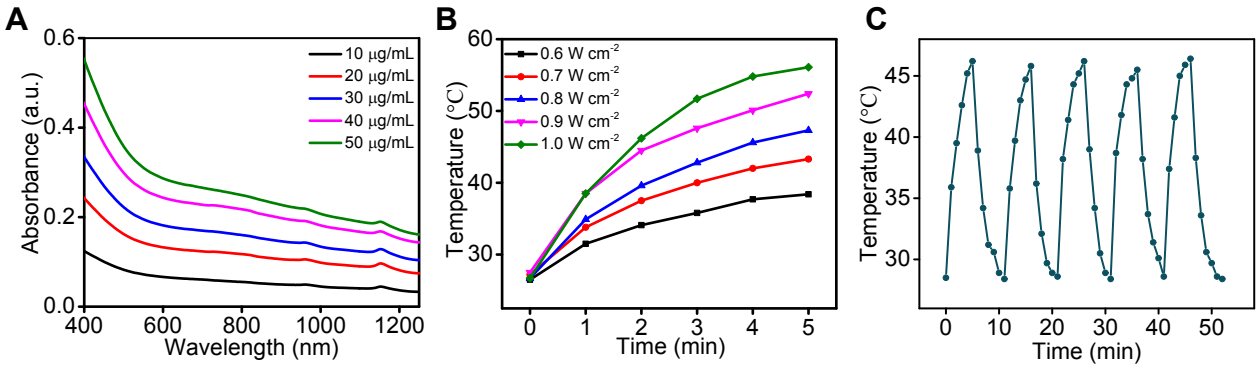

Figure S2. A) UV-vis-NIR absorption spectra of ultrasmall **CS-RuO$_2$NPs** at different concentrations. B) Photothermal heating curves with varied power densities. C) Heating curves of ultrasmall **CS-RuO$_2$NPs** for five times laser on/off cycles (1.0 W cm^{-2}) under the irradiation with 1064nm laser.

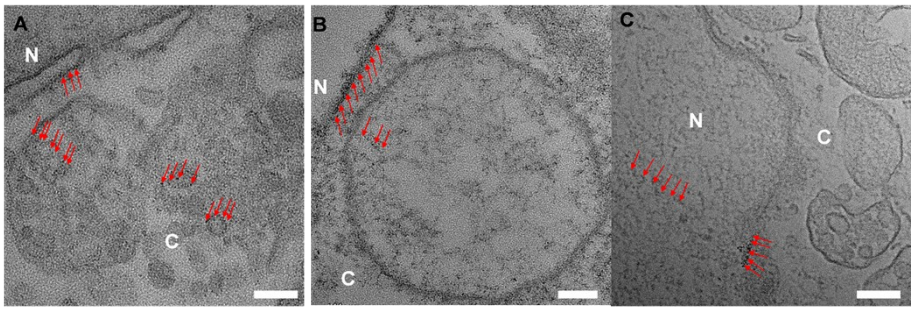

Figure S3. Localization of ultrasmall **CS-RuO_2NPs** in MCF-7 cells over time A) 0.5 h, B) 1 h, C) 2 h. Scale bar:100 nm

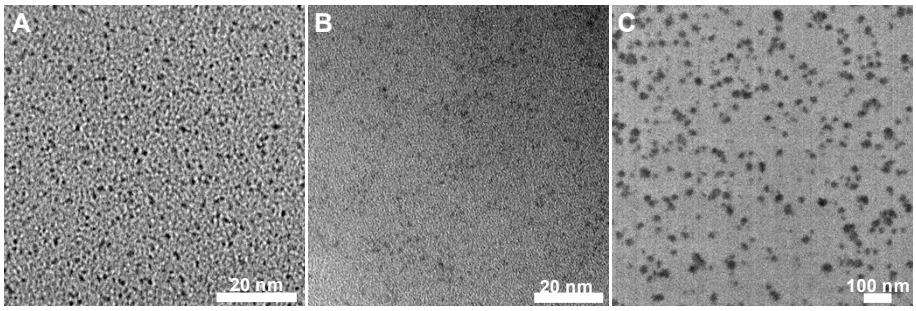

Figure S4. TEM characterization of 2 and 25 nm **RuO_2NPs** with different sizes and surface charges. A) The TEM of 2 nm **PVP-RuO_2NPs**, B) The TEM of 2 nm **BSA- RuO_2NPs**, C) The TEM of 25 nm **CS- RuO_2NPs**.

Table S1. The zeta potentials of RuO_2NPs with different sizes and surface charges.

Sample	Diameter (nm)	Zeta Potential (mV)
CS-RuO_2NPs	2	25.8
PVP-RuO_2NPs	2	2.3×10^{-3}
BSA-RuO_2NPs	2	-18.5
CS-RuO_2NPs	25	23.5

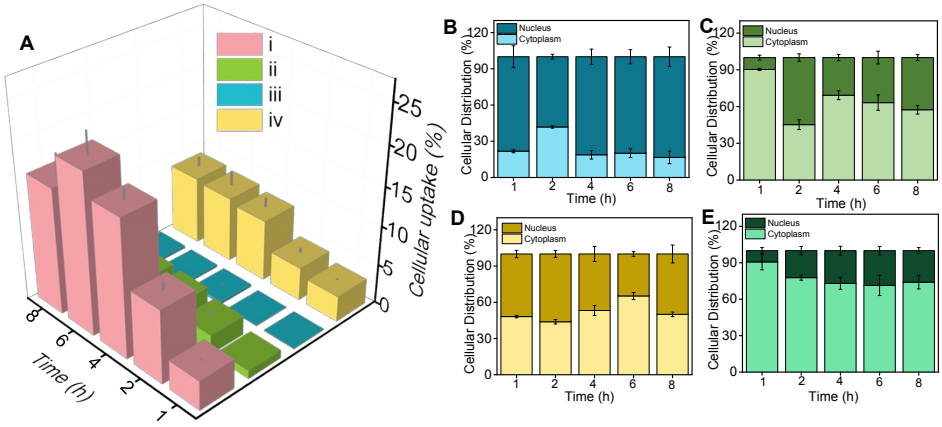

Figure S5. A) The cellular uptake of **RuO_2NPs** at different times. i: 2 nm **CS-RuO_2NPs**, ii: 2 nm **BSA-RuO_2NPs**, iii: 2 nm **PVP-RuO_2NPs**, iv: 25 nm **CS- RuO_2NPs**. B) The cellular distributions of 2 nm **CS-RuO_2NPs.** C**)** The cellular distributions of 2 nm **BSA-RuO_2NPs.** D) The cellular distributions of 2 nm **PVP-RuO_2NPs.** E) The cellular distributions of 25 nm **CS-RuO_2NPs.**

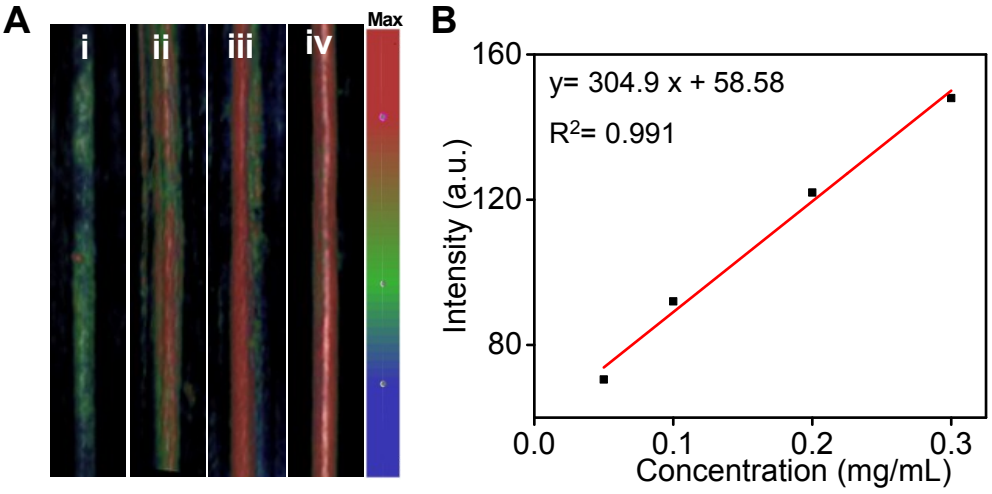

Figure S6. A) *In vitro* PA images, B) the quantitative curve of PA intensity of ultrasmall **CS-RuO_2NPs** at different concentrations under 1064 nm laser source. i. 50 μg/mL, ii. 100μg/mL, iii. 200 μg/mL, iv. 300 μg/mL.

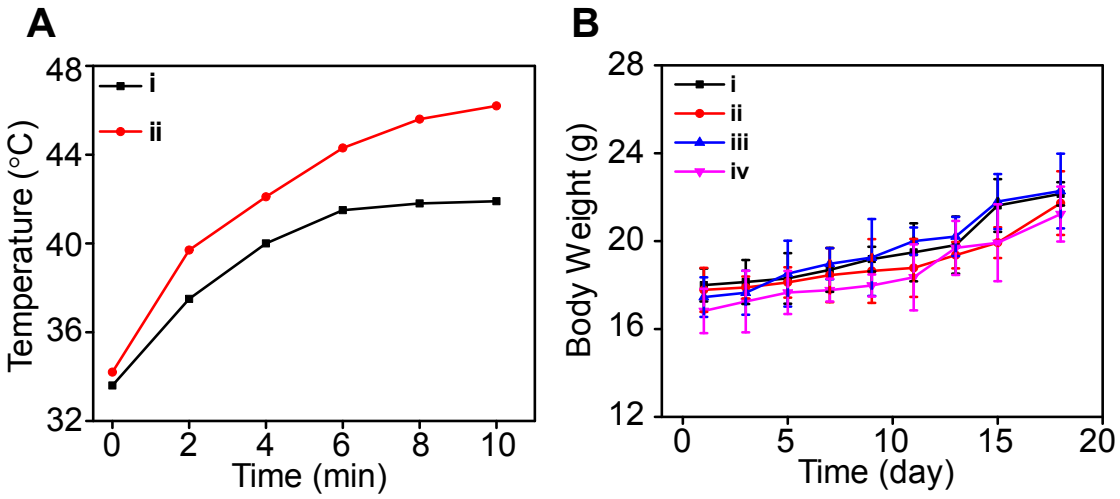

Figure S7. A) Temperature change of tumor site in mice injected with i) PBS or ii) ultrasmall **CS-RuO$_2$NPs**, with 1064 nm laser irritation. B) body weight of mice after different treatments. i) PBS only, ii) PBS with 1064 nm laser irradiation for 10 min, iii) ultrasmall **CS-RuO$_2$NPs**, iv) ultrasmall **CS-RuO$_2$NPs** with 1064 nm laser irritation for 10 min.

Figure S8. Photograph of tumors collected from four groups of mice 18 d after the following treatments: i) PBS, ii) PBS+NIR, iii) ultrasmall **CS-RuO$_2$NPs**, iv) ultrasmall **CS-RuO$_2$NPs**+NIR.

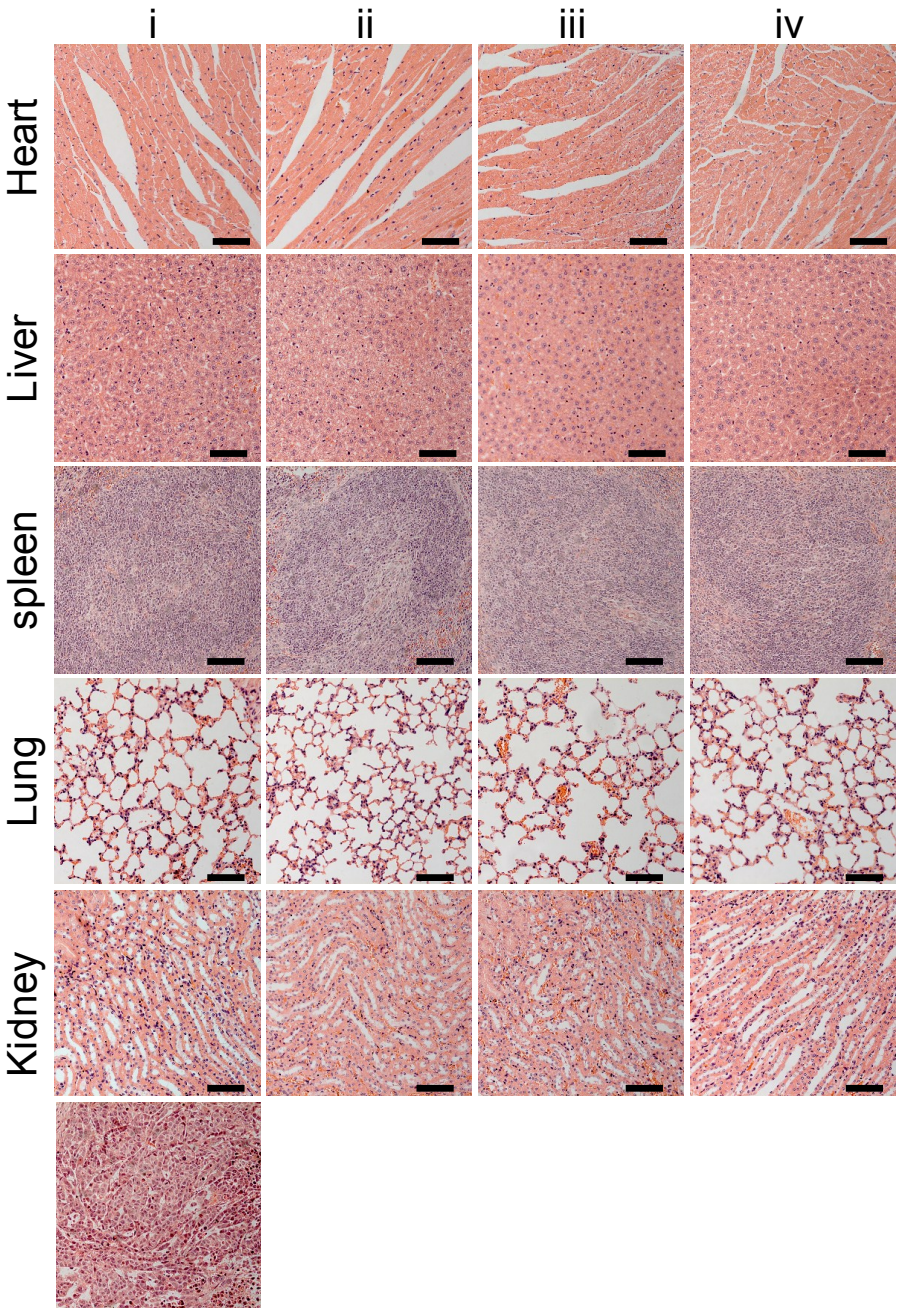

Figure S9. H&E stained slices of organs (heart, liver, spleen, lung, kidney), and tumors from the mice of the following treatment groups: i) PBS, ii) PBS+NIR, iii) ultrasmall **CS-RuO_2NPs**, iv) ultrasmall **CS-RuO_2NPs**+NIR. Scale bar: 50 μm.

References

[1] X. Q. Huang, G. J. Chen, J. L. Pan, X. Chen, N. Huang, X. Wang, J. Liu, *J. Mater. Chem. B.* **2016**, 4, 6258

[2] D. K. Roper, W. Ahn, M. J. Hoepfner, *Phys. Chem. C*. **2007**, 111, 3636.

[3] Y. Cheng, F. Yang, G. Xiang, K. Zhang, Y. Cao, D. Wang, H. Dong, X. Zhang, *Nano letters,* **2019**, 19, 1179-1189.

[4] R. R. Ye, C. P. Tan, Y. N. Lin, L. N. Ji and Z. W. Mao, *Chem. Commun*. **2015**, 51, 8353-8356.