Electronic Supplementary Material (ESI) for Nanoscale.

Cobalt Carbide-Based Theranostics for In Vivo Multimodal Imaging Guided Photothermal Therapy

Dong-Yang Zhang, Han Xu, Ting He, Muhammad Rizwan Younis, Leli Zeng, Hengke Liu, Chao Jiang, Jing Lin and Peng Huang*

Marshall Laboratory of Biomedical Engineering, International Cancer Center, Laboratory of Evolutionary Theranostics (LET), School of Biomedical Engineering, Shenzhen University Health Science Center, Shenzhen, 518060, China

AUTHOR INFORMATION

The authors declare no competing financial interest.

All authors have given approval to the final version of this manuscript.

*Address correspondence to peng.huang@szu.edu.cn.

Experimental section

Materials. Hexadecyl trimethyl ammonium bromide (CTAB) was purchased from Aladdin Reagent (China). Cobalt carbonyl was obtained from Energy Chemical (China). Oleylamine (OM) and chloroform were bought from JK Chemical (China). 1,2-diastearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)] (DSPE-PEG-CH_3) was acquired from Pengshuo biotechnology co., LTD (China). PI/calcein acetomethoxy Assay Kit was bought from Sigma-Aldrich. All reagents were of analytical grade and used without any purification. Deionized water, purified by a Milli-Q water purification system (Millipore, USA) to a minimum resistivity of 18.2 MΩ/cm, was used in all the experiments.

Synthesis of Co_2C NPs. In a three-neck flask, a mixture of OM (14.5 g) and CTAB (0.113 g) was stirred vigorously and degassed under a flow of N_2 at 120 ^oC. The mixture was cooled to 60 ^oC, and then 0.3 g of $Co_2(CO)_8$ was added quickly to the reaction mixture. The mixture was heated to 200 ^oC and kept at this temperature for 20 min. Subsequently, the mixture was further heated to 320 ^oC and kept at this temperature for 30 min before cooled down naturally at room temperature. The as-synthesized NPs were washed with ethanol and hexane and collected for characterization.

Synthesis of Co_2C-PEG. For PEGylation, Co_2C NPs (20 mg) were dispersed in chloroform (45 mL) and mixed with DSPE-PEG-CH_3 (80 mg) in chloroform (5 mL). The mixture was sonicated for 2 h, stirred at room temperature overnight and dried with N_2. Then, the obtained black solid was re-dispersed in H_2O (5 mL). The excess PEG was removed by centrifugation at 12000 rmp. The precipitate was collected and washed three times with H_2O to obtain the Co_2C-PEG NPs. The product was dissolved in phosphate buffered saline (PBS) and stocked at 4 °C for future use.

Characterization. The sample morphology was observed by the transmission electron microscope (TEM, FEI Tecnai G2 Spirit, Holland). The sample crystallinity was analyzed by the powder X-ray diffraction (XRD, Japan RigakuD/max-2500 diffractometer) measurement. The hydrodynamic size was measured by dynamic light scattering (DLS, 90Plus/BI-MAS instrument, Brookhaven Instruments Co., USA). X-

ray photoelectron spectra (XPS) were carried out on an SSI S-Probe XPS spectrometer with Al Kα radiation as X-ray source (1486 eV). The size distribution of Co_2C-PEG NPs was recorded on a laser granulometer (Zetasizer Nano S90, Malvern, UK) equipped with a He-Ne laser. The optical properties were characterized by the UV/vis-NIR absorption spectra (Cary 60, Agilent Technologies, USA) and FT-IR infrared spectra (L16000300 Spectrum TWO LITA, Llantrisant, UK) measurements, respectively. PA imaging was performed on a VisualSonics Vevo LAZR system (VisualSonics Inc. New York, NY).

Measurement of Photothermal Performance. To measure the photothermal performance of Co_2C-PEG NPs, 200 μL Co_2C-PEG NPs (0, 20, 40, 80 μg/mL of Co concentration) aqueous dispersion was irradiated with an 808 nm NIR laser for 5 min at a power density of 1 W/cm^2. Light-induced temperature changes in the solutions were recorded using an infrared thermal imager. The photothermal conversion efficiency of the Co_2C-PEG NPs was calculated by the method reported by Roper *et al*. [1]

Cell Lines and Culture Conditions. The 4T1, U87 and MCF-10A cells were obtained from the Experimental Animal Center of Shenzhen University (Shenzhen, China). Cells were maintained in dulbecco's modified eagle medium (DMEM) (Gibco BRL), which contained 10% FBS (fetal bovine serum, Gibco BRL), 100 μg/mL streptomycin (Gibco BRL), and 100 U/mL penicillin (Gibco BRL). The cells were cultured in a humidified incubator, which provided an atmosphere of 5% CO_2 and 95% air at a constant temperature of 37 °C.

Animals and Tumor Model. Female BALB/c nude and BALB/c mice were obtained from Beijing Vitalriver Experimental Animal Technology Co. Ltd. with body weights of 19~21 g and housed in stainless steel cages under the standard conditions (20 ± 2 ^{o}C room temperature, 60 ± 10% relative humidity) with a 12 h light/dark cycle. Mice were injected with 10^7 4T1 cells (0.1 mL cells in PBS) subcutaneously at the right rear flank region. The tumor sizes were measured using a vernier caliper and calculated as: volume = length × width^2/2.

Cytotoxicity and Hemolysis assays. The 4T1, U87 and MCF-10A cells were seeded in 96-well plates and treated with different concentrations of Co_2C-PEG NPs. After 20 h, 20 μL MTT (5 mg/mL) solution was added to each well. The plates were incubated in the dark for an additional 4 h. The medium was carefully removed and DMSO was added (150 μL per well). The absorbance at 595 nm was measured using a microplate reader. The cells treated under identical conditions in dark were kept as control groups. Cell viability was calculated by the following formula: (viable cells)% = (OD of treated sample/OD of untreated sample) × 100%.

Fresh blood was drawn from healthy mice *via* eyeball blood extraction. The blood was diluted and washed with PBS. The Co_2C-PEG NPs were dispersed in PBS with various concentrations (37.5, 75, 150, 300, and 600 μg/mL of Co). 0.3 mL of the blood were mixed with 1.2 mL sample dispersion and incubated for 3 h at room temperature. After the solutions were centrifuged at 8000 rpm for 5 min, the absorbance at 541 nm of the upper solution was measured on a UV/vis-NIR spectrometer. The hemolysis percent of the RBCs after incubation was determined based on the following formula: hemolysis percentage (%) = $(A_{sample} - A_{PBS})/(A_{DI\ water} - A_{PBS}) \times 100\%$, where A_{sample}, A_{PBS}, and $A_{DI\ water}$ are the 541 nm absorbance values of the sample groups, the PBS group, and the DI water group, respectively.

Photothermal Killing Effect on Cancer Cells. To quantitatively evaluate the photothermal ablation effect, both 4T1 and U87 cells, respectively, were incubated with the different concentrations of Co_2C-PEG NPs dispersion for 4 h, and then irradiated by the 808 nm laser (1 W/cm^2) for 5 min, respectively. After 16 h, 20 μL MTT (5 mg/mL) solution was added to each well. The plates were incubated in the dark for an additional 4 h. The medium was carefully removed and DMSO was added (150 μL per well). The absorbance at 490 nm was measured using a microplate reader. The cells treated under identical conditions in dark were kept as the control groups. Cell viability was calculated by the following formula: (viable cells)% = (OD of treated sample/OD of untreated sample) × 100%.

To further assess the PTT effect, 4T1 or U87 cells were treated with the Co_2C-PEG NPs dispersion (0, 25, 50 μg/mL) for 4 h, followed by NIR laser irradiation (1

W/cm^2) for 5 min. After stained with calcein AM (2 μM) and PI (4 μM), the live and dead cells were observed under the fluorescence microscope.

***In Vitro* and *In Vivo* Measurements of Photoacoustic Performance.** Co_2C-PEG NPs aqueous dispersions with various concentrations of Co element (0, 10, 20, 40, 67, 80) were filled into plastic pipes to detect their PA signal. PAI was conducted on a Vevo 2100 LAZR system with the following parameters: Frequency: 40 MHz; 2D gain: 0 dB; PA gain: 28 dB; excitation wavelength: 808 nm.

For *in vivo* PAI, Co_2C-PEG NPs (20 mg/kg) were injected *via* tail vein into the mice bearing 4T1 tumors, and PA images at a series of time points (0, 2, 4, 8 and 24 h) were recorded by the photoacoustic system. The parameter setting was the same as *in vitro* experiment.

***In Vitro* and *In Vivo* Measurement of MRI Relaxation Properties.** Measurement of T_2-weighted MRI relaxation was conducted in test tubes with a 3T clinical MRI scanner (United Imaging). Co_2C-PEG NPs with various concentrations of cobalt (0.035-0.56 mM) were dispersed in water. MR images were acquired using a spinecho sequence with the following parameters: TR = 5000 ms, 16 different TEs from 20 to 1000 ms, with the step size set at 50 ms, FOV = 80 × 140 mm^2, slice = 1, matrix size = 201 × 352. Post process was carried out on post-processing software, and the selected region of interests (ROIs) in the T_2-mapping were measured with the same size to obtain the signal intensities for each concentration. Based on the inverse relaxation time ($1/T_2$), the resulting r_2 values were measured as a function of cobalt concentration. T_2-mapping image sequence is TR = 5000 ms, TE = 92.4 ms, FOV =40 × 40 mm^2, slice = 15, slice thickness = 1.5 mm, matrix size = 128 × 128.

When the tumor volume reached about 200 mm^3, mice were administered with Co_2C-PEG NPs (20 mg/kg) *via* tail vein. MR images were acquired after 0, 1, 2, 4, 8 and 24 h post-injection. The T_2-weighted images were obtained in a clinic 3T MRI scanner (United Imaging), and the sequence is TR = 5000 ms, TE =92.4 ms, slice thickness = 1.5 mm.

***In Vivo* Photothermal Therapy.** 4T1 tumor-bearing BALB/c nude mice were randomly divided into the following four groups (n = 5): (a) Control (no treatment),

(b) saline + laser, (c) Co_2C-PEG NPs (20 mg/kg) and (d) Co_2C-PEG NPs + laser (20 mg/kg). After 4 h intravenous injection (*i.v.*) of either saline or Co_2C-PEG NPs dispersions, respectively, the mice in groups (b) and (d) were exposed to the 808 nm laser (1.0 W/cm^2) for 5 min. During the irradiation, the IR thermal camera was used to monitor the real time tumor temperature variations. After laser irradiation, the length and width of the tumor were measured every 2 days. The tumor volume was calculated according to the following formula: (tumor volume) = (length) × (width)^2/2. Relative tumor volumes were normalized by V/V_0 (V_0 is the tumor volume at day 0). After 15 days, tumors in all the groups were dissected, photographed and stored for H&E analysis. Finally, the tumor sections slides were observed by a microscope.

Hematological data collections. Hematological data were collected at 14 days post-injection of 20 mg/kg Co_2C-PEG NPs into BALB/c mice *via* tail.

Statistical Analysis. All the biological experiments were performed at least twice with triplicates in each experiment. Representative results were depicted in this report and the data were presented as means ± standard deviations (SD). $P<0.05$ was considered as statistically significant difference between groups.

Reference:

1. D. K. Roper, W. Ahn, M. Hoepfner, *J. Phys. Chem. C*, 2007, **111**, 3636-3641.

Supporting Figures

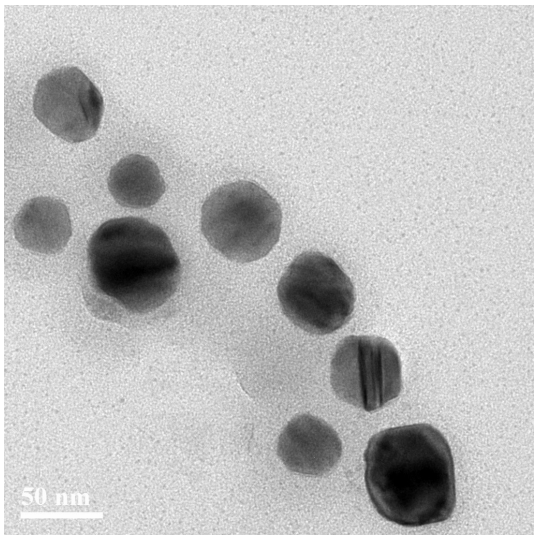

Figure S1. TEM image of Co_2C-PEG NPs. Scale bar: 50 nm.

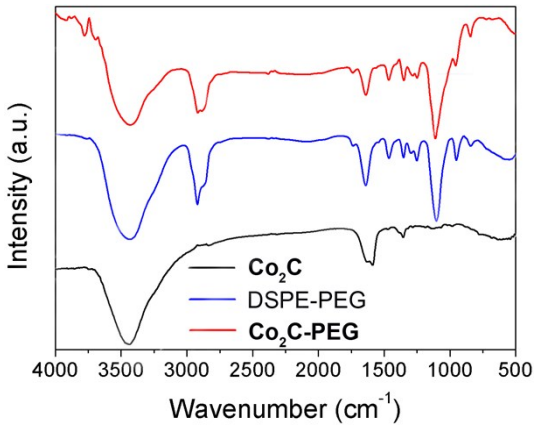

Figure S2. FT-IR spectra of Co_2C NPs, DSPE-PEG and Co_2C-PEG NPs.

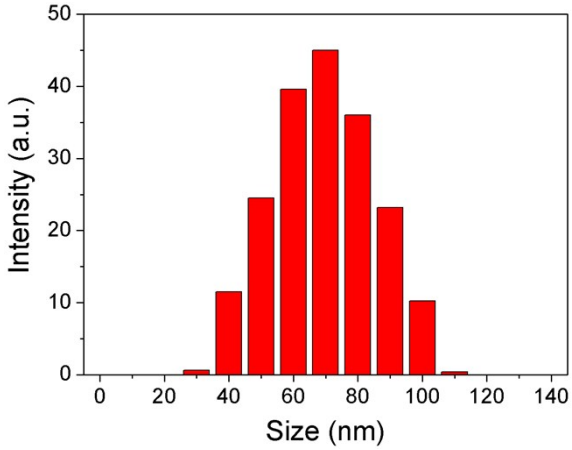

Figure S3. Hydrodynamic diameter of Co_2C-PEG NPs, measured by dynamic light scattering (DLS).

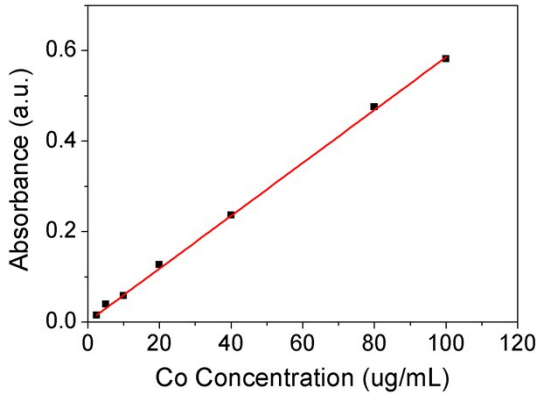

Figure S4. Absorbance at 808 nm for aqueous suspensions of various concentrations of Co_2C-PEG NPs with an optical path of 1 cm. Solid lines are Beer's-law fits for obtaining the molar extinction coefficients.

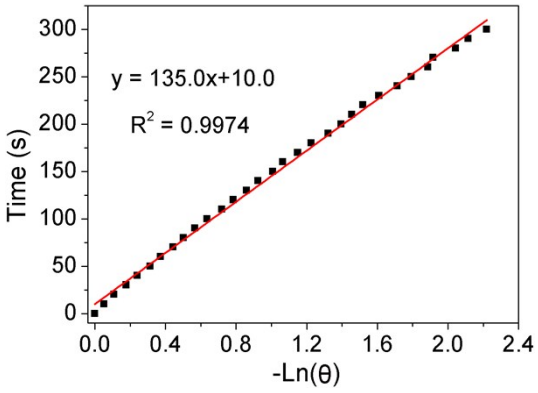

Figure S5. Plot of cooling time versus negative natural logarithm of the temperature driving force which is obtained from the cooling stage. Time constant for heat transfer from the system is determined to be τ_s = 135 s.

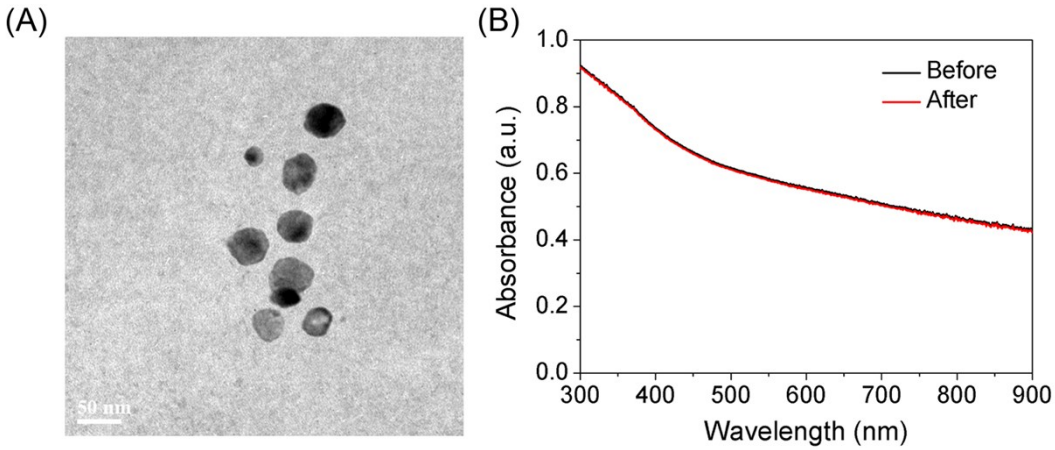

Figure S6. (A) TEM image of Co_2C-PEG NPs irradiated with 808 nm laser at the power density of 1 W/cm^2 for 20 min. (B) UV-vis-NIR spectra of Co_2C-PEG NPs irradiated with 808 nm laser at the power density of 1 W/cm^2 for 20 min.

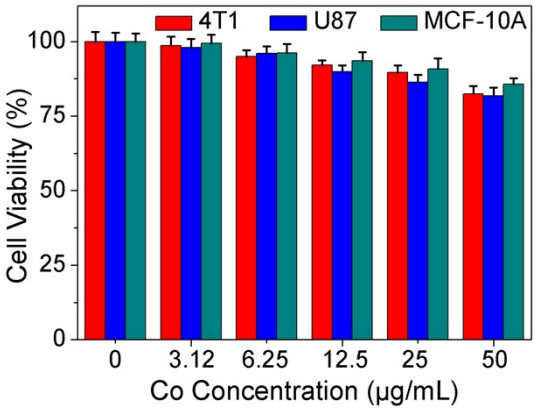

Figure S7. Relative viabilities of 4T1, U87, and MCF-10A cells after incubation with different concentrations of Co_2C-PEG NPs for 24 h.

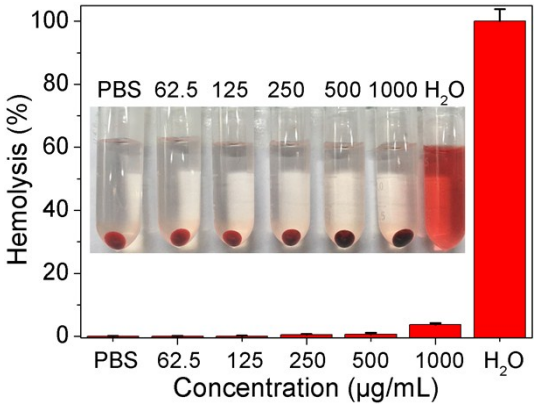

Figure S8. Hemolysis test of the Co_2C-PEG NPs on RBCs.

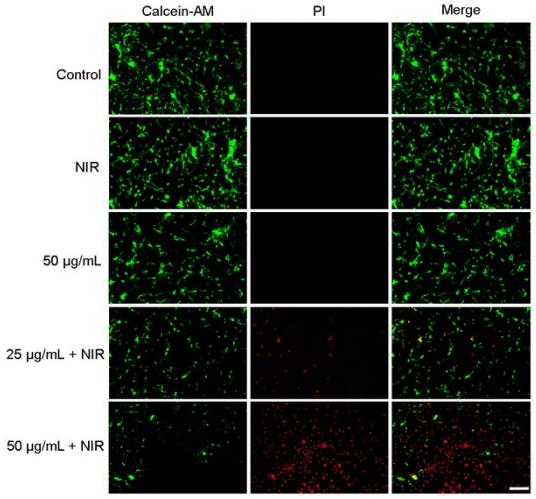

Figure S9. Fluorescence images of U87 cells stained with calcein AM (live cells, green) and PI (dead cells, red) after indicated treatments (scale bar: 100 μm).

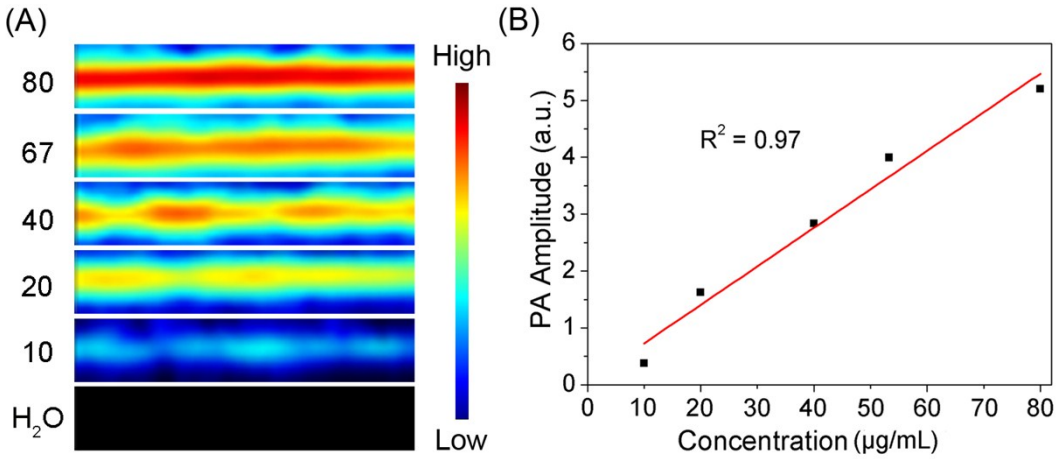

Figure S10. (A) PA images and (B) PA values of the various concentrations of Co_2C-PEG NPs dispersions upon 808 nm laser irradiation.

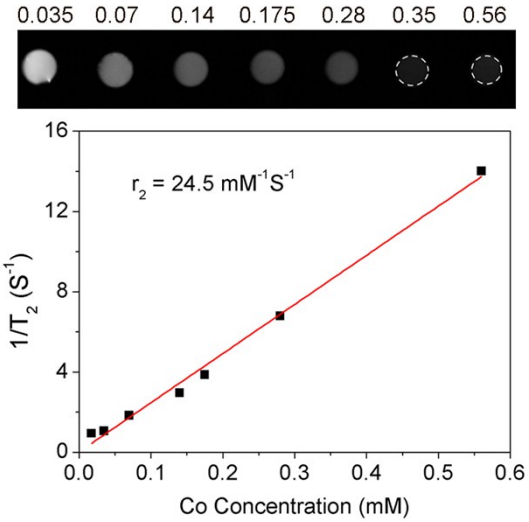

Figure S11. T_2-weighted MR images and T_2 relaxivity of Co_2C-PEG NPs.

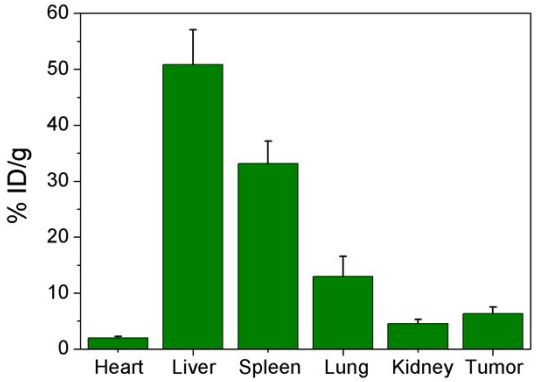

Figure S12. Biodistribution of Co_2C-PEG NPs in major organs and tumor tissue of mice determined by Co concentrations (24 h after *i.v.* injection, 20 mg/kg) measured by ICP-MS.

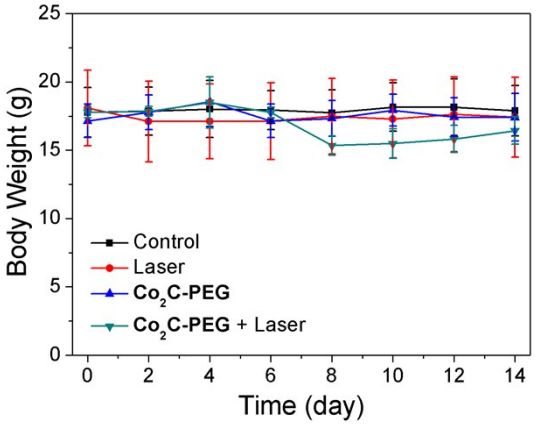

Figure S13. Body weight of mice with various treatments.

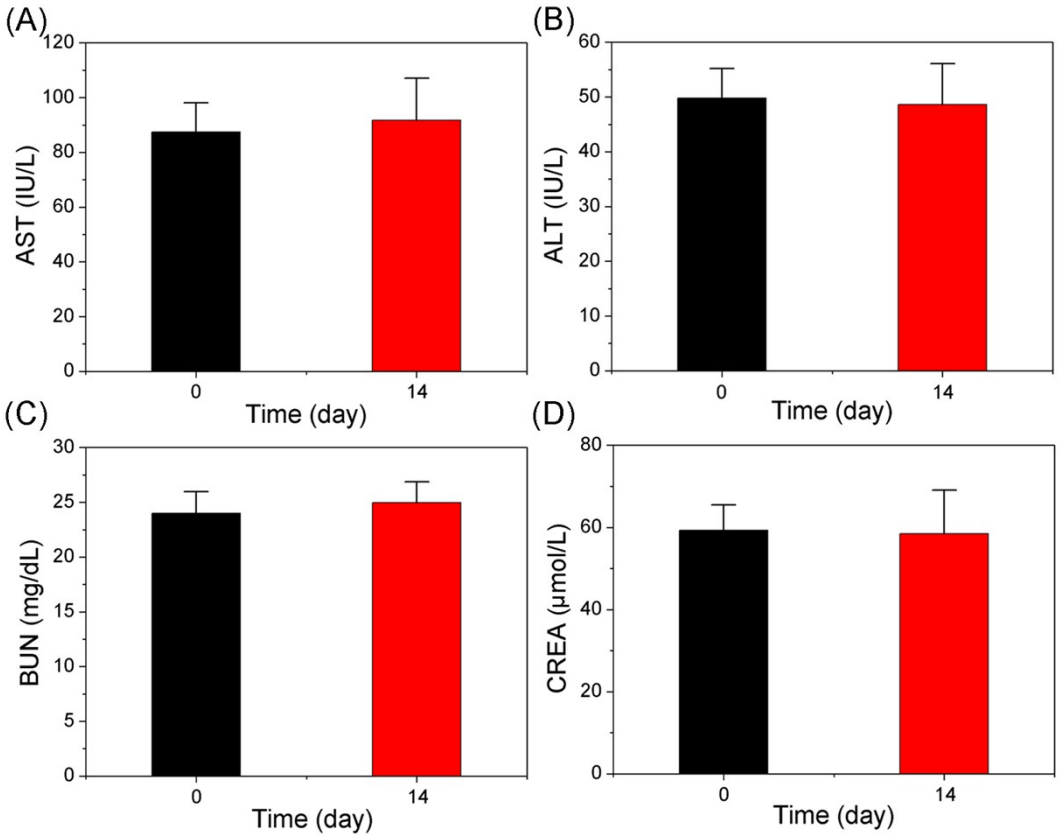

Figure S14. (A-D) Blood biochemistry analysis (AST, ALT, BUN and CREA)of healthy BALB/c mice before (0 day) and after (14 days) *i.v.* injection of Co_2C-PEG.

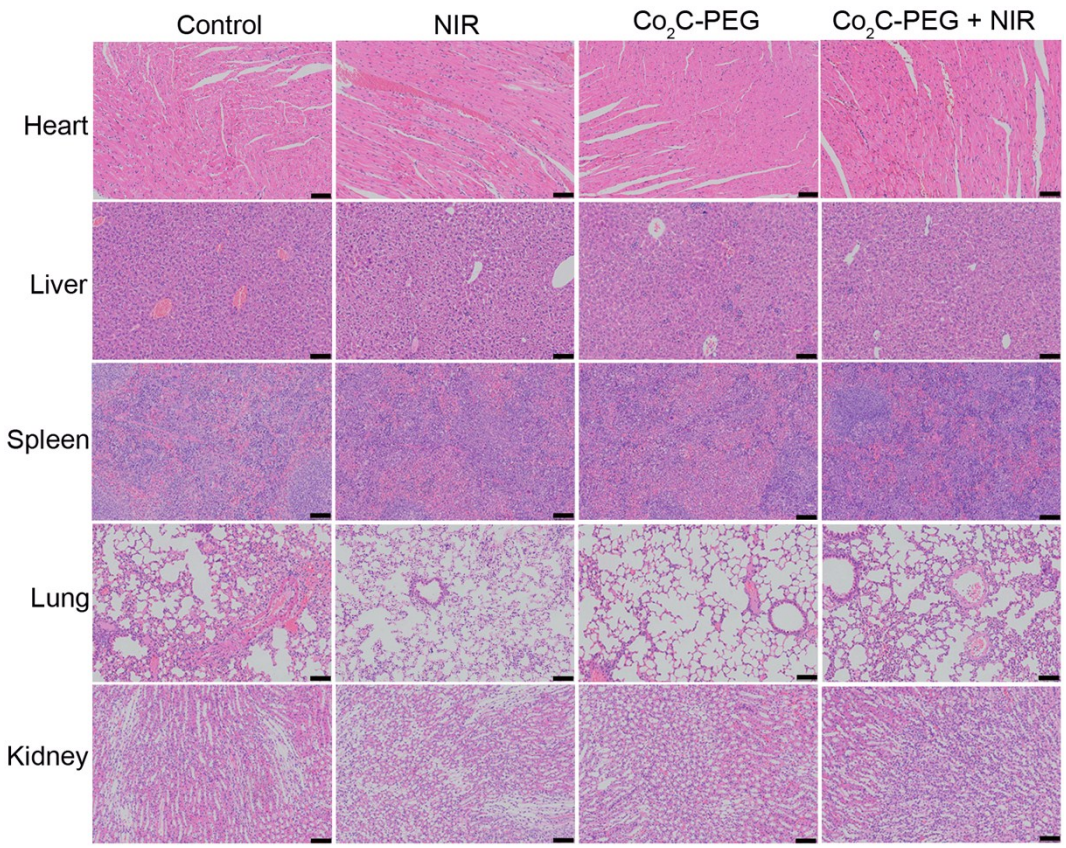

Figure S15. Hematoxylin and eosin (H&E) stained images of major organs (heart, liver, spleen, lung, and kidney) collected from mice treated with different treatments (scale bar: 100 μm).