Supporting Information

Platinum-Carbon Integrated Nanozyme for Enhanced Tumor Photodynamic and Photothermal Therapy

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MATERIALS AND METHODS

Materials

$Zn(NO_3)_2 \cdot 6H_2O$, 2-Methylimidazole, Hexadecyl trimethyl ammonium bromide (CTAB), Fluorescein diacetat (FDA), propidium iodide (PI), ethylenediaminetetraacetic-acid (EDTA), cell counting kit-8 (CCK-8) and tetraethyl orthosilicate (TEOS) were obtained from Sigma-Aldrich (USA). PBS and fetal bovine serum (FBS) were purchased from Thermo-Fisher (USA). DCFH-DA was purchased from Beyotime Company (China). All reagents used in this work were analytical reagents (A.R.) and used without any further purification.

Synthesis of ZIF-8 nanoparticles

A solution of $Zn(NO_3)_2 \cdot 6H_2O$ (32 mmol in 500 mL CH_3OH) was poured into a solution containing 135 mmol 2-methylimidazole and 0.3 mmol CTAB in methanol (400 mL), and the resulting solution stirred (800 rpm) for 2 h at room temperature. ZIF-8 nanoparticles with different diameter were prepared by adjusting stirring speed (600 rpm or 700 rpm). The white solid precipitate was separated by centrifugation and washed with methanol.

Synthesis of ZIF-8@$mSiO_2$ core@shell nanoparticles

ZIF-8 was dispersed in 240 mL of a 10 vol% methanol solution which had already been adjusted to pH 11 with NaOH, and then 6 mL of an aqueous CTAB (0.068 mol/L) solution was added. TEOS (1.2 mL) was then added dropwise into the above solution, and the resulting dispersion was stirred for 0.5 h. The resulting core-shell nanoparticles were separated by centrifugation and washed with ethanol.

Synthesis of porphyrin-like mesoporous carbon nanozyme

The ZIF-8@$mSiO_2$ core@shell sample was pyrolyzed at 800 °C for 2 h under flowing N_2, and then allowed to cool slowly to room temperature. The pyrolyzed sample was then etched with 4 M NaOH solution to remove the $mSiO_2$ shell, followed by centrifugation and washing with deionized water several times until the supernatant was neutral.

Synthesis of Pt-carbon nanozyme

Carbon nanozyme (~100 mg) was dispersed into DI water to form a 5 mL dispersion and then the aqueous solutions of 0.05 mmol $HPtCl_4$ was added with stirring for 1 h. A subsequent addition of 50 mg $NaBH_4$ dissolved in 1 mL deionized water was followed and the mixture was continued to shake for another 1 h. After the metal ions were reduced completely, the products were washed by DI water for 3 times to remove free metal particles.

Characterization

The morphology of nanozyme was characterized using a transmission electron microscope (TEM; JEM-2010 ES500W, Japan) and field emission scanning electron

microscopy (Zeiss Merlin Compact). UV-vis-NIR spectra for carbon nanozyme and Pt-carbon nanozyme were collected on a Cary 5000 UV-vis-NIR spectrometer. Dynamic light scattering analysis was carried out on Zetasizer (Nano-ZS90, Malvern). The valence state of the element was determined through X-ray photoelectron spectrometer (ESCALAB250Xi, Thermo Fisher Scientific). The presence of Pt element in the Pt-carbon nanozyme nanoparticles was proved by the scanning electron microscopy-energy dispersive X-ray (SEM-EDX) element mapping and spectroscopy analysis, which was also confirmed by inductively coupled plasma mass spectrometry (ICP-MS) analysis, revealing that the Pt content in Pt-carbon nanozyme was about 20%.

Photothermal conversion efficiency

The optical absorption spectra of the carbon nanozyme and Pt-carbon nanozyme nanoparticles were acquired on a UV-visible-NIR spectrophotometer. An 808 nm NIR laser (Changchun New Industries Tech. Co., Ltd, China) with irradiation powers was used to stimulate the different concentrations (50, 100, 200 μg/mL) of carbon nanozyme and Pt-carbon nanozyme in an aqueous medium. The photothermal images of the carbon nanozyme nanoparticles and Pt-carbon nanozyme based suspensions during laser irradiation were recorded every 30s using an infrared thermal imaging system. The NIR laser source was equipped with a 4 mm diameter laser module with an adjustable power. The photothermal conversion efficiency, is calculated using Equation:

$$\eta = \frac{hS(T_{max} - T_{surr}) - Q_0}{I(1 - 10^{-A_\lambda})}$$

Where h is the heat transfer coefficient, S is the surface of the container, T_{max} and T_{surr} were the equilibrium temperature and ambient temperature, respectively. Q_0 is the heat associated with the light absorbance of the solvent, A_λ is the absorbance of carbon nanozyme and Pt-carbon nanozyme at 808 nm, and I is the laser power density. According to the above equation, the η value of carbon nanozyme nanoparticles was determined to be about 32%, the η value of Pt-carbon nanozyme was determined to be about 39%.

$^1O_2$ -Generation Detection

9,10-anthracenediyl-bis-(methylene)-dimalonic acid (ABDA) was used as the $^1O_2$-monitoring agent. In the experiments, 200 μL of ABDA stock solution (7.5 mM) was added to Pt-carbon nanozyme or carbon nanozyme. The Carbon nanozyme concentration was 100 μg/mL. And NIR laser (0.5 W/cm^2) was employed as the irradiation source. Then the absorption of ABDA at 378 nm was recorded at various irradiation times to obtain the decay rate of the photosensitizing process.

$^1O_2$ -Generation Detection of Pt-carbon nanozyme with different sizes

9,10-anthracenediyl-bis-(methylene)-dimalonic acid (ABDA) was used as the $^1O_2$-monitoring agent. In the experiments, 200 μL of ABDA stock solution (7.5 mM) was added to Pt-carbon nanozyme with different size (122, 180, and 250 nm). The carbon

nanozyme concentration was 100 μg/mL in all groups. And NIR laser (0.5 W/cm^2) was employed as the irradiation source. Then the absorption of ABDA at 378 nm was recorded at various irradiation times to obtain the decay rate of the photosensitizing process.

Cell culture

CT26 mouse colon cancer cell line, U937 human macrophage cell line, RAW 264.7 mouse monocyte macrophage cell line, MCF-7 human breast cancer cell lines and CT26 mouse breast cancer cell line cells were obtained from the Cell Bank of the Chinese Academy of Sciences and incubated in RPMI-1640 medium supplemented with 10% FBS in a humidified atmosphere at 37℃ with 5% CO_2.

Animal models

Female BALC/c mice aged 4-5 weeks were purchased from Vital River Company (Beijing, China). 1×10^6 CT26 cells suspended in 100 μL PBS were subcutaneously injected into each mouse to establish the tumor models. All protocols were approved by Wuhan University Animal Care Facility and National Institutes of Health Guidelines.

Intracellular reactive oxygen species (ROS) generation

For determination of ROS levels via fluorescent imaging, CT26 cells were incubated for 2 h with six different groups: (1) PBS (21% O_2) (2) PBS (1% O_2) (3) carbon nanozyme (21% O_2) (4) Pt-carbon nanozyme (21% O_2) (5) carbon nanozyme (1% O_2) (6) Pt-carbon nanozyme (1% O_2). The carbon nanozyme concentration was 100 μg/mL in group 3, 4, 5 and 6. The hypoxic condition was achieved by pre-incubating the cells in a hypoxic incubator supplied with a hypoxic gas stream (1% O_2, 5% CO_2, and 94% N_2) for 12h. The normoxic condition was achieved by pre-incubating the cells in a 21% O_2 incubator supplied with a normoxic gas stream (21% O_2, 5% CO_2, and 74% N_2) for 12 h. Then, the fluorescent dye, DCFH-DA (10 μmol/L), was added and co-incubated for 20 min at 37 °C. Then, cells in each group were irradiated with the 808 nm laser at a power density of 0.5 W/cm^2 for 3 min. ROS level was determined by fluorescence microscopy (IX81, Olympus, Japan). The fluorescent intensity of each group was calculated by a flow cytometer.

In vitro phototoxicity of Pt-carbon nanozyme

The phototoxicity was measured by CCK-8 assay. CT26 cells were seeded in 96-well plates at a density of 5×10^3 cells per well and incubated for 24 h. Afterwards, CT26 cells were incubated for 2h with six different groups: (1) PBS (21% O_2) (2) PBS (1% O_2) (3) carbon nanozyme (21% O_2) (4) Pt-carbon nanozyme (21% O_2) (5) carbon nanozyme (1% O_2) (6) Pt-carbon nanozyme (1% O_2). The carbon nanozyme concentration was 100 μg/mL in group 3, 4, 5 and 6. The normoxic condition was achieved by pre-incubating the cells in a hypoxic incubator supplied with a hypoxic gas stream (1% O_2, 5% CO_2, and 94% N_2) for 12 h. The normoxic condition was achieved

by pre-incubating the cells in a normoxic incubator supplied with a normoxic gas stream (21% O_2, 5% CO_2, and 74% N_2) for 12 h. Then, cells in each group were irradiated with the 808 nm laser at a power density of 0.5 W/cm^2 for 3 min. At the end of the incubation, 5 mg/mL CCK-8 PBS solution was added, and the plate was incubated for another 4 h. Finally, the absorbance values of the cells were determined by using a microplate reader (Emax Precision, USA) at 450 nm. The background absorbance of the well plate was measured and subtracted. The cytotoxicity was calculated by dividing the optical density (OD) values of treated groups (T) by the OD values of the control (C) (T/C × 100%).

To further visualize the cell phototoxicity of each group, CT26 cells were incubated for 2 h with six different groups: (1) PBS (21% O_2) (2) PBS (1% O_2) (3) carbon nanozyme (21% O_2) (4) Pt-carbon nanozyme (21% O_2) (5) carbon nanozyme (1% O_2) (6) Pt-carbon nanozyme (1% O_2). The carbon nanozyme concentration was 100 μg/mL in groups 3, 4, 5 and 6. The hypoxic condition was achieved by pre-incubating the cells in a hypoxic incubator supplied with a hypoxic gas stream (1% O_2, 5% CO_2, and 94% N_2) for 12 h. The normoxic condition was achieved by pre-incubating the cells in a normoxic incubator supplied with a normoxiac gas stream (21% O_2, 5% CO_2, and 74% N_2) for 12 h. Then, cells in each group were irradiated with the 808 nm laser at a power density of 0.5 W/cm^2 for 3 min. Then, all cells were washed with PBS, treated with FDA and PI according to the manufacturer's protocol, and detected under a fluorescent microscope (IX81, Olympus, Japan). The fluorescent intensity of each group was calculated by ImageJ software.

Biocompatibility of Pt-carbon nanozyme

We then tested the biocompatibility of Pt-carbon nanozyme to other types of cells. Huh-7 human liver cancer cells, U937 human macrophage cells, RAW 264.7 mouse monocyte-macrophage cells, MCF-7 human breast cancer cells were seeded in 96-well plates at a density of 5×10^3 cells per well and incubated for 24 h. Afterward, cells were incubated for 6h with different concentrations of Pt-carbon nanozyme (122, 180, 250 nm)(0, 50, 100 and 200 μg/mL carbon nanozyme). At the end of the incubation, 5 mg/mL CCK-8 PBS solution was added, and the plate was incubated for another 4 h. Finally, the absorbance values of the cells were determined by using a microplate reader (Emax Precision, USA) at 450 nm. The background absorbance of the well plate was measured and subtracted. The cytotoxicity was calculated by dividing the optical density (OD) values of treated groups (T) by the OD values of the control (C) (T/C × 100%).

Hemolysis Assay

A hemolysis assay was carried out to evaluate the cytotoxicity of Pt-carbon nanozyme in vitro. Rabbit heart blood (5 mL) was stabilized by ethylenediamine tetraacetic acid (EDTA) (0.2 mL), an anticoagulant agent. Then red blood cells (RBCs) were obtained from the rabbit heart blood by centrifugation and washing with PBS (2%). 0.5 mL of the RBC solution was then mixed with 0.5 mL Pt-carbon nanozyme PBS solution at different concentrations (50, 100 and 200 μg/mL carbon nanozyme). Water and PBS

were used as positive and negative controls, respectively. All samples were mixed gently and kept at room temperature for 3 h. The absorbance of each supernatant, obtained by centrifugation, was measured at 570 nm on a JASCO UV-vis photospectrometer. The hemolysis ratio was calculated by using the formula: hemolysis ratio = (sample absorbance-negative control absorbance) / (positive control absorbance − negative control absorbance) × 100.

In vivo biodistribution evaluation

The experimental Balb/c mice were intravenously injected with Pt-carbon nanozyme (100 μL, 20 mg/kg carbon nanozyme). Then the mice (N = 3) were euthanized at different points in time (12 h, 24 h, and 48 h). The major organs including heart, liver, spleen, lung, and kidney as well as tumors were collected in a flask to be weighed. All the organs were treated with concentrated nitric acid and H_2O_2 (v/v =1:2) on heating (70 °C) until the solutions. The amounts of Pt in the solutions were measured by ICP-MS, and the concentrations in each organ were calculated.

***In* Vivo intratumoral blood oxygen saturation**

The CT26 tumor-bearing mice were intravenously injected with PBS, carbon nanozyme and Pt-carbon nanozyme (100 μL, containing 5 mg/kg carbon nanozyme). After 24 h post-injection, the PA images of oxygen saturation were collected.

Intracellular H_2O_2 Catalytic Test

Intracellular H_2O_2 was detected by ROS fluorescence probe of DCFH-DA. The 4T1 cells pre-seeded in 24-well plates were incubated with 100 μg/mL of Pt-carbon nanozyme or Carbon nanozyme for 12 h. The cells were then washed with PBS and re-cultured with 1 mL of fresh medium containing 100 μM H_2O_2 for 30 min. After that, the above medium was removed, washed with PBS, and stained with 100 mM DCFH-DA for 15 min. The fluorescence was detected by a fluorescent microscope (Leica DMI3000).

The hypoxia changes within the tumor microenvironment

To study the hypoxia change within tumor microenvironment after intravenous injection with different nanoparticles. The CT26 tumor-bearing mice were intravenously injected with PBS, carbon nanozyme, and Pt-carbon nanozyme (100 μL, 5 mg/kg carbon nanozyme). 24 h later, the mice were intraperitoneally injected with pimonidazole hydrochloride (Hypoxyprobe, USA) at a dose of 30 mg kg^{-1} according to the procedure provided by the manufacturer. 1.5 h later, tumors were surgically excised from the mice and then stained with mouse antipimonidazole monoclonal antibody as primary antibodies to label tumor hypoxia regions. Then, the slices were stained with Alex 488-conjugated goat antimouse secondary antibody and DAPI. Finally, the images of stained slides were characterized using CLSM (Zeiss LSM 710). The hypoxia region of each slice was statistically analyzed using the Imaging-J software.

ROS detection in vivo

When the tumor volume reached 200 mm^3, mice were intravenously injected with (1) PBS, (2) carbon nanozyme, (3) Pt-carbon nanozyme (100 μL,5 mg/kg carbon nanozyme). After 24 h post-injection, the tumor site of mice in all groups was irradiated by an 808 nm laser (0.5 W/cm^2, 10 min). Then the fluorescent dye, DCFH-DA (10 μmol/L, 50 μL) was injected intratumorally. Subsequently, tumors from each group were dissected. The cryosections were observed by an inverted fluorescence microscope (Olympus IX71).

In vivo antitumor study

1×10^6 CT26 cells suspended in 100μL PBS were subcutaneously injected into each mouse to establish the tumor models. When tumor size reached approximately 200 mm^3, the mice were divided randomly into 5 groups (each group included 5 mice): i) a control group (PBS injection); ii) NIR (laser only irradiation, 0.5 W/cm^2, 5 min); iii) carbon nanozyme group (i.v. injection, 5 mg/kg carbon nanozyme); iv) Pt-carbon nanozyme group (i.v. injection, containing 5 mg/kg carbon nanozyme). v) carbon nanozyme + NIR group (i.v. injection, 5 mg/kg carbon nanozyme, 0.5 W/cm^2, 5 min) and vi) Pt-carbon nanozyme +NIR group (carbon nanozyme i.v. injection, containing 5 mg/kg carbon nanozyme, 0.5 W/cm^2, 5 min) The semiconductor laser device at 808 nm was used as a source of laser light. An infrared thermal imaging camera (Fotric 225) was used to acquire the images and determine temperature changes. The treatment was conducted every 2 days for 14 days. Mice body weight and tumor volume in all groups were monitored every 2 days. A caliper was employed to measure the tumor length and tumor width and the tumor volume was calculated according to the following formμLa. Tumor volume = tumor length × tumor $width^2$ / 2. After 14 days of treatment, all the mice were sacrificed. Five main organs (heart, liver, spleen, lung, and kidney) of all mice were harvested, washed with PBS, and fixed with paraformaldehyde for histology analysis. And the tumor tissues were weighed, and fixed in 4% neutral buffered formalin, processed routinely into paraffin, and sectioned at 4 μm. Then the sections were stained with hematoxylin and eosin (H&E), Ki-67, and terminal deoxynucleotidyl (TUNEL) staining and finally examined by using an optical microscope (BX51, Olympus, Japan).

Statistical Analysis

Data analyses were conducted using the GraphPad Prism 5.0 software. The one-way analysis of variance (ANOVA) followed by the post-Tukey comparison tests was used for statistical analysis. P-value of < 0.05 indicates statistical difference.

Supplementary Figures

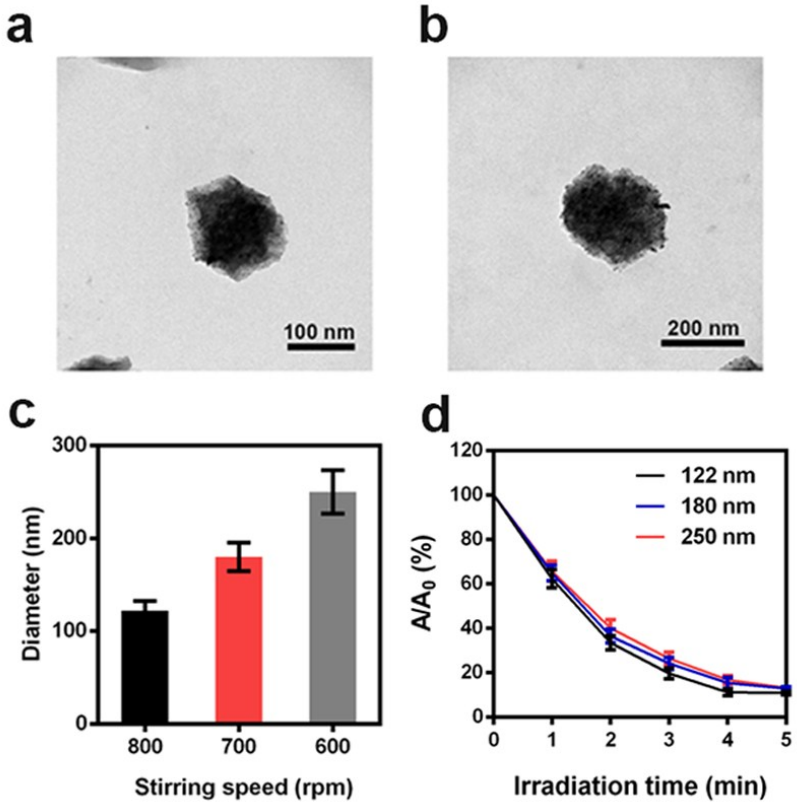

Figure S1. a) TEM image of Pt-carbon nanozyme (700 rpm); b) TEM image of Pt-carbon nanozyme (600 rpm); c) The particle size distribution of Pt-carbon nanozyme formed at different speeds (800, 700, 600 rpm); d) The decomposition rates of ABDA in Pt-carbon nanozyme of different sizes (122, 180, 250 nm) under light irradiation.

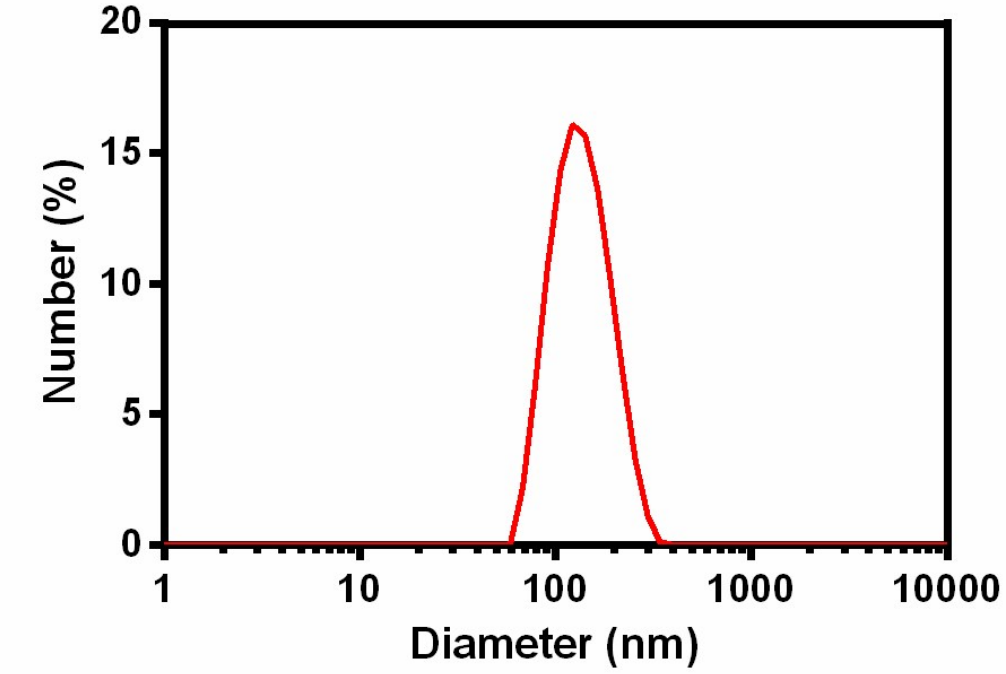

Figure S2. The hydrodynamic sizes of Pt-carbon nanozyme.

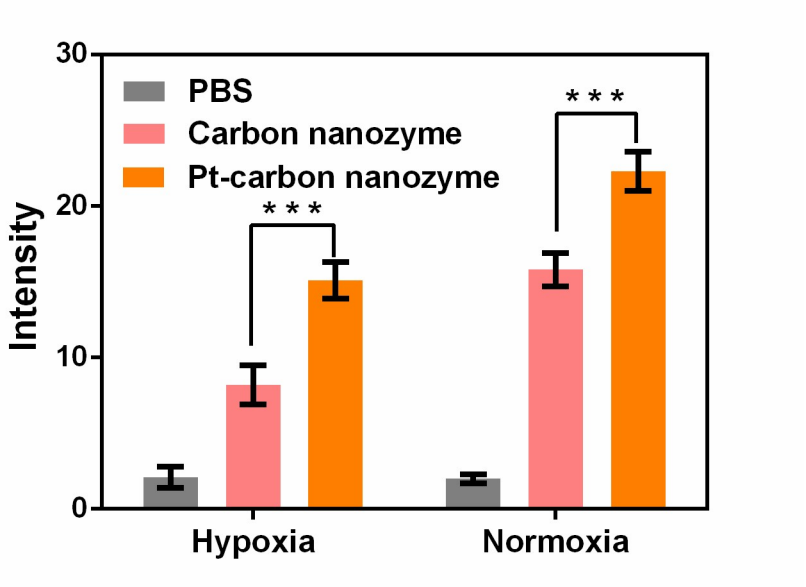

Figure S3. PI fluorescence intensity in **Figure 3a** was quantified using the ImageJ software.

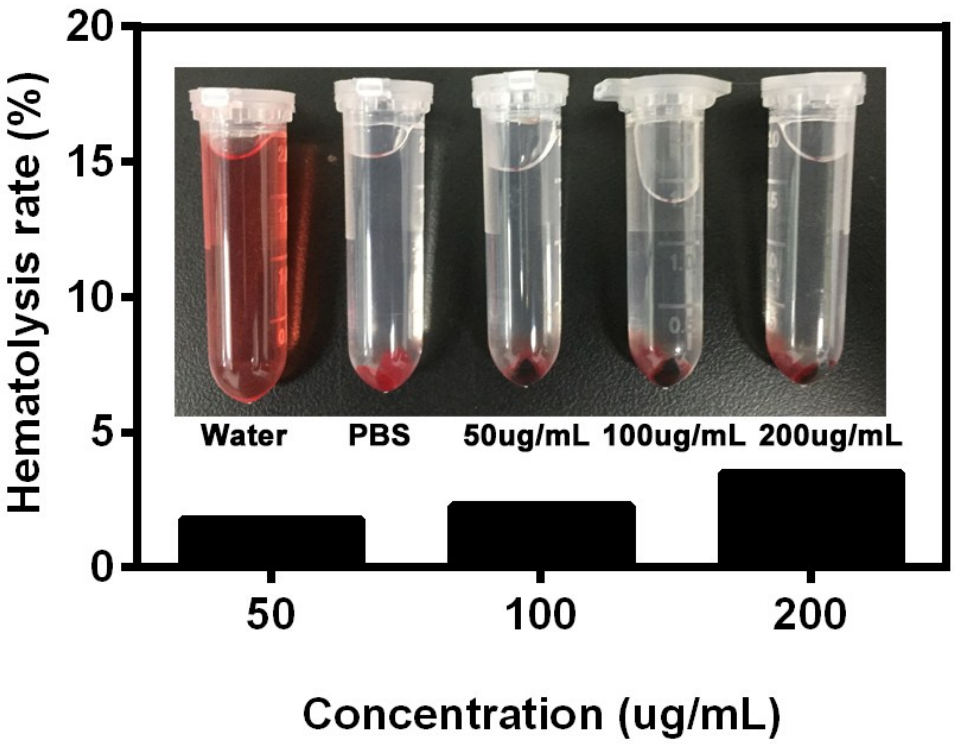

Figure S4. Hemolysis ratio of Pt-carbon nanozyme at different concentrations. The inset shows the corresponding hemolysis images.

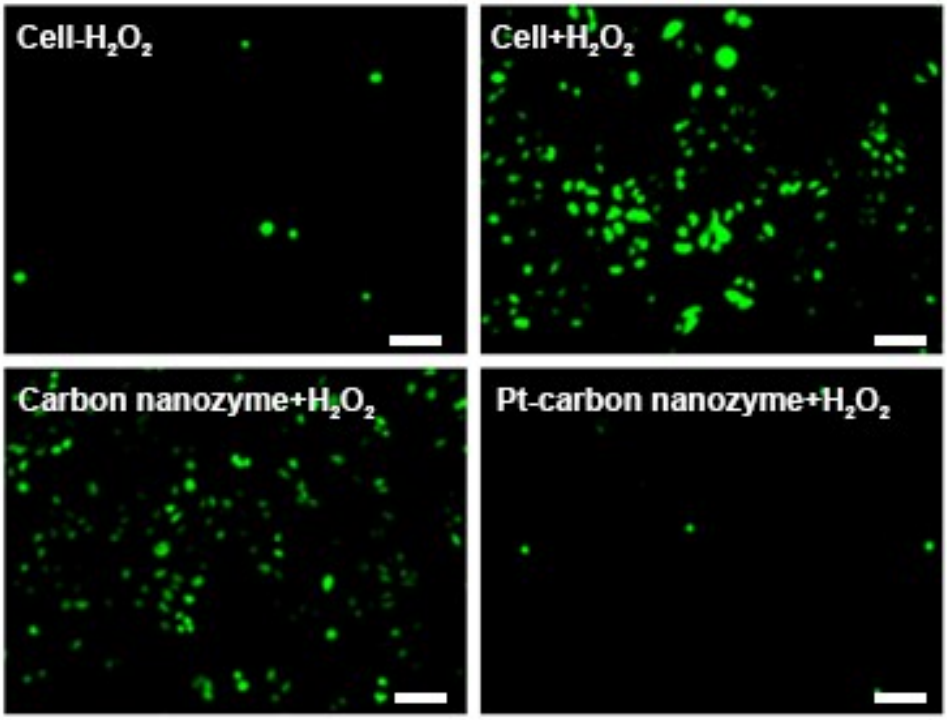

Figure S5. Fluorescence analysis of H_2O_2 level determined by DCFH-DA in CT26 cells treated with PBS, H_2O_2 (100 μM), Carbon nanozyme (100 μg/mL) + H_2O_2, or Pt-carbon nanozyme (100 μg/mL) + H_2O_2. Scale bar = 50 μm.

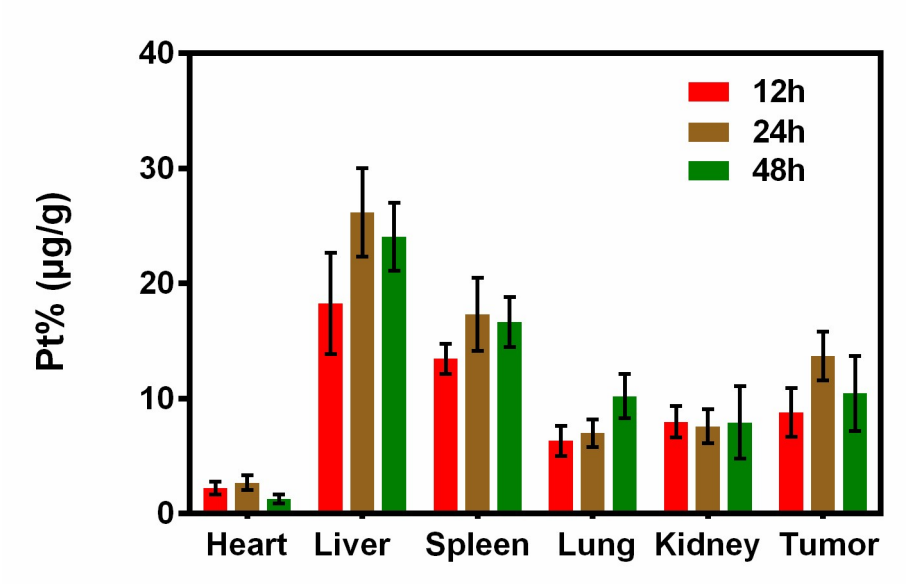

Figure S6. Biodistribution of Pt in tumor and main organs after injection with Pt-carbon nanozyme intravenously for 12, 24 and 48 h.

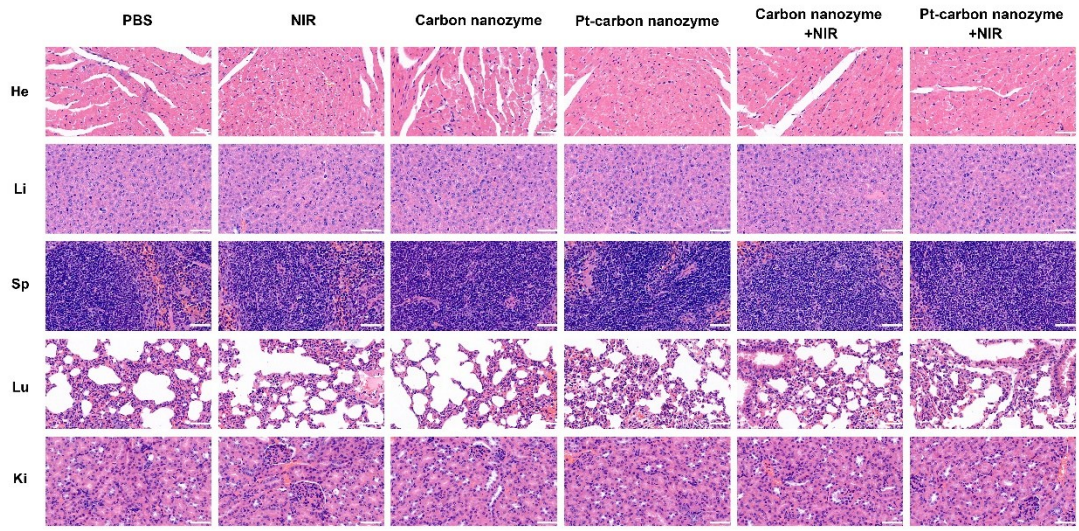

Figure S7. Histopathologic examination of the tissues including heart (He), liver (Li), spleen (Sp), lung (Lu), and kidney (Ki) from BALB/c mice after different treatment. Scale bars = 100 μm.