Supporting Information

Prevention of Hepatic Ischemia-reperfusion Injury by Carbohydrates-derived Nano-antioxidants

Yin Long,[1,†] Hao Wei,[2,,3,5,†] Jun Li,[1] Mengting Li,[2,3,5] Yizhan Wang,[1] Ziyi Zhang,[1] Tianye Cao,[2,5] Corey Carlos,[1] Lazarus G German,[1] Dawei Jiang,[2,5] Tuanwei Sun,[2,5] Jonathan W. Engle,[2] Xiaoli Lan,[3] Yadong Jiang,[4] Weibo Cai,[2,5*] and Xudong Wang,[1*]*

[1]Department of Material Science and Engineering, University of Wisconsin - Madison, Wisconsin 53706, United States

[2]Departments of Radiology and Medical Physics, University of Wisconsin - Madison, Wisconsin 53705, United States

[3]Department of Nuclear Medicine, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430073, China

[4]State Key Laboratory of Thin Films and Integrated Devices, School of Optical Science and Engineering, University of Electronic Science and Technology of China, Chengdu 610054, China

[5]University of Wisconsin Carbone Cancer Center, Madison, Wisconsin 53705, United States

Correspondence and requests for materials should be addressed to H.W. (email: hwei52@wisc.edu), W.B.C. (email: wcai@uwhealthy.org) and X.D.W. (email: xudong.wang@wisc.edu).

†These authors contributed equally to this work.

SI-Part 1 Methods

Synthesis and Characterizations of C-NPs. The C-NPs were synthesized by a simple one-step hydrothermal approach. 1g glucose was added into 50 ml DI water at continuous stirring at room temperature for 0.5 h. The solution was then transferred into a 100 ml Teflon autoclave and heated at 180 ℃ for 6 h. Then the autoclave was naturally cooled down to room temperature. The C-NPs suspension was collected by 10 min of centrifugation at 15000 rpm, washed with DI water and ethanol for three times, and finally dispersed in DI water or PBS solution for further use.

Morphology of the synthesized C-NPs were observed using a Zeiss LEO 1530 field-emission scanning electron microscope (SEM) and a FEI TF30 transmission electron microscope (TEM). Atomic force microscopy (AFM) tomography images were obtained using an XE-70 Park System. X-ray photoelectron spectroscopy (XPS) spectrum was measured by a Thermo Scientific Kα XPS instrument with a 100 μm spot size. X-ray diffraction (XRD) measurements were performed on a Bruker D8 diffractometer with Cu Kα radiation. The surfaced zeta potential and hydrodynamic size were measured using a Malvern Zetasizer Nano ZS. Fourier transform infrared (FTIR) spectra were acquired by a Thermo Nicolet iZ10.

***In vitro* ROS-scavenging capability in cell-free system.**

Electron spin resonance (ESR) spectroscopy: All ESR measurements were carried out at ambient temperature using a Bruker ELEXSYS E500 ESR spectrometer. To analyze the free radical-scavenging capability of C-NPs, 100 μL, 5, 10, and 15 ppm of C-NPs were added to a cell-free radical system, which was generated by Fenton reaction (70 μL of 0.735 mM $FeSO_4$ and 25 μL of 0.315 mM H_2O_2). DMPO (5 μL, 98%) was selected as the spin trapping agent. The peak intensity of ESR spectra in each group was recorded and compared as DMPO/·OH adducts indicated the ROS scavenging effect of C-NPs.

Raman spectroscopy. C-NPs were deposited on a silicon wafer and 5 μL H_2O_2 (37%) was dropped on the silicon wafer. The wafer was scanned by a Raman microscope (Thermo Scientific DXRxi) at 0, 15, and 45 minutes after adding H_2O_2 under a 505 nm laser excitation.

UV-vis spectroscopy. 1 mL C-NPs solution (1 mg/mL) was added with 0 – 450 μM H_2O_2 and maintained for 3 days. The solution was then scanned by UV-vis spectrometer within the wavelength range of 200-600 nm.

ROS assay. All experiments were conducted according to the protocols of different assays. $O_2^{\bullet-}$-scavenging ability was evaluated by a superoxide dismutase (SOD) assay kit (Sigma-Aldrich, USA). $^{\bullet}OH$-scavenging performance was measured with a hydroxyl radical antioxidant capacity (HORAC) assay kit (Cell Biolabs, Inc., USA). H_2O_2-scarvenging activity was measured with the Amplex® red hydrogen peroxide/peroxidase (ARHP) assay kit (Molecular Probes, Inc., USA).

***In Vitro* cell cytotoxicity, uptake, and ROS scavenging studies in cell system.**

Cell cytotoxicity. The cell cytotoxicity of C-NPs was assessed with a MTT assay using human embryonic kidney 293 (HEK293) cells (American Type Culture Collection). Generally, the cells were seeded in a 96-well plate (5×10^3 cells/well) and incubated for 24 h at 37°C and 5% CO_2, and then different concentrations of C-NPs (0, 50, 100, 200, 500 and 1000 μg/mL) in culture media solutions were added to the wells. After incubation for 20, 44 and 68 h, 20 μL MTT solution was added to each well and incubated for another 4 hours. The absorbance of each well was measured at 490 nm using ClarioStar Plate Reader. Untreated cells were used as control, and the relative cell viability was expressed as $(Abs_{sample}\text{-}Abs_{blank})/(Abs_{control}\text{-}Abs_{blank})\times 100\%$, where Abs_{blank} is the optical density of the wells without any cells.

Cell uptake. RAW264.7 cells were seeded in 96-well plates (10^4 cells per well). After 12 h, C-NPs were added to incubate with cells for 0, 1, 3, 6, 12, and 24 h at a final concentration of 50 ug/mL.

Then, all wells were washed with 1 x PBS for 3 times to remove all residue C-NPs in the culture media or on the surface of the cells. The plate was reading using a PolarStar plate reader at 360 nm for absorption and 0 h for 80 nm C-NPs was used as baseline control.

Cell ROS scavenging ability. For ROS scavenging assay, culture media of the C-NPs with different concentrations (5, 15, and 25 μg/mL) were added to all wells (n = 4) and incubated for 12 h. The cells were treated with H_2O_2 (250 μM) and incubated for another 24 h at 37 ℃ under 5% CO_2. Cells treated with C-NPs only or H_2O_2 only were used as controls. The cell viability was measured by MTT assay. To visualize ROS in live cells, the HepG2 (human hepatocellular carcinoma) cells were cultured with/without adding C-NPs for 12 h with 5% CO_2 at 37 ℃. 250 and 375 μM were added to the culture dish and incubated for another 24 h under the same condition. Then 100 μL Hoechst (5 μg/mL) and 10 μM of CellROX® Green were added to stain the nuclei (blue) and the ROS (green), respectively. After 0.5 h incubation at 37 ℃, the cells were washed with PBS for three times, and finally confocal imaging was conducted with a Nikon A1RS confocal microscope.

^{89}Zr radiolabeling and *in vivo* PET imaging. ^{89}Zr-oxalate was produced by the University of Wisconsin-Madison cyclotron group. Firstly, 1 mCi (or 37 MBq) of ^{89}Zr-oxalate was added to 300 μL of C-NPs dispersed in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer and the final pH value of the solution was adjusted to 7-8 with 1 M Na_2CO_3. After shaking for 2 h at 37 or 55 ℃, ^{89}Zr-C-NPs could be easily collected through PD-10 column with PBS as the mobile phase. Radiolabeling yield was monitored and quantified by using thin layer chromatography (TLC) with subsequent autoradiography. Then, 150 μL (~0.5 mCi) of ^{89}Zr-C-NPs in PBS was intravenously (*i.v.*) injected into C57BL/6 mice (n = 3). Serial PET images were scanned at various time points within 120 h *p.i.* of ^{89}Zr-C-NPs. Region of interest (ROI) analysis was performed to calculate the percentage of injected dose per gram of tissue (%ID/g) in mouse main organs, using

vendor software (Inveon Research Workplace [IRW]) on decay-corrected whole-body images. After the last PET imaging at 120 h *p.i.*, all major organs and tissues were collected and the radioactivity were measured using a gamma counter (Perkin Elmer 2480 automatic gamma counter).

***In vivo* toxicity study of C-NPs.** 150 μL (1.8 mg/kg) C-NPs was *i.v.* injected into C57BL/6 mice (n = 5) and mice receiving PBS injection were used as the control group. The mice were sacrificed to collect blood for serum biochemistry assays on both day 1 and day 15 *p.i.*. At the same time, major organs from each mouse were harvested and fixed in 4% paraformaldehyde solution for 1 day. These tissues were then embedded in a paraffin, sliced and stained with hematoxylin and eosin (H&E) and examined using a digital microscope (Nikon Eclipse Ti). Examined tissues included the heart, liver, spleen, lung, and kidney. The complete blood panel data from the C-NPs-injected and PBS control groups were measured by the University of Wisconsin-Madison Veterinary Hospital.

Hepatic IRI treatment and evaluation. *Treatment of hepatic IRI.* All animal studies were conducted under a protocol approved by the University of Wisconsin Institutional Animal Care and Use Committee. The hepatic IRI model was generated as follows: the mice were firstly anesthetized with isoflurane, then all structures in the portal triad (hepatic artery, bile duct, and portal vein) to the left and median liver lobes were blocked with a microvascular clamp for 60 min, followed with reperfusion by removing the clamp. In the sham group, the healthy mice underwent anesthesia, laparotomy, and exposure of the portal triad without hepatic ischemia. Blood and liver tissues were harvested for further analysis at 12 and 60 hours after reperfusion. For treatment of IRI mice, the C-NPs (0.4 mg/kg) or PBS (1 x) were *i.v.* injected into C57BL/6 mice (n = 5) at 1 day before the surgery. The mice in the sham group and healthy mice treated with the C-NPs (0.4

mg/kg) or PBS (1 x) were used as control (n = 5). After 12 h of the hepatic IRI model induction, their liver function was evaluated. For a long-term assessment of liver function, two groups of IRI mice treated with C-NPs or PBS (1 x) (n = 5) were sacrificed at 60 h after treatment.

Evaluation with Liver function test and H&E staining. Liver function test and H&E staining were performed to evaluate the treatment of IRI. Mice in all groups were sacrificed, and blood samples were collected and sent for analysis of liver functions as previously described in *in vivo* toxicity assessment. Livers were also collected at 12 and 60 h after the model induction and fixed with paraformaldehyde (4% in PBS), then sent to the University of Wisconsin Carbone Cancer Center Experimental Pathology Laboratory for sectioning and H&E staining. Blood samples were collected in pediatric heparin tubes (BD Biosciences, San Jose, CA, USA), centrifuged (2,000 g, 5 min, 4 ℃) to obtain the plasma, and then sent to Clinical Pathology Laboratory in Veterinary Medical Teaching Hospital at the University of Wisconsin – Madison for analysis of liver functional profiles (alanine aminotransferase (ALT) levels and aspartate aminotransferase (AST) levels). Livers from each group were frozen and stored at -80 ^{o}C until usage. Liver homogenates were prepared according to the protocol of superoxide dismutase (SOD) assay. SOD level was assessed with a SOD assay kit (Sigma-Aldrich, USA).

Immunofluorescence staining. The frozen liver section slides were firstly warmed up in fresh PBS solution for 3 mins, and 4% PFA solution for 10 mins at room temperature. After two washings with $PBST_{0.2}$ (PBS with 0.2% Triton), slides were further permeabilized in $PBST_2$ (PBS with 2% Triton) for 15 mins. After $PBST_{0.2}$ washing, samples were blocked by incubating in blocking buffer (PBS with 5% horse serum and 0.3% Triton) for 1 hour. Antibodies including anti-mouse F4/80, CD31, Ly6G (Biolegend), CLEC4F (R&D System) and Caspase 3 (Abcam) were diluted in blocking buffer by different combinations and incubated with liver sections overnight at

4 ℃. After that, slides were washed with blocking buffer and then incubated in blocking buffer containing secondary antibodies with fluorescence Alexa Fluor 488, 594 and 647 for another 1 h. Finally, the liver sections were mounted via the vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). Slides were examined and the confocal images were obtained *via* a Nikon A1R confocal microscope (Nikon Instruments, Melville, NY, USA).

Cytokines Measurements by Enzyme-linked Immunosorbent Assay (ELISA). Obtained livers were cut into small pieces and homogenized in the radioimmunoprecipitation assay (RIPA) buffer (Boston Bio Products) containing 1 x protein inhibitor (Pierce Protease inhibitor tablets, Thermofisher) at a final concentration of 200 mg/mL. All process was conducted on ice. Then, lysates were obtained after 20000 g centrifugation for 20 mins at 4 ℃ and stored in -80 ℃ until use. Before tests, samples were gentlely defrozened on ice and diluted in a serial of dilutions (1:10 to 1:640). The following measurements of secreted cytokines by ELISA were conducted according to the manufacturer's instructions (Mouse IL-1β ELISA development Kit, PromoKine; Mouse TNF-α ELISA Kit, Cayman; Mouse IL-12, IFN-γ and IL-6 ELISA Kit, Bioleagend; Mouse Myeloperoxidase ELISA Kit, R&D system; Mouse NOS2 ELISA Kit, G-Bioscience).

SI-Part 2 Supporting Figures and Tables

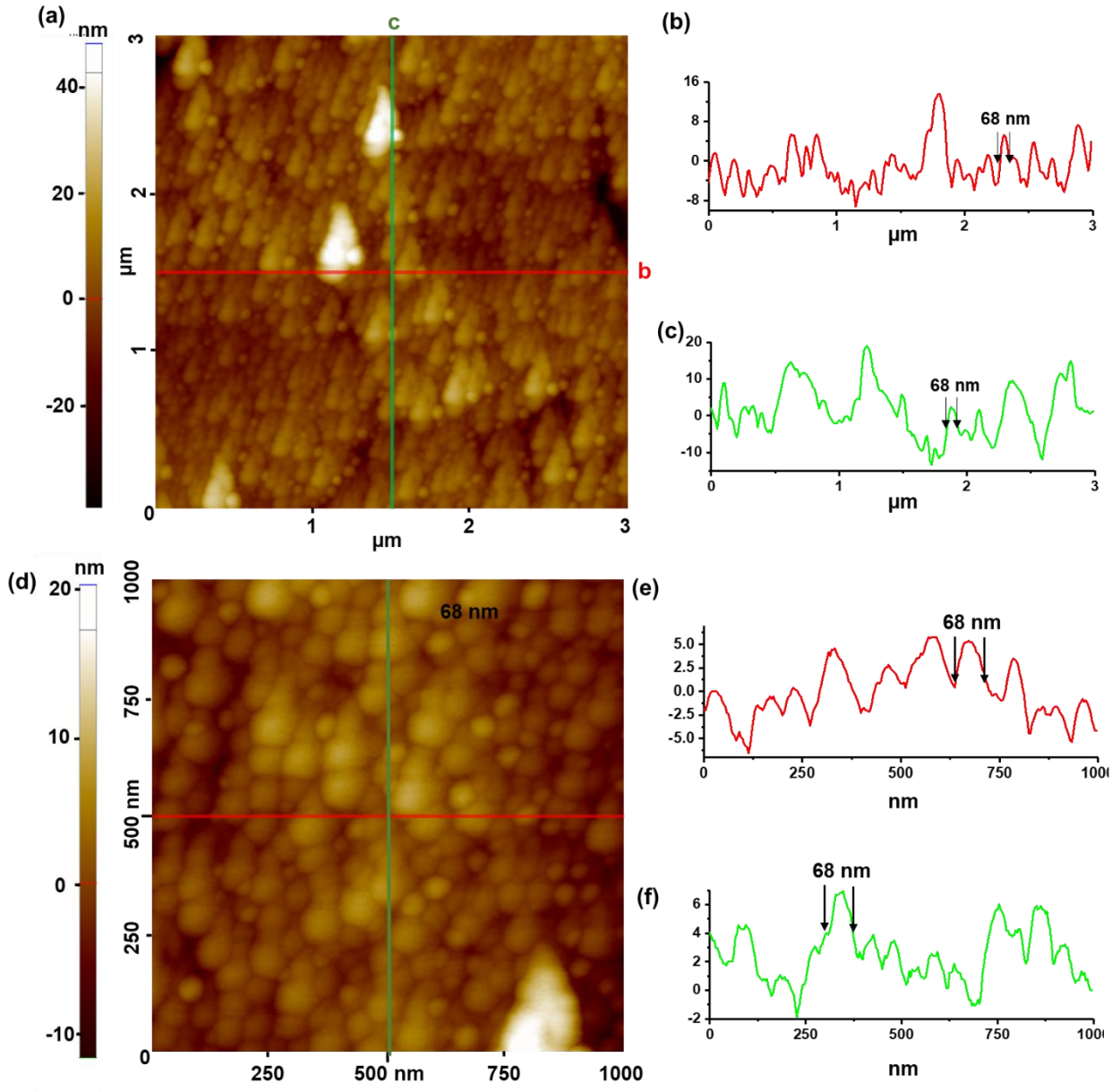

Figure S1. Atomic force microscopic image of C-NPs. **(a)** AFM topographic image of C-NPs. **(b)** and **(c)** are height profiles along different directions in (a). **(d)** Higher resolution AFM topographic image of C-NPS. **(e)** and **(f)** are height profiles along different directions in (d).

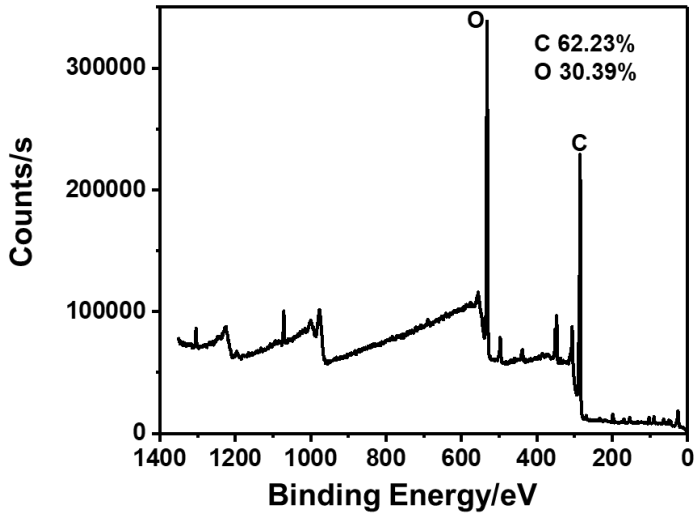

Figure S2. XPS spectra of as-prepared C-NPs.

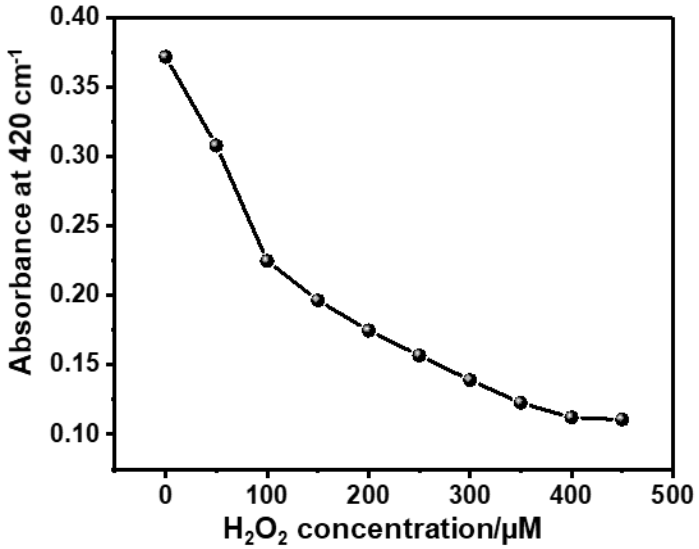

Figure S4. UV-vis absorbance at 420 cm^{-1} as a function of H_2O_2 concentration.

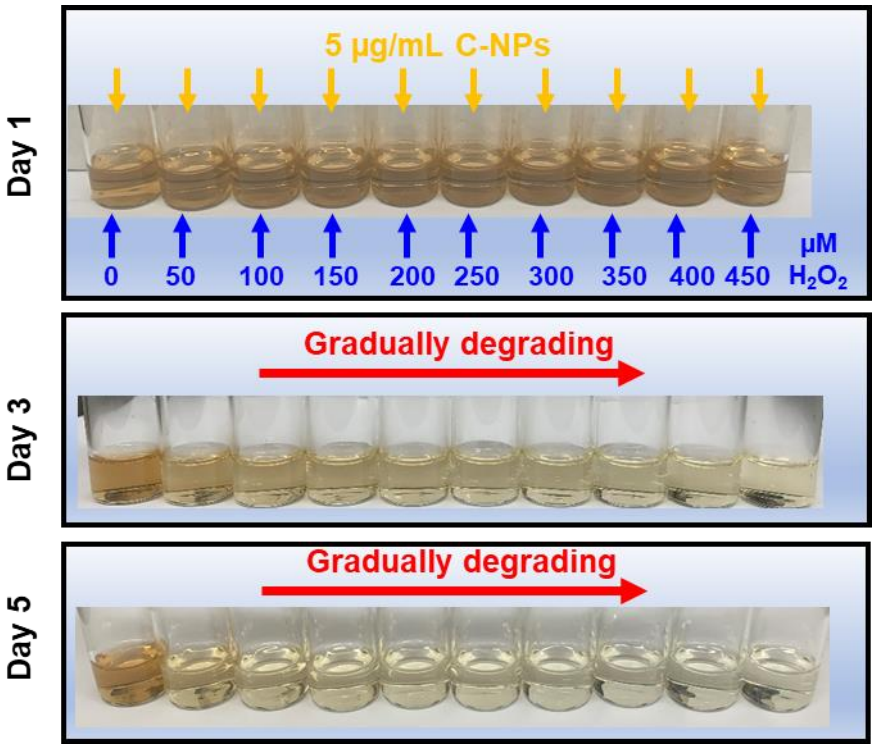

Figure S4. Photographs of C-NPs degrading after adding H_2O_2

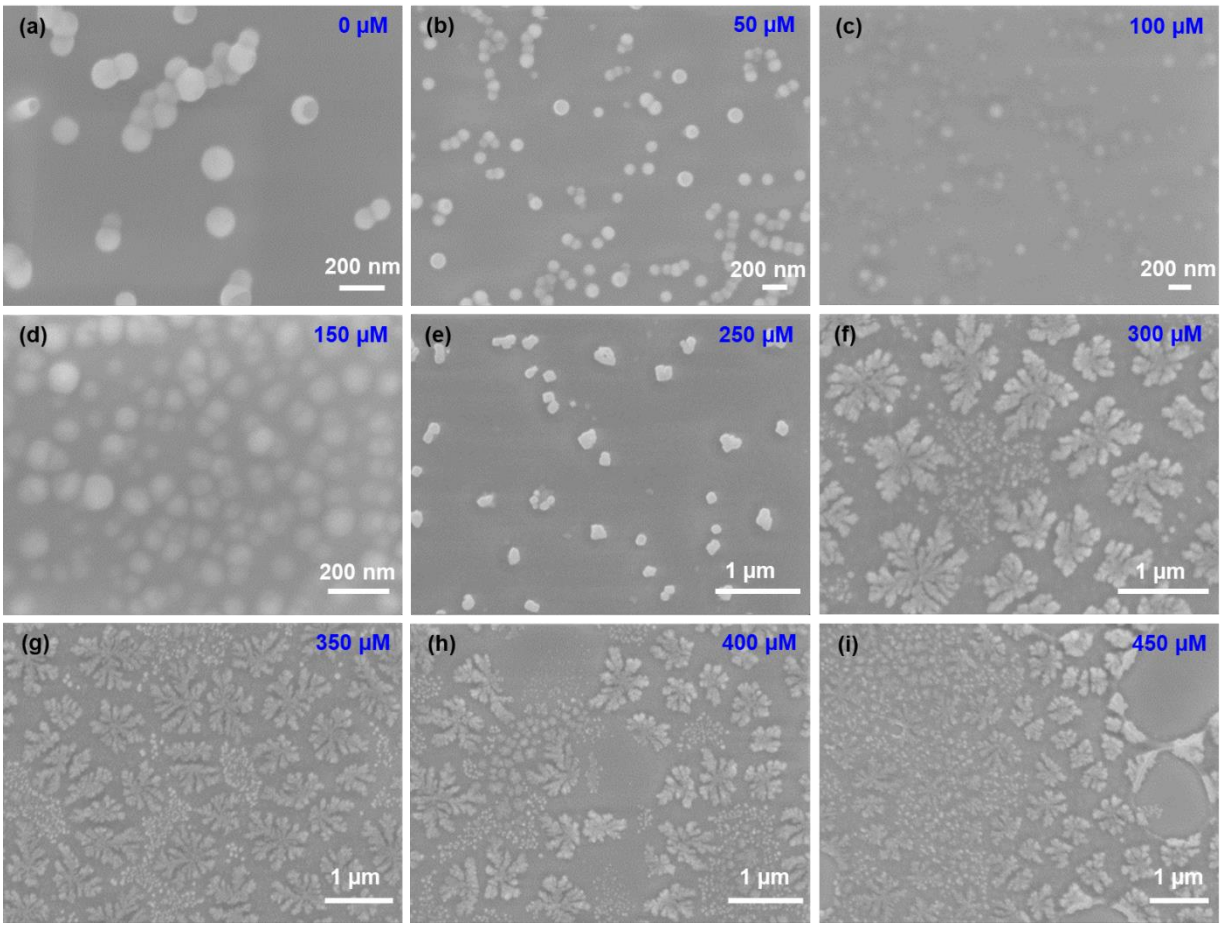

Figure S5. SEM images of H_2O_2 oxidized C-NPs. (a) As-synthesized C-NPs. Oxidized C-NPs by adding H_2O_2 with a concentration of **(b)** 50 μM, **(c)** 100 μM, **(d)** 150 μM, **(e)** 250 μM, **(f)** 300 μM, **(g)** 350 μM, **(h)** 400 μM H_2O_2, and **(i)** 450 μM.

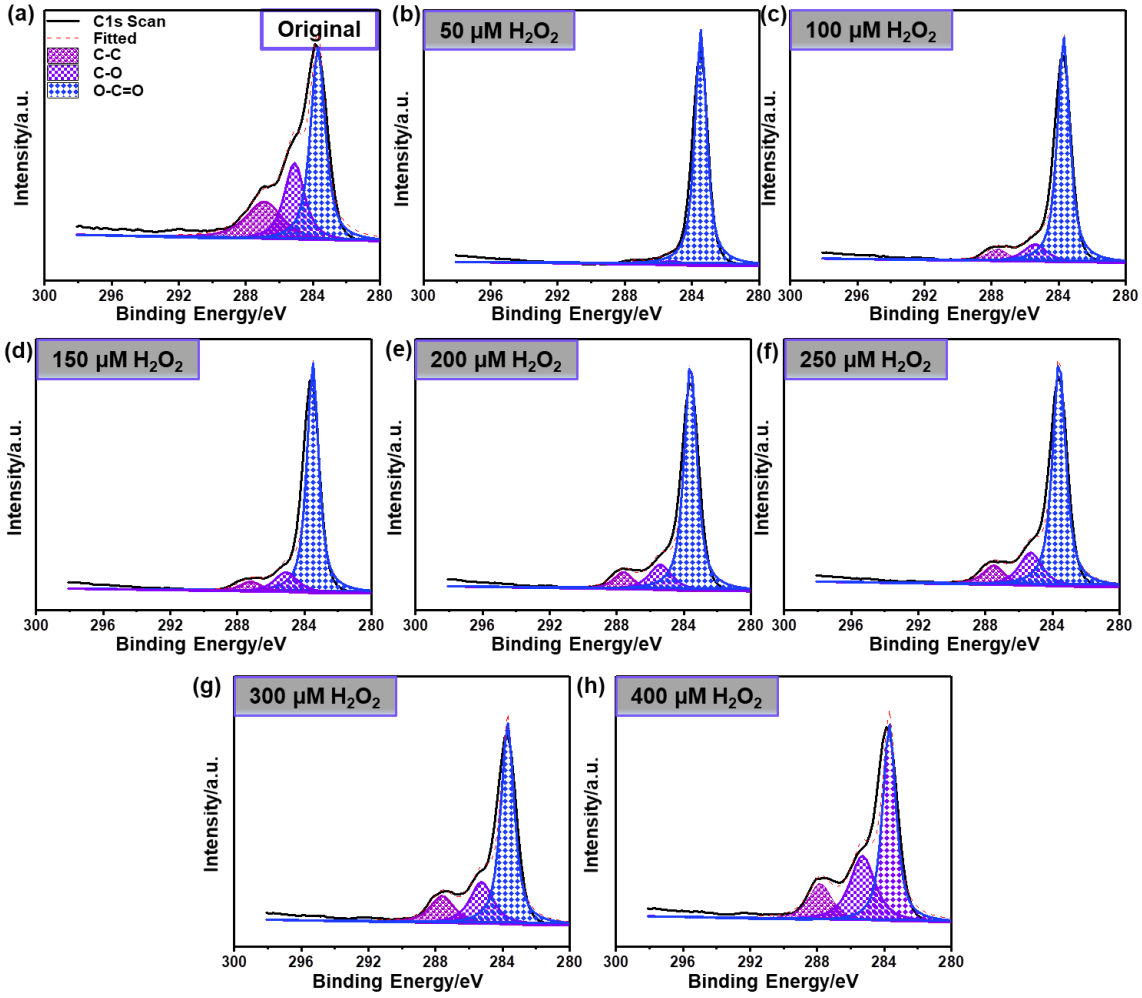

Figure S6. XPS spectra of H_2O_2 oxidized C-NPs. C 1s scan of **(a)** original C-NPs and oxidized C-NPs with **(b)** 50 μM, **(c)** 100 μM, **(d)** 150 μM, **(e)** 200 μM, **(f)** 250 μM, **(g)** 300 μM, **(h)** 400 μM H_2O_2,

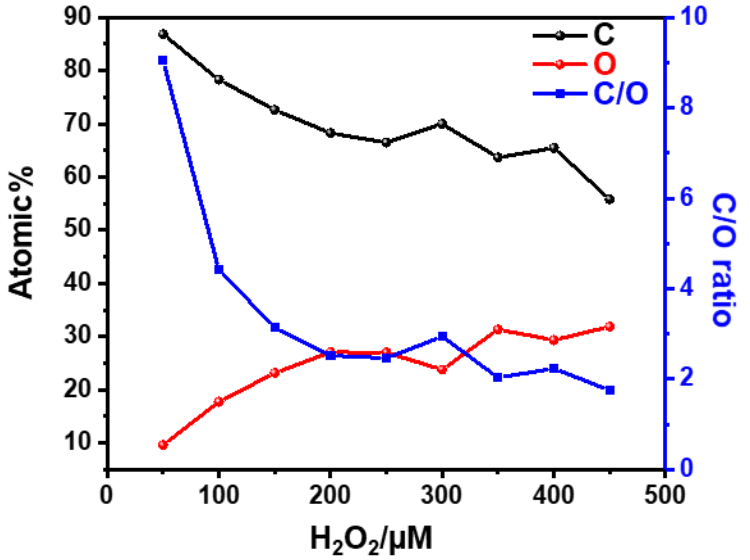

Figure S7. Atomic ratio from XPS spectra of H_2O_2 oxidized C-NPs. Carbon (black), oxygen (red) and C/O (blue) atomic ratio from XPS results of pristine C-NPs and oxidized C-NPs with different concentrations of H_2O_2,

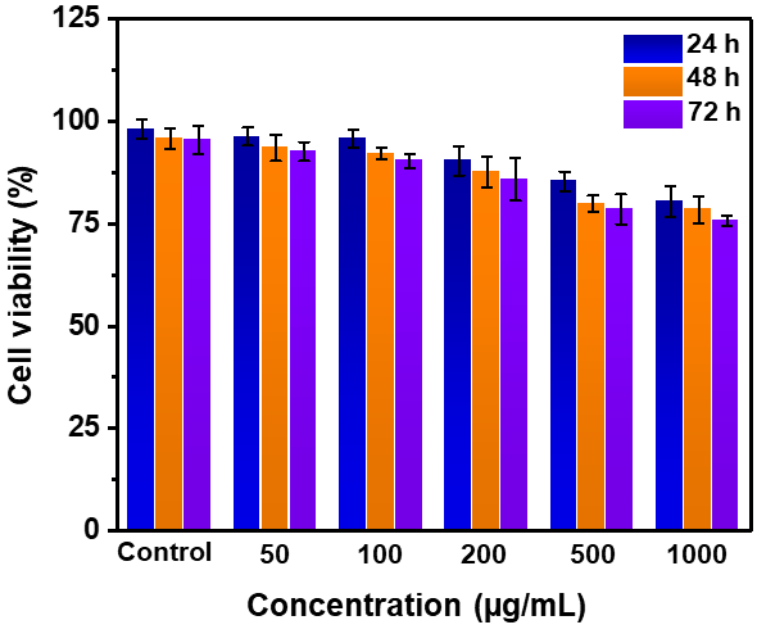

Figure S8. *In vitro* cell viabilities of HEK293 cells incubated with different concentrations of C-NPs for 24, 48 and 72h.

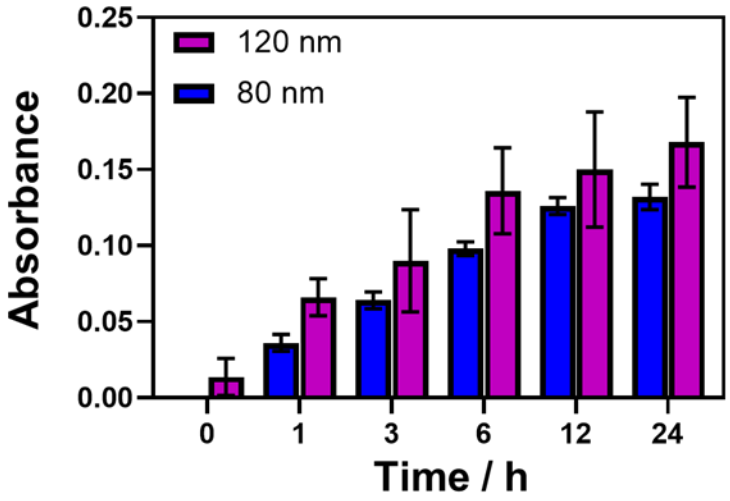

Figure S9. Time-dependent cellular uptake of RAW264.7 cells to 120 nm (purple bar) and 80 nm (blue bar) C-NPs at a concentration of 50 μg/mL.

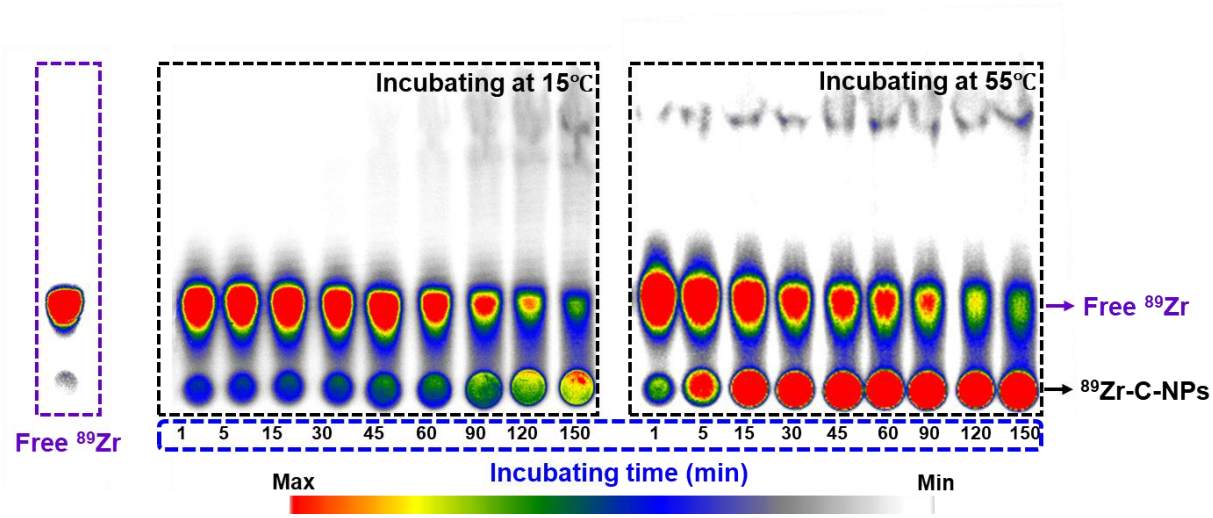

Figure S10. Autoradiography of thin layer chromatography (TLC) plates of ^{89}Zr-C-NPs (bottom) and free ^{89}Zr (top) at varied time and temperature.

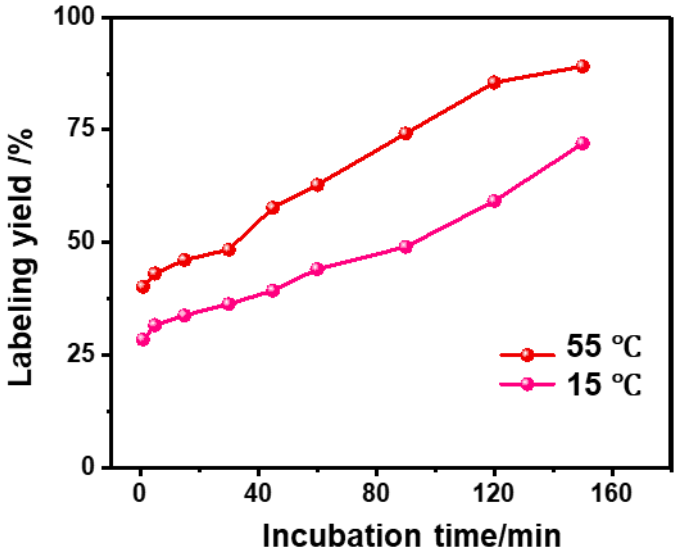

Figure S11. ^{89}Zr labeling yields in ^{89}Zr-C-NPs as a function of incubation time at a temperature of 15 °C (pink) and 55 °C (red).

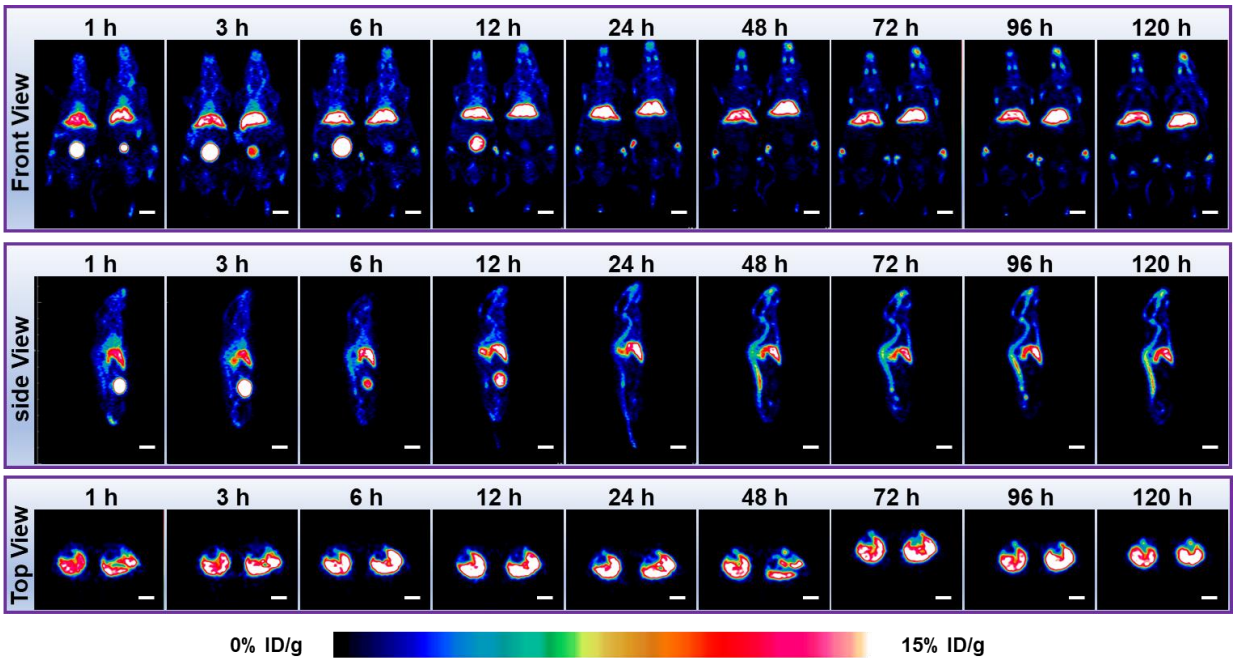

Figure S12. PET images of mice *i.v.* injected with ^{89}Zr-C-NPs at different time points. Scale bar: 1 cm.

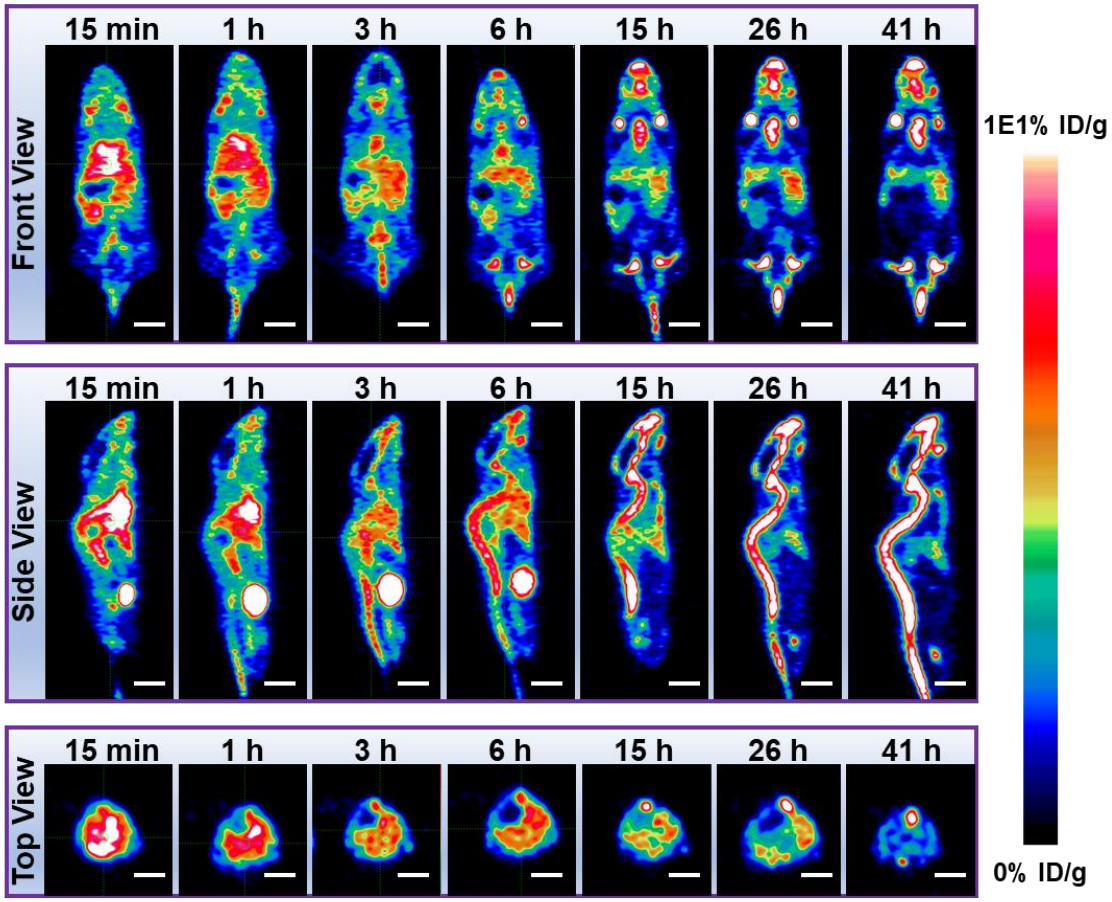

Figure S13. PET images of mice *i.v.* injected with free ^{89}Zr at different time points. Scale bar: 1 cm.

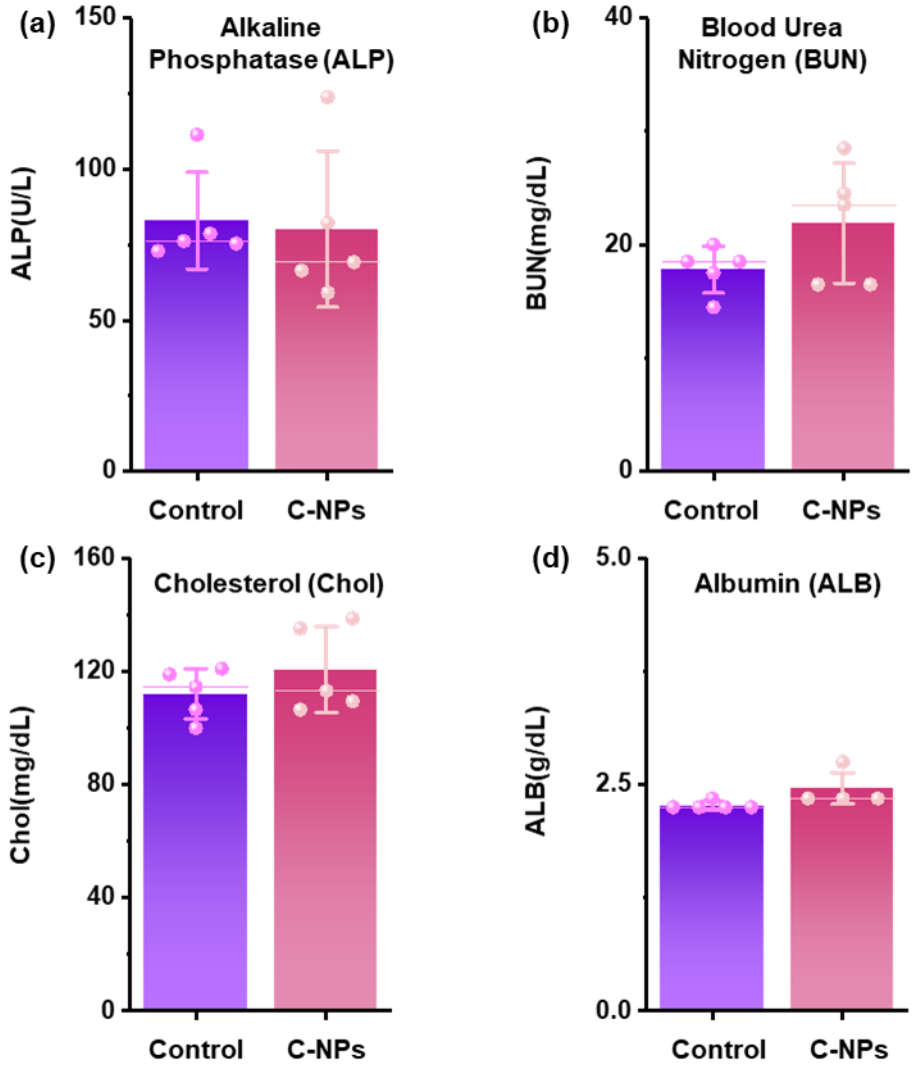

Figure S14. **Cytotoxicity evaluation of C-NPs on liver.** Indicators of **(a)** alkaline phosphatase (ALP), **(b)** blood urea nitrogen (BUN), **(c)** cholesterol (Chol), **(d)** albumin (ALB) of healthy mice with or without *i.v.* injection of C-NPs (n = 5, Data represent mean ± standard deviation).

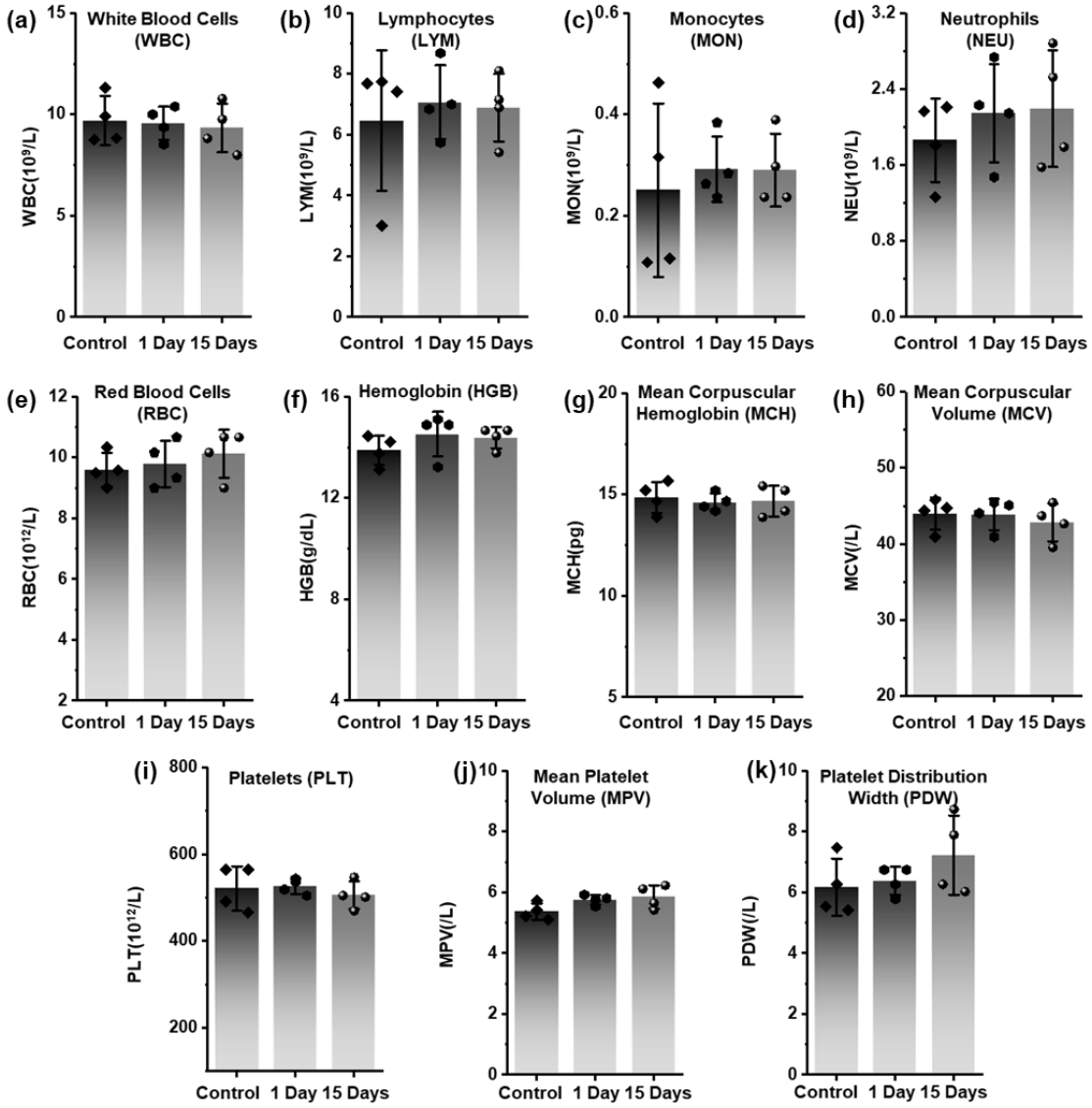

Figure S15. Long-term cytotoxicity test on blood serum. Numbers of **(a)** white blood cells (WBC), **(b)** lymphocytes (LYM), **(c)** monocytes (MON), **(d)** neutrophils (NEU), **(e)** redblood cells (RBC), **(f)** hemoglobin (HGB), **(g)** mean corpuscular hemoglobin (MCH), **(h)** mean corpuscular volume (MCV), **(i)** platelets (PLT), **(j)** mean platelet volume (MPV) and **(k)** platelet distribution (PDW) in healthy mice before and after *i.v.* injection of C-NPs (n = 4, Data represent mean ± standard deviation).

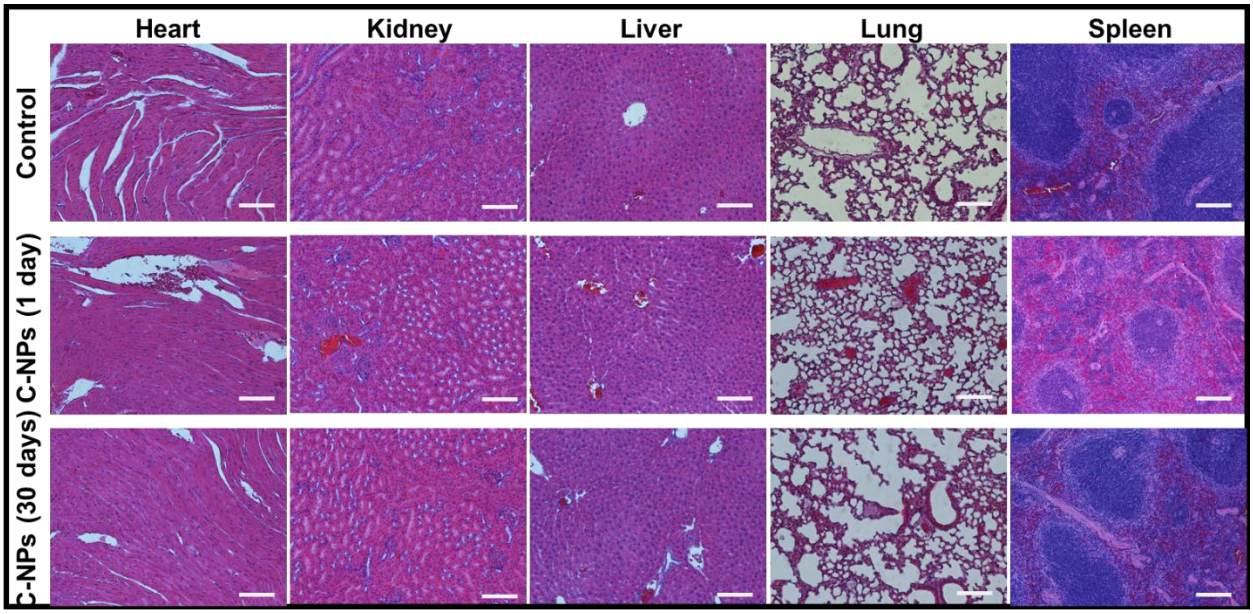

Figure S16. Microscopic photographs of H&E-stained tissues from major organs (heart, kidney, liver, lung and spleen) of healthy mice and mice at 1 day or 30 days after *i.v.* injection of C-NPs. Scale bar: 100 μm.

Table S1. Interpretation of Suzuki Score for the Assessment of Liver Damage Following Hepatic Ischemia/Reperfusion

Score	**Congestion**	**Vacuolization**	**Necrosis**
0	None	None	None
1	Minimal	Minimal	Single cell necrosis
2	Mild	Mild	-30%
3	Moderate	Moderate	-60%
4	Severe	Severe	>60%

Table S2. Suzuki Score for the Assessment of Liver Damage Following Hepatic Ischemia/Reperfusion

Group	Mice 1	Mice 2	Mice 3	Mice 4	Mice 5
Sham	0	0	0	0	0
NPs	0	0	0	0	0
IRI (12h)	3	3	2	3	3
IRI (60h)	2	2	1	2	3
IRI+NPs (12h)	1	1	1	0	1
IRI+NPs (60h)	1	0	0	1	0

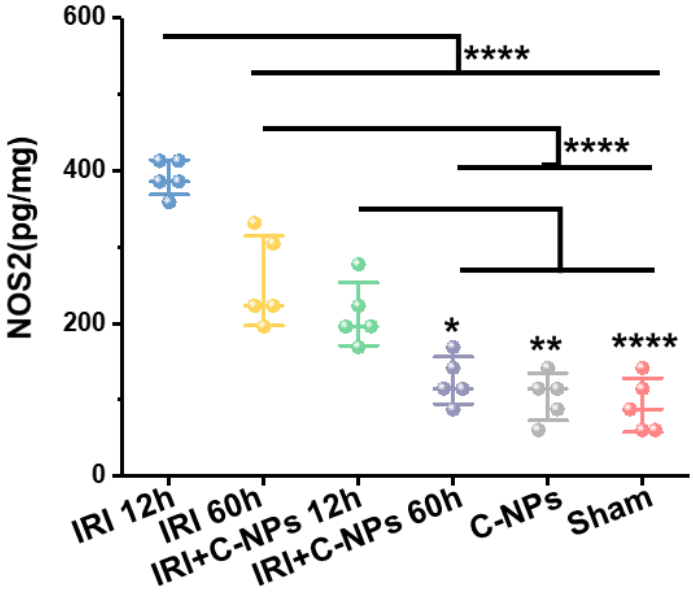

Figure S17 Detection of NOS2 from activated Kupffer cells in liver homogenates from each group. Data represent means ± *s.d.* from five independent replicates, and *P* values were calculated by one-way ANOVA with Tukey's honest significant difference post-hoc (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$).

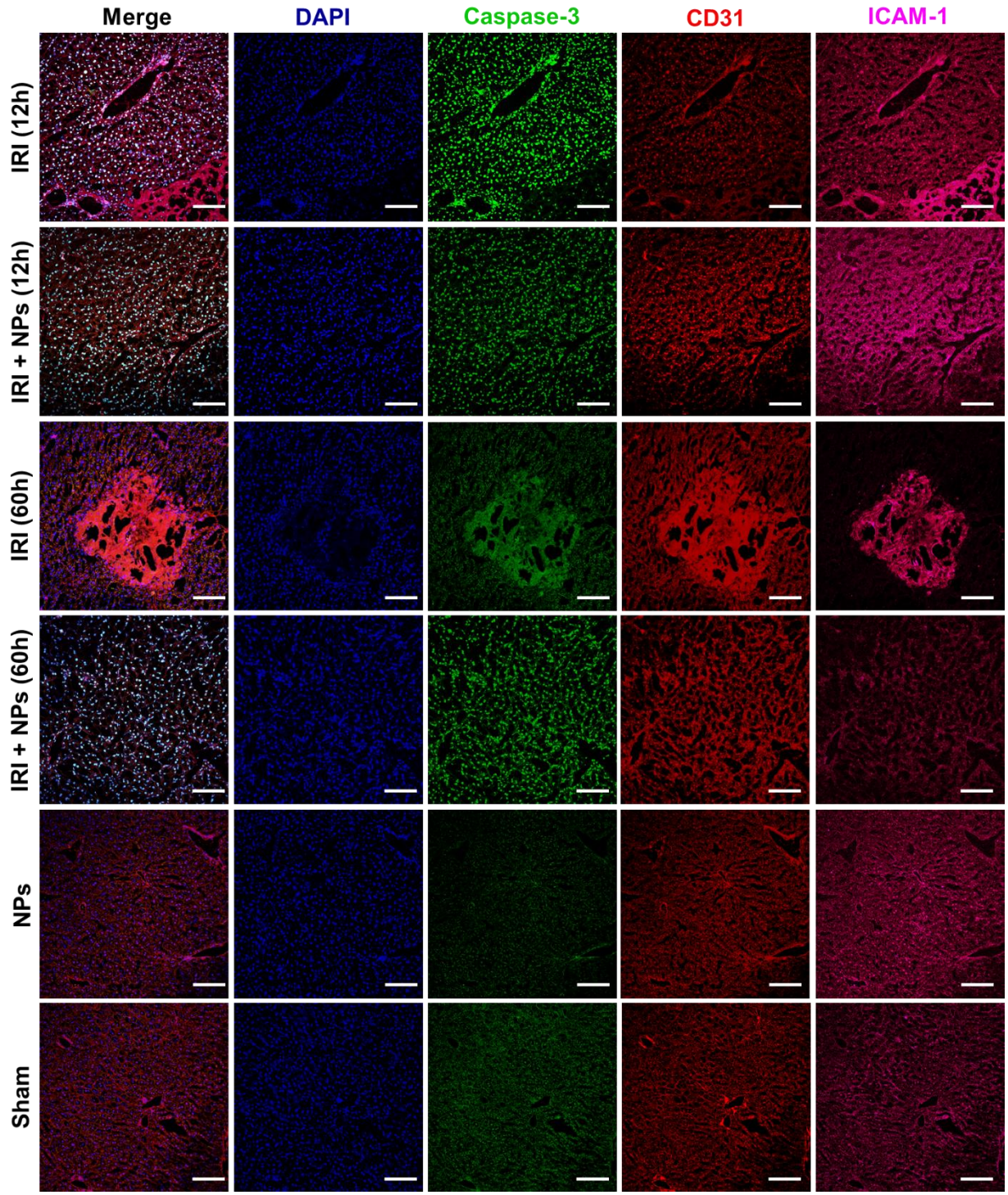

Figure S18. Immunofluorescence staining of liver samples by using DAPI (blue) for nuclear stainging, anti-Caspase-3 antibody (green) as cell apoptosis marker, anti-CD31 antibody (red) as an endothelial marker, and anti-ICAM-1 antibody (pink) as marker of intracellular adhesion in liver tissues from each group. Scale bar: 100 μm.

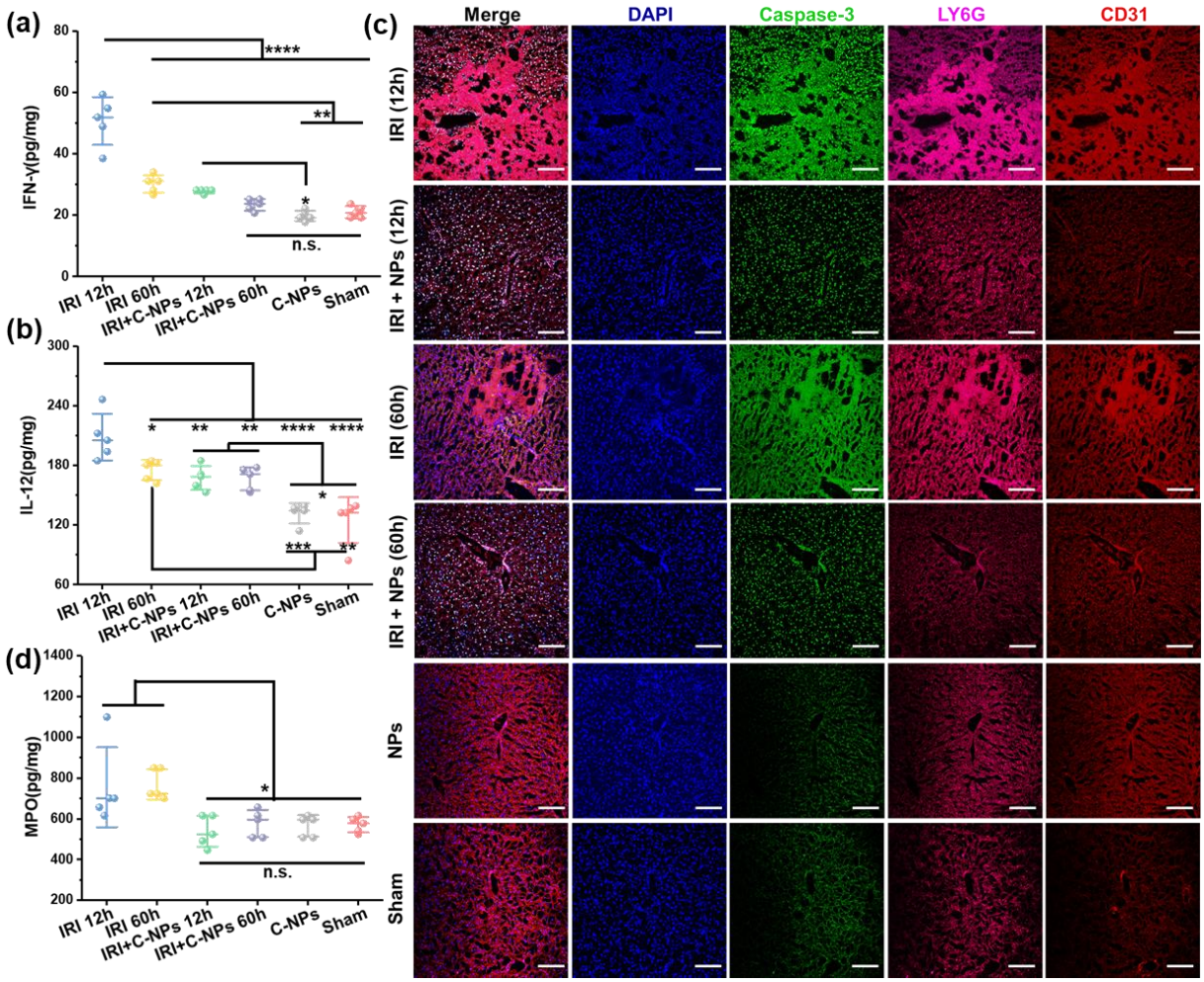

Figure S19. Immunofluorescence staining and dection of cytokines of liver samples. Cytokines of INF-γ **(a)**, IL-12 **(b)**, and MPO **(d)** from activated Kupffer cells and infiltrated neutrophils were measured in livers hemogenates from each group. Data represent means ± *s.d.* from five independent replicates, and *P* values were calculated by one-way ANOVA with Tukey's honest significant difference post-hoc (*$p < 0.05$; **$p < 0.01$; *** $p < 0.001$; ****$p < 0.0001$). **(c)** Immunofluorescence staining of liver samples by using DAPI (blue) for nuclear stainging, anti-caspase-3 antibody (green) as cell apoptosis marker, anti-Ly6G antibody (pink) as a neutrophil marker, and anti-CD31 antibody (red) as an endothelial marker in liver tissues from each group. Scale bar: 100 μm.