Supporting Information

An antioxidant nanodrug protects against hepatic ischemia-reperfusion injury by attenuating oxidative stress and inflammation

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Experiment section

Materials

Cerium(III) nitrate hexahydrate (99%), manganese chloride and 3,3',5,5'-Tetramethylbenzidine (TMB) were purchased from Shanghai Aladdin Reagent Co. Bovine serum albumin (BSA) was purchased from Beijing Gentihold Technology Co. Glutathione (GSH) were purchased from Sigma-Aldrich (MO, America). The CCK-8 was purchased from Changchun Sanbang Pharmaceutical Technology Co (Changchun, China).

Characterization

Morphology of the CM NCs were observed by a TECNAI G2 high resolution transmission electron microscope (TEM), X-ray diffraction (XRD) pattern was performed on a D8 ADVANCE X-ray diffraction with Cu Kα radiation. X-ray photoelectron spectroscopy (XPS) spectra were performed on an ESCALAB-MKII 250 photoelectron spectrometer (VG Co.). The hydrodynamic size and surfaced zeta potential measurements were conducted on a Nano-Zetesizer (Malvern Instruments Ltd). The UV-vis absorption spectra were measured on spectrometer (SHIMADZU, UV-3600). Inductively coupled plasma atomic emission spectrometer (ICP-AES) was obtained from Varian Liberty 200 spectrophotometer to analysis elemental concentrations.

Synthesis of CeO_2-MnO_2 nanocomposites (CM NCs). Bovine serum albumin (BSA) (0.24g) was dissolved in ultrapure water (5ml), and reacted with glutathione (GSH) solution (12.5mg mL^{-1}, 4ml). After 20 min, $Ce(NO_3)_3$ solution (100mM, 0.6ml) and $MnCl_2$ solution (100mM, 1.2ml) was added dropwise into the above solution. Then a NaOH solution (2.0 M) was added into mixture until the pH was adjusted to 10. The reaction was stirred for 12 h. After that, ethanol (5mL) was introduced into reaction system, and the mixture was stirred for 40 min. Finally, excess precursors were removed by dialysis against ultrapure water for 24 h (membrane cutoff MW: 100 KD). The CM NCs were further concentrated and purified using an ultrafiltration tube (Millipore, USA, MWCO 10 kDa).

Hydroxyl radical scavenging capacity of CM NCs. Using Fenton reaction of $FeSO_4$ (1.8 mM) and H_2O_2 (5 mM) generated the ·OH which was used as control group. The CM NCs with different amounts were added to solution containing ·OH at a final Ce concentration of 0.1, 0.2 and 0.4mM). Then the 3,3',5,5'-Tetramethylbenzidine (TMB) dissolved in DMF (20mM) were added to reaction system for color development, and detected the absorbance at 650 nm with UV absorption spectrometer.

Catalase mimic activity of CM NCs. Different amounts of CM NCs (12.5, 25, 50 and 100 μM final Ce concentration) were added into 20ml deionized water including 10mM H_2O_2 under constant stirring. The dissolved oxygen meters was used to monitor generated O_2 concentration and recorded every 10 seconds.

Electron spin resonance (ESR) spectra of CM NCs. The scavenging ROS (·OH and $\cdot O_2^-$) activities were quantitatively detected by ESR measurements. Hydroxyl radicals were generated in the Fenton reaction by adding 70ul $FeSO_4$ (0.735mM) and 25ul H_2O_2 (0.315 mM). The $\cdot O_2^-$ were generated in the hypoxanthine/xanthine oxidase (HYP 0.5mM, XOD 0.1U/ml) system. Then 100ul CM NCs (50 and 100ppm) were added to radical system. 10ul DMPO were added as the spin trapping agent. The scavenging activity of $\cdot O_2^-$ was also measured with SOD assay kit (Nanjing Jiancheng Bioengineering Institute).

Cell experiment

Cytotoxicity assessment of CM NCs. Human hepatocarcinoma (HepG2) cells and human embryonic kidney 293T (HEK293T) cells were selected for cytotoxicity assessment of CM NCs. Generally, the cells were plated into 96-well plates at 5×10^3 cells/well and cultured for 24 h at 37 ℃and 5% CO_2 conditions. Then different concentrations of CM NCs (0, 25, 50, 100, 200, 400, 800, 1000 and 1500mg mL^{-1}) in culture media solution were added to each well of a 96-wells plate. Then, cells were washed with PBS after incubation for 24 h, CCK-8 solution (100 μl/well) was added into each well and incubated for 1.5 h. The absorption values at 450 nm of each well were measured by a microplate reader. For the hemolysis assay, the mouse erythrocytes were obtained by centrifugation at 3000 rpm. 150 μl of erythrocytes were

incubated with distilled water, PBS and CM NCs solution with different concentrations for 8 h at 37 ℃, respectively. Finally, the mixtures were centrifuged to spin down erythrocytes.

Time-dependent cellular uptake of CM NCs. The RAW264.7 cells were plated into 96-well plates. Then cells incubated with fluorescein isothiocyanate (FITC)-labeled CM NCs at concentrations of 800μg mL^{-1} for 2, 4 and 6 h under 37 ℃and 5% CO_2 conditions. Finally, a fluorescence microscope was used to detected intracellular fluorescence.

Intracellular ROS scavenging with CM NCs. The HepG2 cells were plated into 96-well plates. Then cells incubated with CM NCs of different concentrations (200, 400 and 800μg mL^{-1}) in culture media for 12 h under 37 ℃and 5% CO_2 conditions. The cells were treated with culture medium containing H_2O_2 (1 mM) and incubated for another 24 h. The cells co-incubated with CM NCs only or H_2O_2 only were used as a control. Then the cell viability was evaluated via the CCK-8 assay.

Detection of intracellular ROS by HPF. HepG2 cells were plated in a 6-well plate and cultured for 12 h. The cells were treated with CM NCs (800μg ml^{-1}) in serum-free medium for another 12 h. Then culture medium with H_2O_2 of different concentrations (250 μM and 375 μM) were added to each well. After further incubation for 4 h, cells were washed with PBS, and co-incubated with hydroxyphenyl fluorescein (HPF) for 30 min at 37 ℃. Finally, a fluorescence microscope was used to detected intracellular fluorescence.

Animal experiment

In vivo toxicity assessment of CM NCs. ICR mice (n=6) were treated with CM NCs (100μl 5mg mL^{-1}) once a day for two consecutive days, and the mice (n=3) injected with PBS (1×) were used as the control group. Mice were sacrificed 24h and 30 days after the last administration. The blood samples from three groups were collected into EDTA anticoagulation tubes for further whole blood routine test. The major organs, including heart, liver, spleen, lungs and kidneys, were fixed in 4% paraformaldehyde

for preparation of staining with Hematoxylin-Eosin (H&E).

Biodistribution of CM NCs. The healthy ICR mice (n=27) were sacrificed without pain after intravenously injected with CM NCs (100μl, 10mg mL^{-1}) at various time points within 14 days. The major organs (heart, liver, spleen, lungs, and kidneys) were dissected and weighted. And the abovementioned tissues were soaked in the digesting aquaregia (HCl: HNO_3=3:1) for 48h. The content of Ce was measured by ICP-AES.

Establishment of the hepatic IRI model. All of the animal experiments were conducted under a protocol approved by the Jilin University Animal Care and Use Committee. Male ICR mice (7 weeks old) were purchased from Laboratory Animal Center of Jilin University (Changchun, China). The surgical procedure of the hepatic IRI model was performed as follows. Firstly, mice were anesthetized via inhalation of isoflurane, the limbs were fixed on a thermostatic pad, and the skin was prepared. A median abdominal incision was chosen to expose the abdominal cavity. A microvascular clamp was applied to the portal canal (hepatic artery, portal vein, and bile duct) to the median and left liver lobes, yielding 70% hepatic ischemia. For sham group, the mice were performed under the same surgery procedure without hepatic ischemia. After 60 minutes of liver ischemia, the clamp was removed and reperfusion established visually.

Hepatic IRI treatment. For treatment of hepatic IRI mice, the ICR mice (n=5) were treated with CM NCs (5 mg mL^{-1}) by tail vein injection once a day for two consecutive days prior to surgery. The mice in the sham group and healthy ICR mice treated with the CM NCs (5 mg mL^{-1}) or PBS (1×) were used as control (n=5). After 12 h of the reperfusion, the mice were sacrificed without pain. For a long-term evaluation of liver function, one group of IRI model mice treated with the CM NCs were sacrificed at 7 days after reperfusion.

Liver function evaluation. Liver functional profiles (alanine aminotransferase (ALT) levels and aspartate aminotransferase (AST) levels) and liver tissue H&E staining were conducted to evaluate the severity of IRI. Mice blood samples were collected and centrifuged (3000 rpm at 4 ℃) for 15 minutes to obtain the serum, which was

sent to the Wuhan servicebio technology CO.,LTD for analysis of AST levels and ALT levels. Livers were dissected at 12 h and 7 days after surgery, and fixed with 4% paraformaldehyde and sent to the Wuhan servicebio technology CO.,LTD for further sectioning and H&E staining. MDA levels of all groups were assessed using a MDA assay kit (Nanjing Jiancheng Bioengineering Institute, China).

Level of superoxide in liver tissue evaluation. To investigate superoxide level in liver tissues by fluorescent staing, livers from each group were dissected to prepare the frozen liver tissue slices. Subsequently, the slices were stained with dihydroethidium (DHE) for 30 min to detect superoxide. Finally, a fluorescence microscope was used to detected intracellular fluorescence.

Enzyme-linked Immunosorbent Assay (ELISA) for detection cytokines. Collected liver tissues were weighted and trimmed into small pieces. The samples were homogenized at 4 ℃with a homogenizer in RIPA buffer (Beyotime Biotechnology Co., Ltd) containing 1× protein inhibitor (Protease and phosphatase inhibitor cocktail) at a final concentration of 100 mg ml^{-1}. Then, lysates were obtained after 16099 g centrifugation for 15 min at 4 ℃ and stored in a liquid nitrogen tank until further use. The following measurements of cytokines were conducted according to the ELISA Kit Operation Instruction (Mouse IL-6 Precoated ELISA Kit, Dakewe; Mouse IL-12 Precoated ELISA Kit, Dakewe; Mouse TNF-α Precoated ELISA Kit, Dakewe; Mouse IL-1β Precoated ELISA Kit, Dakewe; Mouse IFN-γ Precoated ELISA Kit, Dakewe; Mouse Myeloperoxidase ELISA Kit, Boster)

Results and discussion

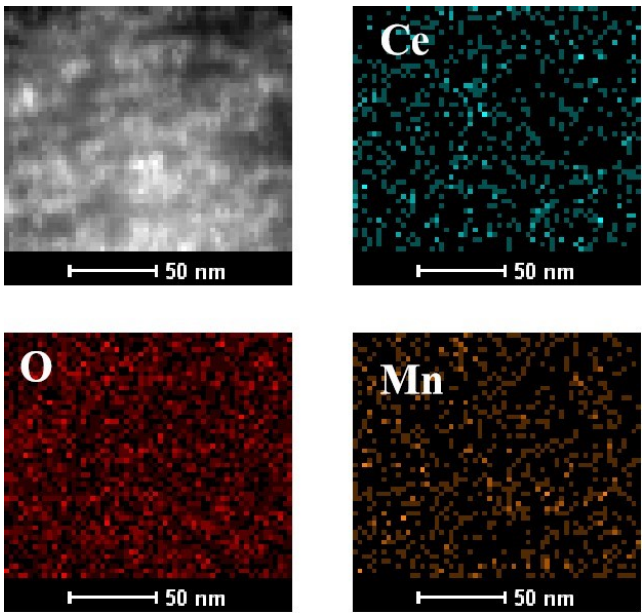

Figure S1. STEM image and EDS elemental mapping of CM NCs.

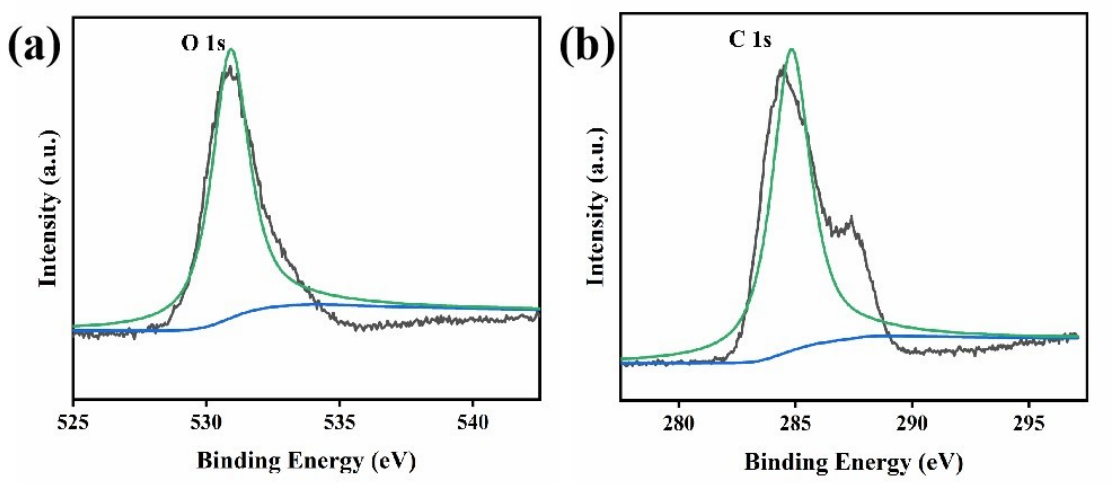

Figure S2. XPS survey spectrum of CM NCs. (a, b) O, C element XPS spectra, together with their corresponding fitting curves.

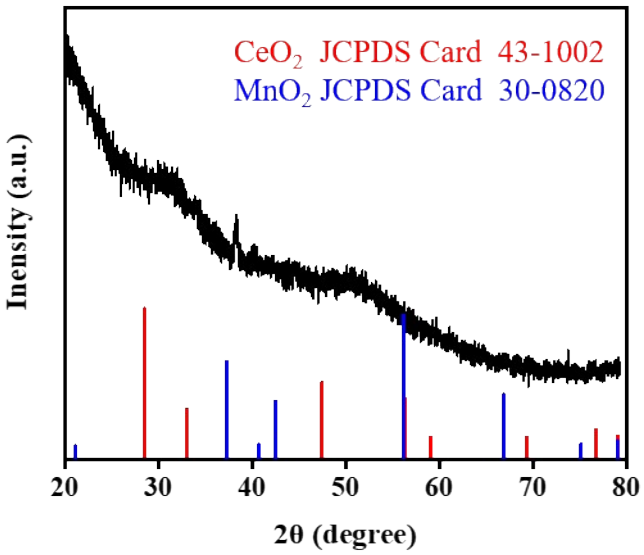

Figure S3. The XRD spectrum of CM NCs.

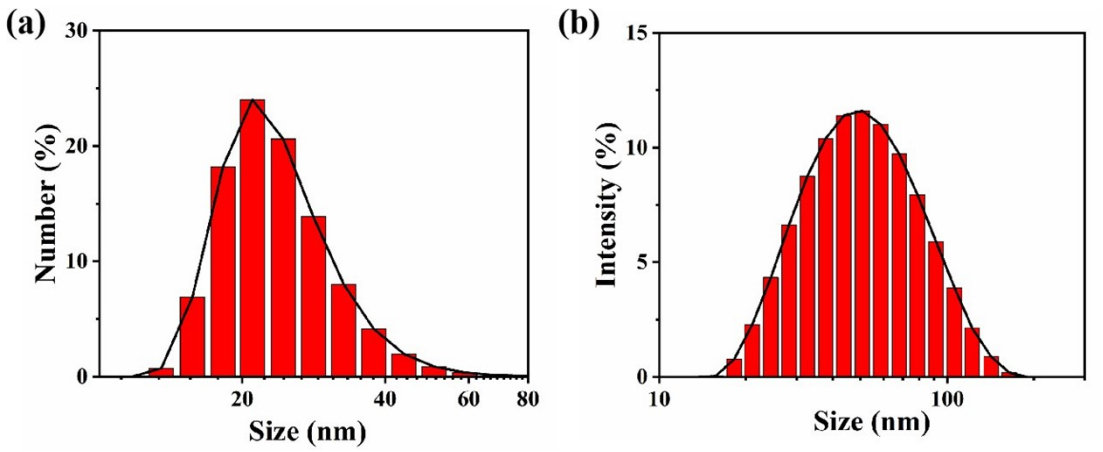

Figure S4. DLS size distribution by number (a) and intensity (b) of CM NCs in water.

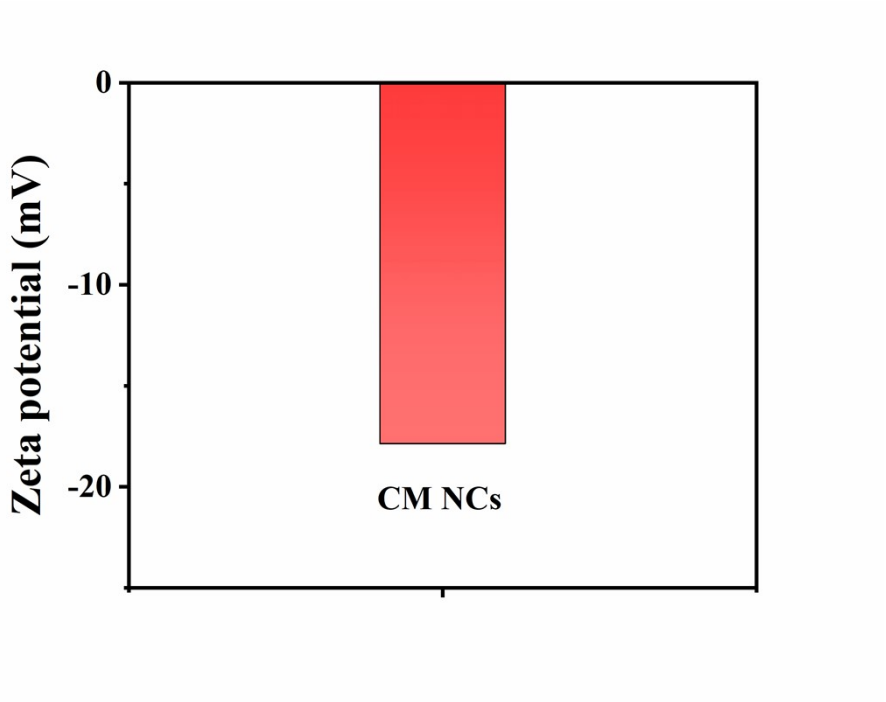

Figure S5. Zeta potential of CM NCs.

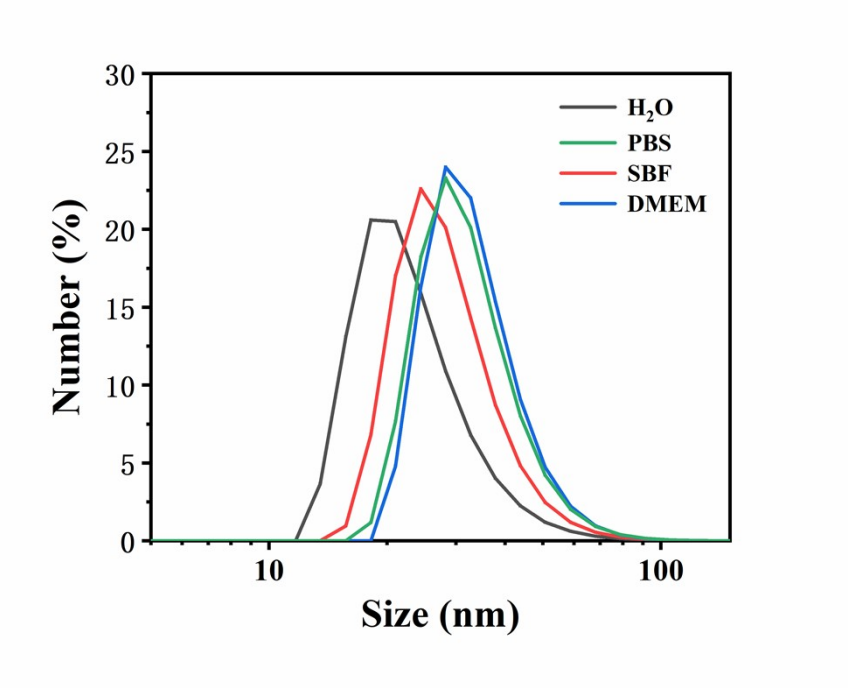

Figure S6. DLS size distribution of CM NCs dispersed in H_2O, PBS, SBF and DMEM for 7 days.

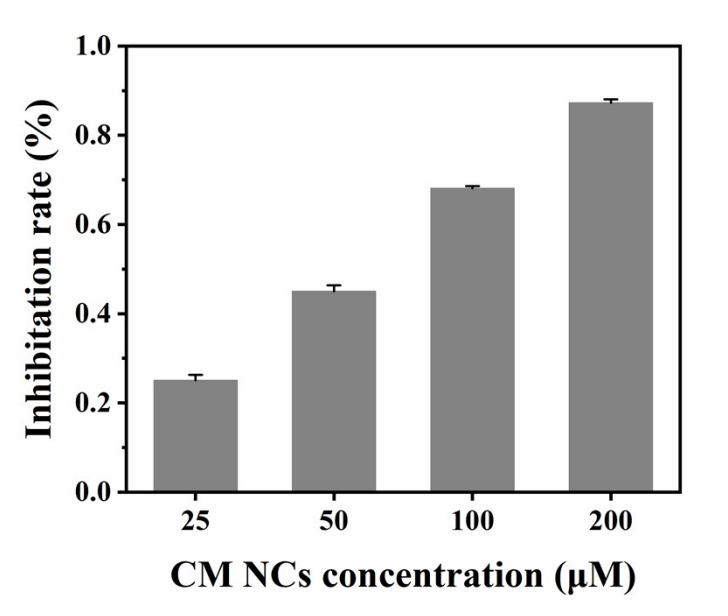

Figure S7. •O_2^- scavenging efficiency of CM NCs with different concentrations. The concentration of CM NCs are expressed in forms of the molar concentration of Ce.

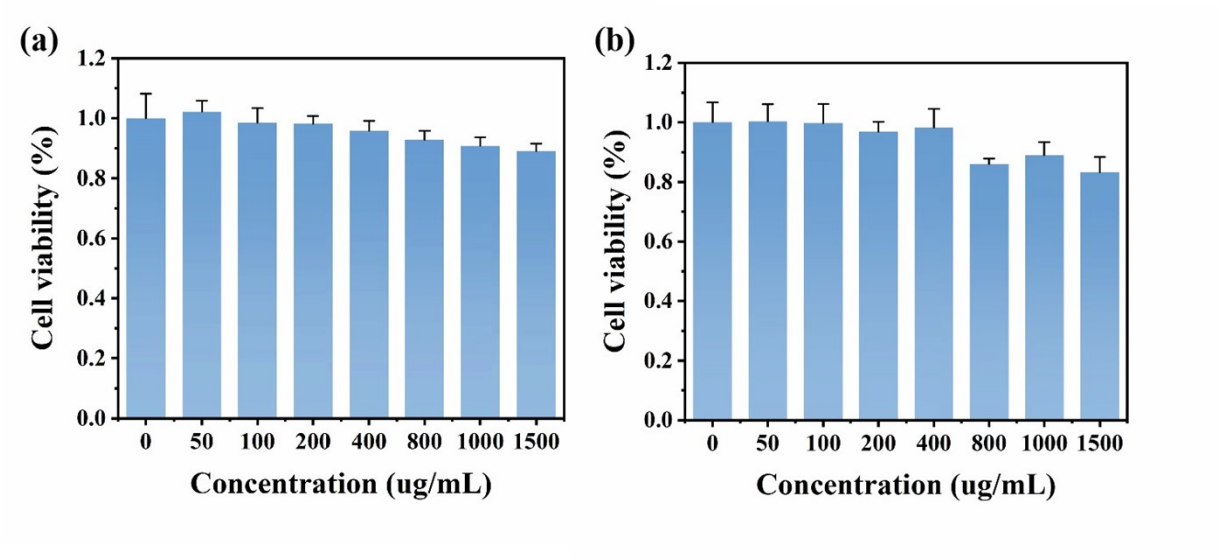

Figure S8. Cell viability of HepG2 cells (a) and HEK293T cells (b) respectively with different concentration of CM NCs.

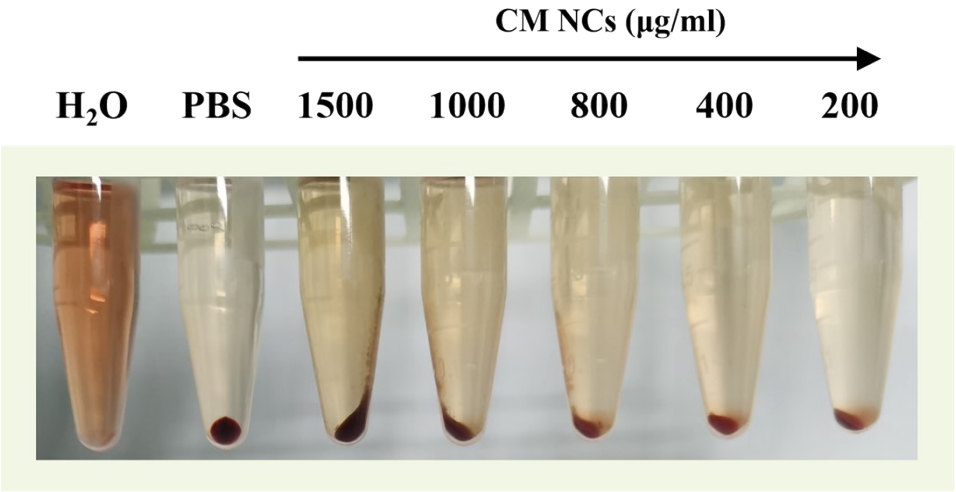

Figure S9. Hemolysis assay of mouse red blood cells incubated with different concentrations of CM NCs solution.

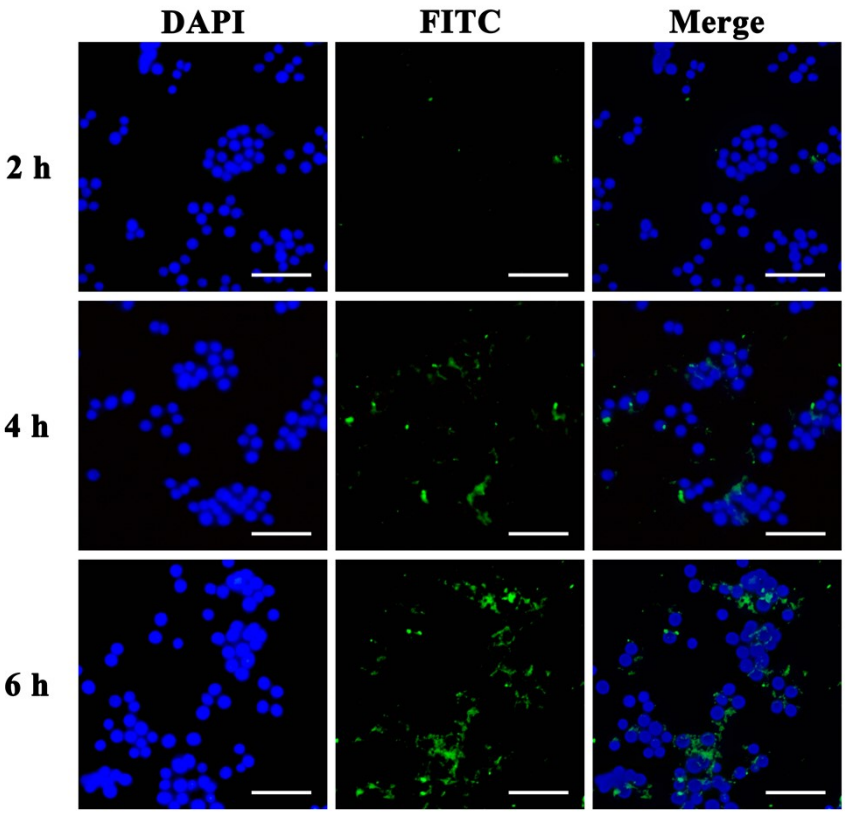

Figure S10. Time-dependent cellular uptake of CM NCs in RAW264.7 cells. Scale bar: 100 μm.

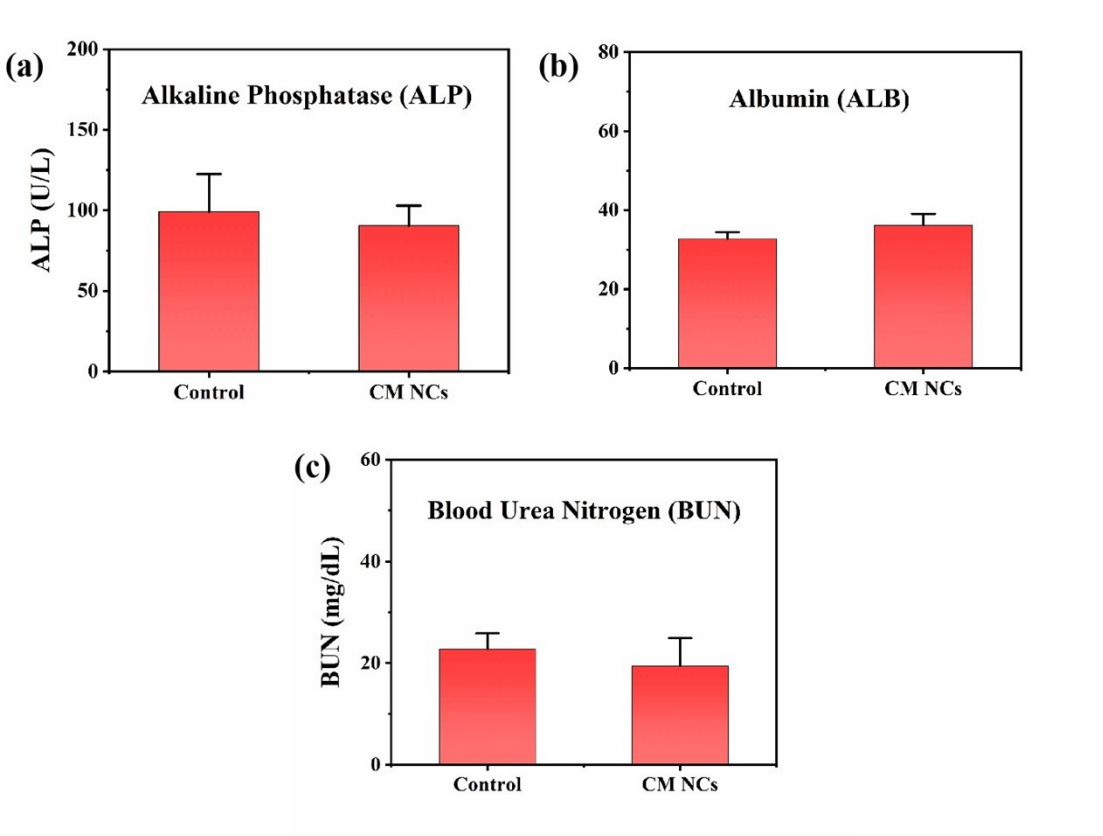

Figure S11. Indicators of (a) alkaline phosphatase (ALP), (b) blood urea nitrogen (BUN), (c) albumin (ALB) of healthy mice with or without i.v. injection of CM NCs (n=5).

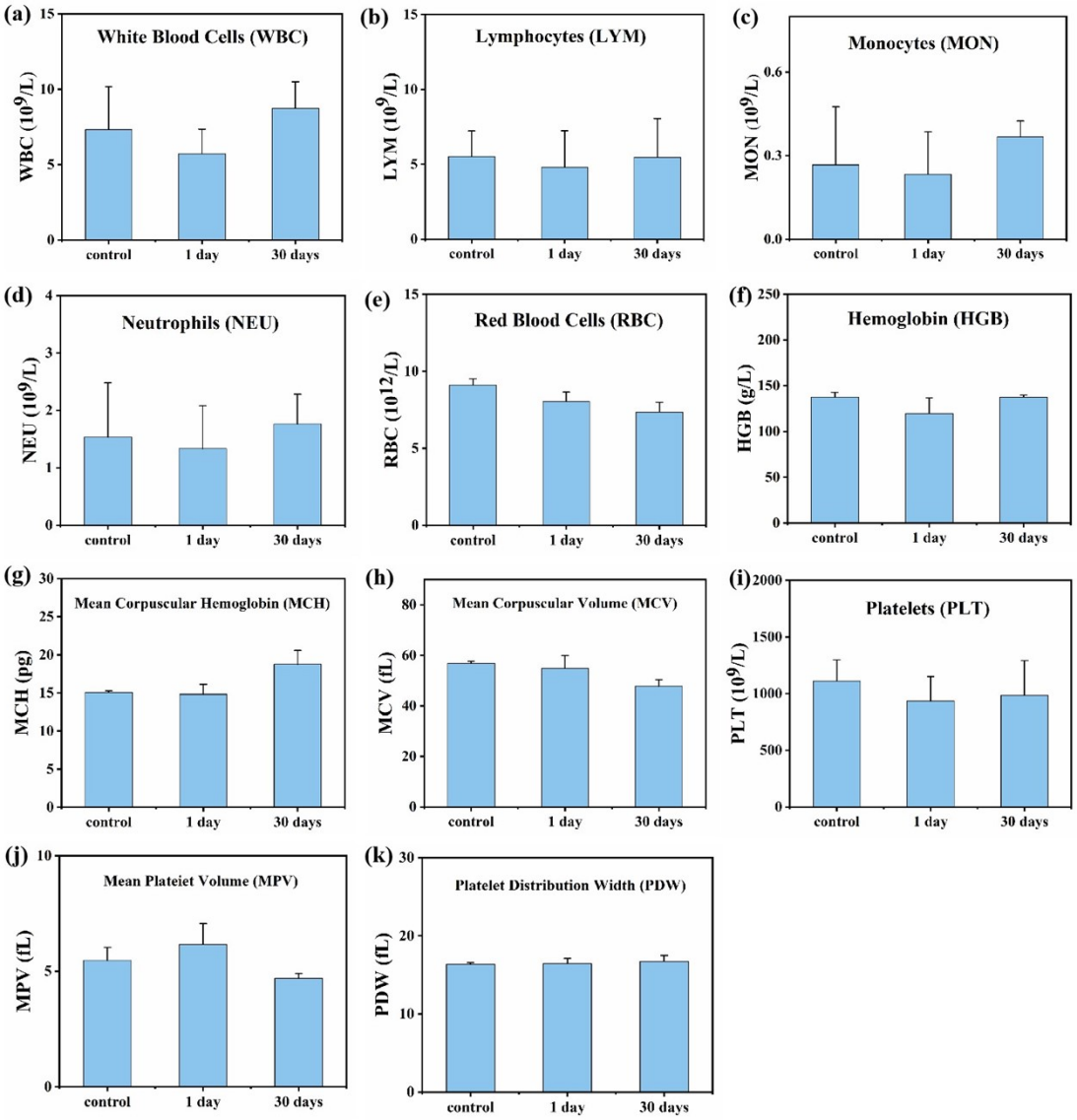

Figure S12. Complete blood count analysis for assessment of long-term cytotoxicity. Number of (a) white blood cells (WBC), (b) lymphocytes (LYM), (c) monocytes (MON), (d) neutrophils (NEU), (e) red blood cells (RBC), (f) hemoglobin (HGB), (g) mean corpuscular hemoglobin (MCH), (h) mean corpuscular volume (MCV), (i) platelets (PLT), (j) mean platelet volume (MPV) and (k) platelet distribution (PDW) in healthy mice with or without i.v. injection of CM NCs (n=3).

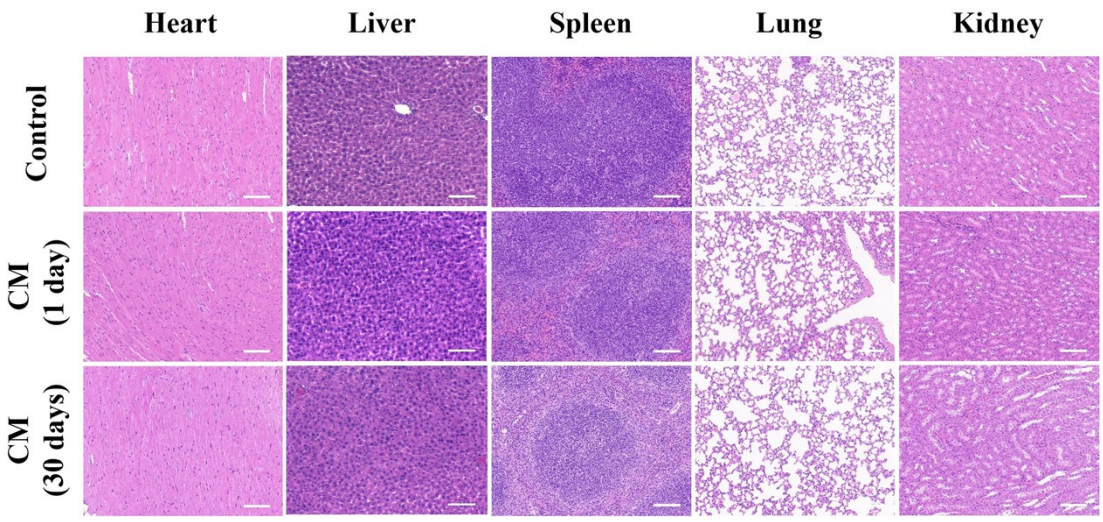

Figure S13. H&E-stained slices of major organs (heart, liver, spleen, lung and kidney) in healthy mice with or without i.v. injection of CM NCs. Scale bar: 100 μm.

Table R1. Criteria of Suzuki score for histological evaluation of hepatic injury

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%

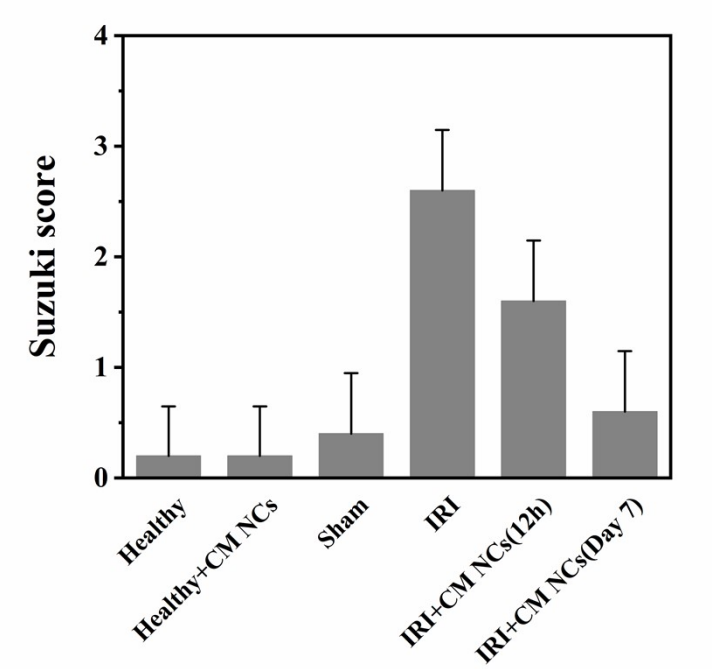

Figure S14. The pathological Suzuki score of liver tissue from each group.