Electronic Supplementary Information (ESI) for

Targeted Inhibition of Pyroptosis *via* A Carbonized Nanoinhibitor for Alleviating Drug-Induced Acute Kidney Injury

Yanjun Ji,[ab] Huan Wang,*[a] Xinchen Liu,[c] Zitong Zhu,[ab] Anjun Song,[ab] Li Chen,[ab] Jinsong Ren*[ab]

a. State Key Laboratory of Rare Earth Resources Utilization and Laboratory of Chemical Biology, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun 130022, P. R. China

b. School of Applied Chemistry and Engineering, University of Science and Technology of China, Hefei, Anhui 230026, P. R. China

c. Jilin Provincial Key Laboratory of Tooth Development and Bone Remodeling, Hospital of Stomatology, Jilin University, Changchun 130021, P. R. China

E-mail: huanwang@ciac.ac.cn; jren@ciac.ac.cn

Methods

Chemicals and reagents.

$K_2S_2O_8$ (≥99.5%), hydrogen peroxide (H_2O_2, 30%), and $NH_3 \cdot H_2O$ (25~28%) were purchased from XiLONG Scientific. Citric acid (≥99.5%), 3,4',5-trihydroxystilbene (99%), and cis-Diammineplatinum dichloride (cisplatin, 99.5%) were purchased from Aladdin Reagent. The mPEG-DSPE (M_W = 5000) and Cy5-PEG-DSPE (M_W = 5000) were ordered from ToYong Biotechnology. 2,2'-azinobis (3-ethylbenzothiazoline 6-sulfonate) (ABTS, ≥98%), (-)-Riboflavin (≥98%) were purchased from Sigma Aldrich. 1,1-diphenyl-2-picrylhydrazyl free radical (DPPH·, 96%) and Nitrotetrazolium Blue chloride (NBT, ＞98%) were purchased from Macklin. Terephthalic Acid (＞99%) was purchased from TCI. Ultrapure water was prepared using Milli-Q-Plus water system (18.2 $M\Omega \cdot cm^{-1}$) and used in all the experiments. Cell Counting Kit-8 was purchased from Invigentech. Reactive oxygen species indicator DCFH-DA was purchased from Sigma Aldrich. DAPI was purchased from MCE. LDH Cytotoxicity Assay Kit and TUNEL Apoptosis Assay Kit were ordered from Beyotime. Caspase 3 Antibody, DFNA5/GSDME Antibody, β-actin Antibody, STAT3 Antibody were purchased from Proteintech Group, Inc.. Annexin V-FITC Apoptosis Detection Kit was purchased from Bestbio. DMEM/F-12 were purchased from Gibco (Thermo Fisher Scientific). FBS was purchased from Inner Mongolia Opcel Biotechnology Co., Ltd.. Super ECL Detection Reagent and Hifair II 1st Strand cDNA Synthesis SuperMix for qPCR (gDNA digester plus) were purchased from Yeasen Biotechnology (Shanghai) Co., Ltd.. SYBR Green qPCR Mix was purchased from Biosharp Life Sciences. Primers were ordered from Sangon Biotech (Shanghai) Co., Ltd.. RNAiso Plus was purchased from TAKARA BIO INC. Quick Start Bradford protein assay Kit was purchased from Bio-Rad Laboratories, Inc.. Penicillin/streptomycin were purchased from Hyclone Laboratories, Inc.. Immobilon®-P Membrane, PVDF, 0.45 μm was purchased from Millipore. All other reagents were of analytical grade and used as received without further purification.

Characterization.

TEM measurements were carried out on a FEI TECNAI G^2F20 high-resolution transmission electron microscope at 200 kV. FT-IR measurements were carried out on a BRUKER Vertex 70 FT-IR spectrometer, and 32 scans were taken with a spectral resolution of 2 cm^{-1}. The ζ potential measurements were detected on Malvern Nano ZS-90 at 25 ℃. XPS measurements were performed on a Thermo Fisher Scientific ESCALAB 250Xi XPS system. Confocal imaging was carried out on a Nikon A1R confocal microscope. UV-vis-NIR absorbance measurements were carried out on an Agilent Cary 300 UV-Vis spectrophotometer. Fluorescence spectra were recorded on a JASCO FP-6500 spectrofluorometer. MALDI-TOF MS analysis was recorded on a BRUKER AutoflexIII smartbeam mass spectrometer. TGA was recorded on a Perkin-Elmer Pyris Diamond TG/DTA with a heating rate of 10 ℃/minute in the temperature range of 35 ℃~910 ℃ in N_2 atmosphere. The ^1H NMR analysis was recorded on a Bruker AVNEO 600 spectrometer in dimethyl sulfoxide-d_6. The ^{13}C solid-state NMR spectra were recorded on a Bruker AVANCE

NEO 400 WB spectrometer. Raman spectrum was recorded on a Jobin Yvon T64000 Raman spectrometer with an excitation of 532 nm.

Synthesis of RCDs from 3,4',5-trihydroxystilbene.

Briefly, 170 mg of the precursor 3,4',5-trihydroxystilbene and 3 mL of ammonia solution (25~28%) were dissolved in ultrapure water (27 mL) by sonication to obtain a solution of 3,4',5-trihydroxystilbene. Then, every 12 mL of the solution of 3,4',5-trihydroxystilbene was added to a Teflon-lined, stainless-steel autoclave and heated in an oven at 200 ℃ for 240 min. After the reactor was cooled to room temperature naturally, the reaction solution was filtered through a 0.22 μm microporous membrane to remove the large tracts, and a transparent brown solution was separated. The solution was first dialyzed in a dialysis bag with a molecular weight cut-off of 1 kDa against ultrapure water to remove residual small molecular species as well as the ammonia, and subsequently was dialyzed against diluted ammonia solution (pH ≈ 9) to fully remove residual small molecular species. Finally, the solution was dialyzed against ultrapure water again to remove the residual ammonia, and purified RCDs were obtained. A fixed volume of the solution was taken out and freeze-dried to determine the concentration of the RCDs.

Synthesis of PEGylated RCDs (P-RCDs).

RCDs (0.5 mg/mL) were sonicated in a solution containing mPEG-DSPE (1 mg/mL) for 0.5 h followed by dialyzed against UP water, yielding a suspension of PEGylated RCDs with non-covalent mPEG-DSPE coating. The concentrations of P-RCDs used in the experiments were calculated based on the content of RCDs in P-RCDs.

Synthesis of fluorescent Cy5-P-RCDs.

RCDs were sonicated in a solution containing Cy5-PEG-DSPE with a mass ratio of 1:2 for 0.5 h followed by dialyzed against UP water, yielding a suspension of Cy5-P-RCDs with non-covalent Cy5-PEG-DSPE coating.

Synthesis of CDs from citric acid as a control.

The graphitic citric acid-derived CDs were prepared by a pyrolysis process of 100 g of citric acid in a muffle furnace at 220 °C for 48 h. After the heating process, the product was dispersed in ultrapure water, and subsequently, the as-formed suspension was neutralized to pH 7 by the addition of NaOH aqueous solution (5 M), and the resulting suspension was filtered through a 0.22 μm microporous membrane to remove the large tract. Finally, the as-prepared suspension was purified *via* dialysis in a dialysis bag with a molecular weight cut-off of 1 kDa and was further filtered through a 0.22 μm microporous membrane. Then, the CDs were obtained by freeze-drying.

Synthesis of P-CDs.

Ultrapure water containing CDs (0.5 mg/mL) and mPEG-DSPE (1 mg/mL) was sonicated for 40 min, followed by dialyzed against ultrapure water, yielding PEGylated CDs with noncovalent mPEG-DSPE coating, which were named as P-CDs. The concentrations of P-CDs used in the experiments were calculated based on the content of CDs in P-CDs. The CDs possessed an average diameter of ca. 3~5 nm and a zeta potential value of −27.80 ± 2.31 mV as

demonstrated in our previous study (*ACS Appl. Mater. Interfaces* 2023, 15, 21854-21865). These citric acid-derived CDs composed of graphitic carbon cores and surface oxygenated groups including C=O, COOH, and C-OH. After PEGylation, a significantly increased zeta potential value for P-CDs could be observed.

DPPH· scavenging activity of RCDs.

Freshly prepared stock DPPH· methanolic solution was mixed with different concentrations of aqueous solutions of CDs (0-25 μg/ml) and incubated in the dark for a fixed period of time. Afterwards, UV-Vis-NIR absorption spectra of the above solutions of DPPH· were monitored, and the absorbance at 520 nm was recorded. Finally, DPPH· scavenging efficacy was calculated by using the ratio of neutralized DPPH· to the overall DPPH· radicals.

$ABTS^{+}$· scavenging activity of RCDs.

ABTS (7 mM) was dissolved in water containing $K_2S_2O_8$ (2.45 mM) to produce $ABTS^{+}$·, and the above mixture was kept in the dark at room temperature for 24 h before use, which was named as the stock solution. Then, the concentration of $ABTS^{+}$· of the above stock solution was diluted with PBS (10 mM, pH 7.4). After the addition of various concentrations of RCDs (0-6.25 μg/ml), the above solutions were further incubated in the dark for a fixed period of time. Finally, UV-Vis-NIR absorption spectra of the above solutions of $ABTS^{+}$·were monitored, and the absorbance at 734 nm was recorded. $ABTS^{+}$·scavenging efficacy was calculated by using the ratio of neutralized $ABTS^{+}$· to the overall $ABTS^{+}$·.

·OH scavenging activity of RCDs

A terephthalic acid (TA) fluorescence experiment was carried out to clarify the ·OH scavenging activity of RCDs by monitoring the production of hydroxyl radicals (·OH). Solutions containing TA (0.5 mM), H_2O_2 (20 mM), and RCDs (0-25 μg/mL) were illuminated by a UV lamp with a constant light intensity for a fixed period of time. TA without fluorescence could be converted into 2-hydroxyterephthalic acid (TAOH) with high fluorescence at ~425 nm in the presence of ·OH when excited at 315 nm. The ·OH scavenging efficacy was calculated by using the ratio of neutralized ·OH to the overall ·OH.

·O_2^- scavenging activity of RCDs.

The ·O_2^- scavenging activity was tested by measuring the inhibition of the photoreduction of NBT. Solutions containing riboflavin (20 μM), L-methionine (0.013 M), NBT (75 μM), and RCDs (0-50 μg /mL) were illuminated by a UV lamp with a constant light intensity for a fixed period of time. Then, the absorbance at 560 nm was measured immediately. The ·O^{2-} scavenging efficacy was calculated by using the ratio of neutralized ·O_2^- to the overall ·O_2^-.

Comparison of the ROS scavenging activity.

The ROS scavenging activities of various tested materials were investigated by using the same concentrations of the precursor 3,4',5-trihydroxystilbene, RCDs, P-RCDs, CDs, and P-CDs as the antioxidants within the same reaction time.

Cell culture.

Human Kidney Tubular Epithelial Cells (HK-2 cells) were obtained from the American Type Culture Collection (ATCC) and cultured at 37 °C under 5% CO_2 in an incubator. Media was Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) containing FBS (10%), and penicillin/streptomycin (1%, W/V).

Cell viability study.

To investigate the detoxification activities of P-RCDs against cisplatin-induced cytotoxicity, HK-2 cells (1×10^4 cells per well) were seeded in 96-well plates and incubated overnight. After incubating with cisplatin (15 μg/mL) together with different concentrations of P-RCDs for 24 h, MTT was added to the culture medium and incubated for 4 h at 37 °C in 5 % CO_2. Then, 200 μL of DMSO was added to dissolve the formazan crystals, and a microplate reader was used to measure the absorbance at 570 nm.

Intracellular ROS detection.

Intracellular ROS level was detected by flow cytometry with DCFH-DA. Briefly, HK-2 cells (5×10^5 cells per well) were seeded in 6-well plates and incubated overnight. After incubating with cisplatin (15 μg/mL) together with P-RCDs (12.5 μg/mL) for 24 h, cells were stained with DCFH-DA for 20 min at 37 °C in 5 % CO_2. Then, the cells were collected for fluorescence intensity detection by flow cytometry.

Western blotting.

Samples were first lysed in RIPA buffer supplemented with the protease inhibitor cocktail at 4 °C, and the protein concentration was determined by a Quick Start Bradford protein assay Kit. Afterwards, 25 μg of protein from each sample was resolved by SDS-PAGE (8 %-10 % SDS- PAGE gels), and subsequently transferred to the polyvinylidene difluoride (PVDF) membranes. Membranes were then blocked in 5 % skim milk in TBST and probed with specific primary antibodies. HRP-conjugated secondary antibodies and ECL Chemiluminescence Detection Kit were used for protein detection. The level of β-actin immunoreactivity was used as a control to monitor equal protein loading**.**

Immunofluorescence Assays.

To visualize proteins, samples were subjected to immunofluorescence. Cells grown on coverslips and fixed with formalin at room temperature for 20 min. After PBS rinses and permeabilization in 0.5% Triton/PBS for 10 min, samples were blocked in 10% Goat Serum for 2 hours. Subsequently, cells on glass coverslips were incubated with the primary antibody at 4 °C for 12 h and labeled with DyLight 550-conjugated secondary antibodies at 4 °C for 12 h. Finally, samples were stained with DAPI for 10 min and imaged by a confocal microscope (Nikon A1R).

qRT-PCR.

Total RNA was extracted from cells with RNAiso Plus. Then, cDNA was synthesized with Hifair II 1st Strand cDNA Synthesis SuperMix for qPCR (gDNA digester plus). The qRT-PCR was performed using SYBR Green qPCR Mix. Triplicate samples were run on an Analytik Jena qTOWER^3 Series qPCR system according to the manufacturer's

protocol. The threshold cycle (Ct) values for each gene were normalized to those of β-actin, and the $2^{-\Delta\Delta Ct}$ method was used for quantitative analysis. Primer sequences are listed below.

GSDME-F: GATCTCTGAGCACATGCAGGTC

GSDME-R: GTTGGAGTCCTTGGTGACATTCC

IL-1β-F: CCACAGACCTTCCAGGAGAATG

IL-1β-R: GTGCAGTTCAGTGATCGTACAGG

TNF-α-F: CTCTTCTGCCTGCTGCACTTTG

TNF-α-R: ATGGGCTACAGGCTTGTCACTC

CCL2-F: AGAATCACCAGCAGCAAGTGTCC

CCL2-R: TCCTGAACCCACTTCTGCTTGG

Cisplatin-induced AKI model.

All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Changchun Institute of Applied Chemistry Chinese Academy of Sciences and approved by the Institutional Animal Care and Use Committee of Changchun Institute of Applied Chemistry Chinese Academy of Sciences (Permit Number: 20220102). Female balb/c mice were obtained from the Laboratory Animal Centre of Jilin University (Changchun, China). All Balb/c mice were adapted for 7 days before being received intraperitoneal injections of cisplatin (15 mg/kg). The time point at 24 h post-injection of cisplatin was defined as the initiation of AKI.

Hemolysis test of P-RCDs.

400 μL of the whole blood was collected in tubes containing EDTA-K2 from the orbital venous of mice. Then, blood samples were mixed with the proper amount of PBS (10 mM, pH = 7.4), centrifuged for 5 min at 3000 rpm and discarded the supernatant, and repeated 3-4 times until the supernatant became colorless transparent. The precipitated erythrocytes were dispersed in 4 mL PBS (10 mM, pH = 7.4) to get erythrocyte suspensions. The P-RCDs and erythrocyte suspensions were added to the PBS (10 mM, pH = 7.4) respectively to obtain a final concentration of P-RCDs as 12.5, 25, 50, 100, 200, 400 μg mL^{-1}, and only erythrocyte suspension was added in 10 mM PBS (pH = 7.4) to get negative control, and positive control was erythrocyte suspension diluted with ultrapure water. The tubes were incubated for 8 h at 4 ℃, and the hemolysis phenomenon was observed and recorded. Meanwhile, the above mixtures were centrifuged, and the absorbance of the supernatants at 540 nm was determined by UV−Vis spectroscopy. Calculation of the hemolysis rate (HR%): $HR\% = (A_{P\text{-}RCDs}\text{-}A_{NC}) * 100\%/(A_{PC}\text{-}A_{NC})$, where $A_{P\text{-}RCDs}$, A_{PC}, and A_{NC} were the absorbance of the sample, the positive control, and the negative control, respectively.

***Ex vivo* fluorescence imaging of Cy5-P-RCDs.**

For fluorescence imaging studies, AKI mice were intravenously injected with Cy5-P-RCDs. Afterwards, mice were sacrificed and major organs were collected and imaged at different expected time points. Images were analyzed *via*

Image J Software.

***In vivo* bio-safety of P-RCDs.**

Balb/c mice were randomly divided into two groups. The mice without any treatments were defined as the control group, and the mice with intravenous injection of P-RCDs (5 mg RCDs/kg) were defined as the test group. The body weight of mice was measured every day to evaluate the *in vivo* bio-safety. At the indicated time points, the blood of mice in all groups was collected and the samples were used to perform blood biochemistry and hematology analysis. Moreover, the major organs including heart, liver, spleen, lung, and kidney were harvested, fixed in 4% paraformaldehyde, processed into paraffin, sectioned, and stained with hematoxylin and eosin (H&E).

Treatment of AKI in mice.

At the time point of the initiation of AKI, different treatments were performed: group 1 was control healthy mice (n = 5); group 2 was the control AKI mice (n = 5); group 3 was AKI mice treated with P-CDs (5 mg CDs/kg, n = 5); group 4 was AKI mice treated with 3,4',5-trihydroxystilbene (5 mg/kg, n = 5); group 5 was AKI mice treated with P-RCDs (5 mg RCDs/kg, n = 10). On day 3 after various treatments, mice in the groups of 1,2,3,4, and five mice in group 5 were euthanized, and the remaining mice in group 5 were euthanized on day 7. Afterwards, the renal functions of mice in different groups were explored. Moreover, the mice in various experimental groups were sacrificed and kidneys were collected for western blot analysis as well as histological analysis.

Body weight measurement.

Survival curves and body weight of AKI mice were monitored for 7 days after different treatments. According to the guidelines on animal welfare, body weight loss in a mouse higher than 20% was considered to be death and euthanized.

Renal function evaluation.

Renal function tests were performed to evaluate the treatment of AKI. After 3 or 7 days post-injection, mice were sacrificed to collect the blood samples for detecting the BUN and CRE levels.

Histological analysis.

At different indicated time points, mice were sacrificed and kidneys were collected for histological analysis. Harvested kidneys were fixed with paraformaldehyde (4%), dehydrated, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) or TUNEL.

Statistical analysis.

All data were expressed as mean ± standard deviation (SD) and performed in at least 3 specimens. Statistical analysis was performed by the two-tailed *t*-test. A P value <0.05 was considered statistically significant. Asterisks indicated significant differences (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) in *in vitro* studies and significant differences (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$) in *in vivo* studies.

Supplementary Figures

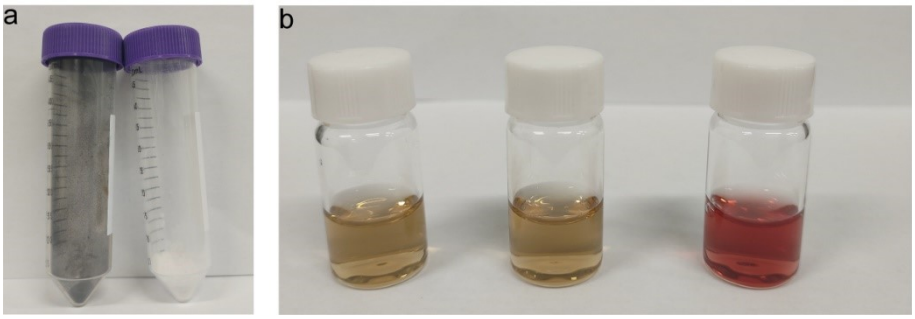

Fig. S1 (a) Typical digital photographs of P-RCDs powder (left) and precursor 3,4',5-trihydroxystilbene powder (right). (b) Typical digital photographs of P-RCDs dispersed in different physiological solutions. From left to right: water, PBS, and DMEM containing 10% FBS.

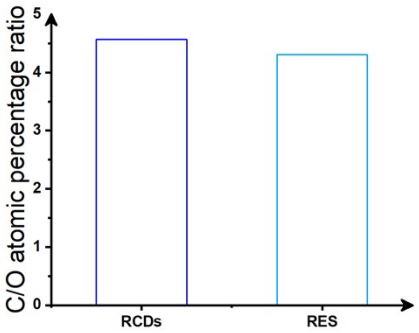

Fig. S2 The C/O atomic percentage ratio in RCDs and RES calculated *via* the deconvolution of the XPS spectra. The O-C=O species in RCDs could be attributed to the occurrence of oxidation caused by the hydrothermal process in the presence of O_2 and H_2O at high temperature (200 ℃).

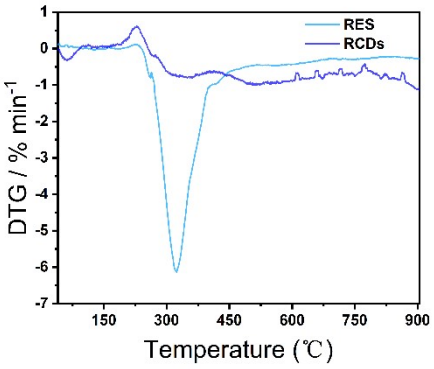

Fig. S3 The DTG profiles for RES and RCDs.

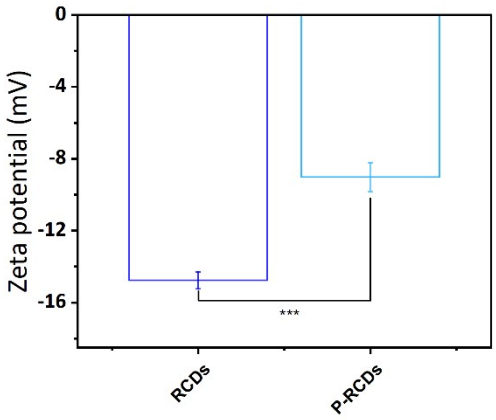

Fig. S4 The ζ-Potential values of RCDs and P-RCDs. Error bars represent standard deviation from the mean (n = 3). Asterisks indicate statistically significant differences (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

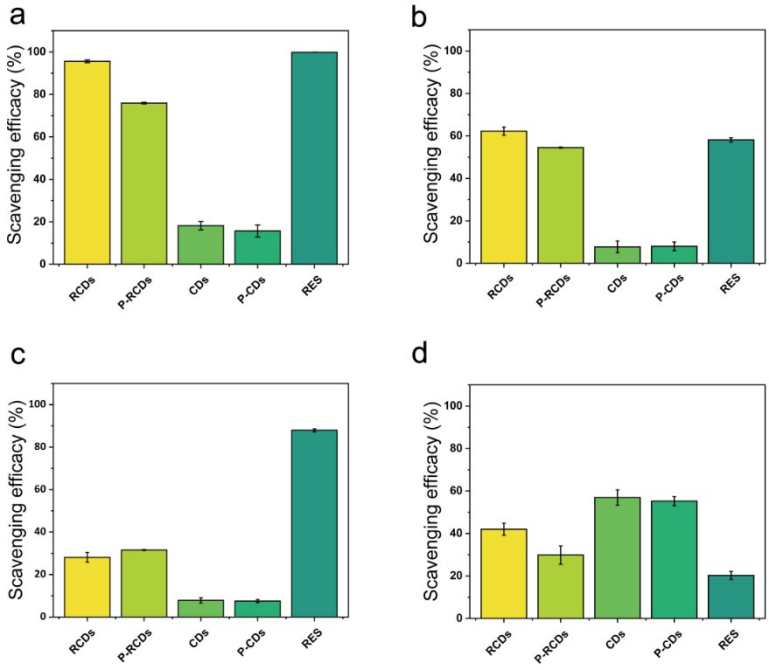

Fig. S5 (a) Comparisons of the scavenging efficacies for $ABTS^{+\cdot}$ of RCDs, P-RCDs, CDs, P-CDs, and RES under the same concentrations of RCDs, CDs, and RES. (b) Comparisons of the scavenging efficacies for DPPH· of RCDs, P-RCDs, CDs, P-CDs, and RES under the same concentrations of RCDs, CDs, and RES. (c) Comparisons of the scavenging efficacies for OH· of RCDs, P-RCDs, CDs, P-CDs, and RES under the same concentrations of RCDs, CDs, and RES. (d) Comparisons of the scavenging efficacies for $\cdot O_2^-$ of RCDs, P-RCDs, CDs, P-CDs, and RES under the same concentrations of RCDs, CDs, and RES. Error bars represent the standard deviation from the mean (n = 3).

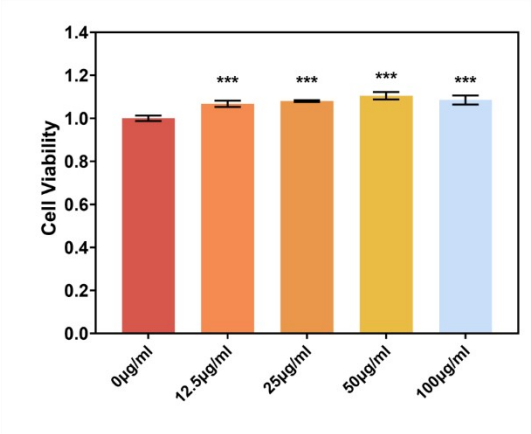

Fig. S6 Cytotoxicity of P-RCDs towards HK-2 cells determined by MTT assay. Error bars represent standard deviation from the mean (n = 4). Asterisks indicate statistically significant differences (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

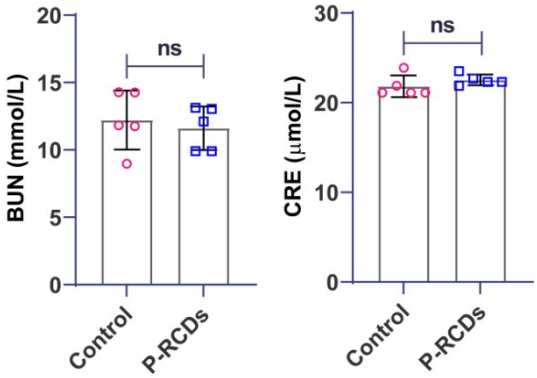

Fig. S7 Serum levels of BUN and CRE of healthy mice after i.v. injection of P-RCDs. Blood was collected at 14 days post-injection. Error bars represent standard deviation from the mean (n = 5).

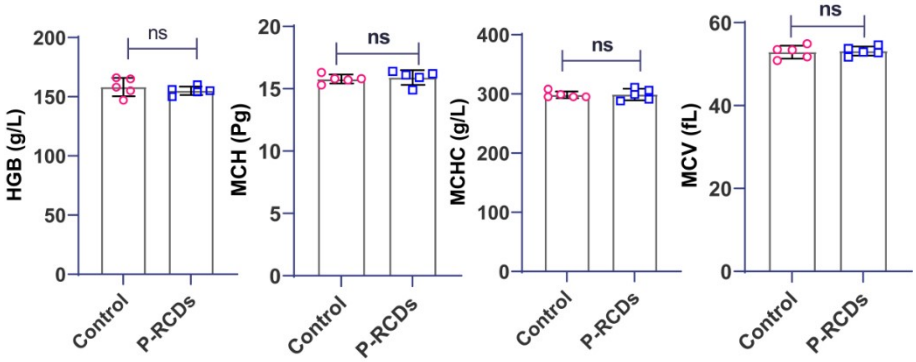

Fig. S8 Levels of HGB, MCH, MCHC, and MCV of healthy mice after i.v. injection of P-RCDs. Blood was collected at 14-day post-injection. Error bars represent standard deviation from the mean (n = 5).

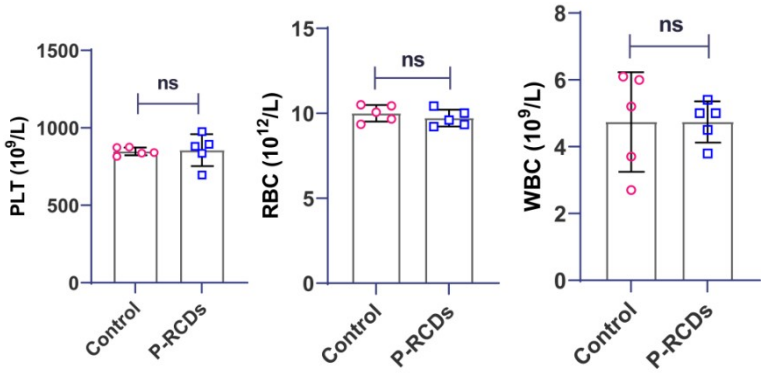

Fig. S9 Numbers of PLT, RBC, and WBC of healthy mice after i.v. injection of P-RCDs. Blood was collected at 14 days post-injection. Error bars represent standard deviation from the mean (n = 5).

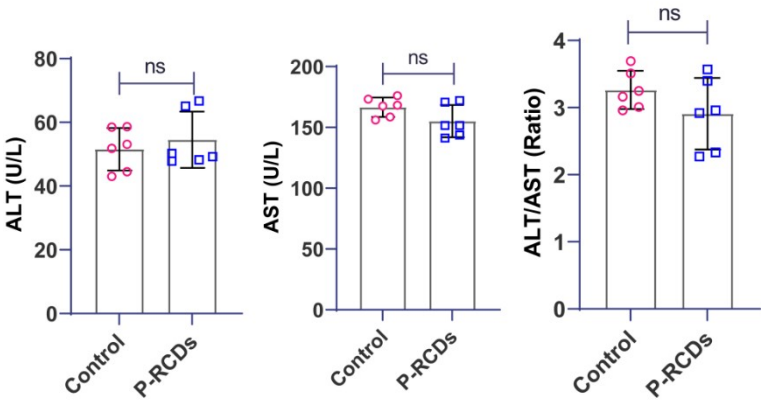

Fig. S10 Serum levels of ALT, AST and AST/ALT of healthy mice after i.v. injection of P-RCDs. Blood was collected at 14 days post-injection. Error bars represent standard deviation from the mean (n = 5).

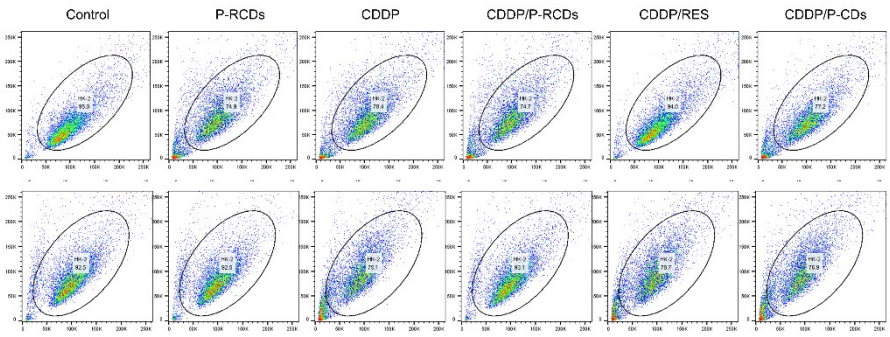

Fig. S11 Related to **Fig. 4d,e**. Top: Analysis of ROS generation in CDDP-treated HK-2 cells after incubating with P-CDs, P-RCDs, and RES at a concentration of 12.5 μg mL^{-1} based on flow cytometry by using DCFH-DA as the indicator. Bottom: Analysis of apoptosis in CDDP-treated HK-2 cells after incubating with P-CDs, P-RCDs, and RES at a concentration of 12.5 μg mL^{-1} based on flow cytometry by using Annexin V-FITC as the indicator.

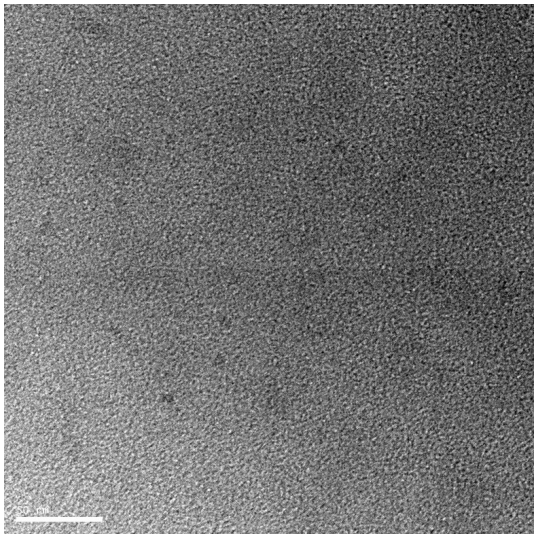

Fig. S12 Related to **Fig. 1c**. Enlarged TEM image of RCDs.