Supporting information

Macrophage membrane-coated hybrid nanoparticles with self-supplied hydrogen peroxide for enhanced chemodynamic tumor therapy

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Materials and methods

Research reagents and instruments

Calcium chloride anhydrous ($CaCl_2$) and zinc acetate dehydrate ($Zn(CH_3COO)_2•2H_2O$) were purchased from Maclin Biochemical Technology Co., Ltd. (Shanghai, China). Hyaluronic acid (HA) was obtained from Huaxi Furuda Biological Medicine Co., Ltd. (Shandong, China). Hydrogen peroxide (H_2O_2, 30 wt%) was provided by Shanghai Lingfeng Chemical Reagent Co., Ltd. (Shanghai, China). 2-Methylimidazole and absolute methanol were purchased from Sigma–Aldrich (St. Louis, Mo. USA). Ferrous chloride tetrahydrate ($FeCl_2•4H_2O$), ammonia solution ($NH_3•H_2O$, 28 wt%), 3,3',5,5'-tetramethylbenzidine (TMB), lipopolysaccharide (LPS) and cell membrane red fluorescent probe (DiD) were obtained from Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). Propidium iodide (PI), an annexin V-FITC apoptosis detection kit, 2′,7′-dichlorofluorescin diacetate (DCFH-DA) and a calcium colorimetric assay kit were purchased from Beyotime (Shanghai, China). Methylene blue (MB), a commassie blue staining kit and ready-to-use DAPI solution were provided by Solarbio Science & Technology Co., Ltd. (Beijing, China). All chemical reagents were purchased commercially and can be used without further purification unless otherwise stated.

The morphology and energy dispersive spectra of the samples were observed on a Field Emission Transmission electron microscope TALOS 200X (TALOS 200X, USA). The zeta potential and size distribution of the samples were measured on a Malvern Zetasizer Nano-ZS instrument (Malvern, ZS90, UK). XPS spectra were

recorded on an X-ray photoelectron spectrometer (ULTRA DLD, USA). XRD analysis was carried out on a Bruker X-ray diffractometer (Bruker D8, Germany). UV–vis absorption spectra were recorded by a BioTek Multimode Reader (Synergy H1, USA). The FTIR spectra of the samples were acquired with a Fourier transform microscopic infrared spectrometer (Bruker V70 & Hyperion1000, Germany). The results of CTG assays were recorded by a Thermo Multimode Reader (Varioskan Flash, USA). The concentrations of Ca and Fe were determined by inductively coupled plasma–mass spectrometry (Jena M90, Germany). Confocal laser scanning microscopy images were acquired using a Zeiss (LSM 800, Germany). *In vivo* fluorescence images were characterized by a PE-fluorescence imaging system of small animals *in vivo* (Lumina III, USA).

Cells and animals

CT26 cell lines were purchased from American Type Culture Collection (ATCC). CT26 cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin at 37 °C with 5% CO_2. RAW264.7 cell lines were purchased from the Stem Cell Bank of the Chinese Academy of Sciences. RAW264.7 cells were cultured in high glucose DMEM supplemented with 10% FBS, 100 U mL^{-1} penicillin and 100 U mL^{-1} streptomycin at 37 °C with 5% CO_2.

BALB/c mice (7-8 weeks old, female) were purchased from the Experimental Animal Management Center of Soochow University. The experimental protocols were carried out according to the Guidelines for Safe Work Practices approved by the Committee on Ethics of Human Specimens and Animal Experiments at Soochow

University. Balb/c mice bearing CT26 cells were obtained by subcutaneous injection of 1×10^6 cells in 50 μL PBS onto the back of each mouse.

Preparation of CaO_2@Fe-ZIF-8 NPs (CFZ NPs)

CaO_2 was prepared using the hydrolysis-precipitation method, with hyaluronic acid as the ligand under basic conditions[1] . In brief, 2 g $CaCl_2$ powder was slowly added into the HA solution (13 mL in water, 12 mg mL^{-1}) under stirring. The mixture was stirred for 5 min, and then 1 mL of $NH_3 \cdot H_2O$ (1 M) and 1.5 mL of H_2O_2 (30 wt%) were sequentially added and reacted for another 3 h. Afterwards, 0.5 mL of NaOH (1 M) was dropped into the mixed solution under ultrasound using a syringe pump at a flow rate of 0.1 mL/min. The HA-modified CaO_2 NPs were washed with NaOH (0.1 M), water and ethanol three times.

CaO_2 (4.5 mg) was dissolved in 11 mL anhydrous methanol. After ultrasonic dispersion, 153.9 mg 2-methylimidazole was added and stirred evenly. Then, 1.24 mg $FeCl_2 \cdot 4H_2O$ and 12.347 mg $Zn(CH_3COO)_2 \cdot 2H_2O$ were sequentially added and stirred at room temperature for 30 min. A yellow–green emulsion solution was obtained. The product CFZ NPs were collected by centrifugation (9000 rpm, 10 min), washed three times with ethanol, and finally stored at 4 ℃ for further use.

CFZ was quantified by commercial H_2O_2 assay kits based on its H_2O_2 production capability. Ca elements in NPs were measured using commercial calcium detection kits from Beyotime (Shanghai, China). Fe in NPs was measured by inductively coupled plasma–mass spectrometry (ICP-MS).

Preparation of macrophage membrane-coated CaO_2@Fe-ZIF-8 NPs (CFZM NPs)

During the culture of RAW264.7 cells, LPS was added to the culture medium at a final concentration of LPS of 1 μg mL^{-1}. The macrophages were incubated in the incubator for 24 h, which allowed for the change into M1-polarized macrophages. Membranes of M1-polarized macrophages were isolated by the repeated freeze thawing procedure[2]. Then macrophages were suspended in 0.1×PBS (pH 7.4), supplemented with protease inhibitors (containing phenylmethylsulfonyl fluoride 35 μg mL^{-1}, EDTA 0.3 mg mL^{-1}, pepstatin 0.7 μg mL^{-1}, and leupeptin 0.5 μg mL^{-1}), frozen in liquid nitrogen, and thawed at 37 °C, and this cycle was repeated ten times. The macrophage membrane fragments were harvested by centrifugation at 10000 × g for 10 min and washed twice with water (pH 7.4). The suspensions of M1-polarized macrophage membranes were stored at 4 °C for further use.

The extracted M1 polarized macrophage membranes were homogenized by an ultrasonic crusher. The CFZ was redispersed in water, then added with M1 polarized macrophage membranes. The mixture was subjected to low-temperature ultrasonication for 1 h at 4 ℃. Then, the mixture was centrifuged at 8000 rpm for 10 min and finally stored at 4 ℃. Fe-ZIF-8@M (FZM) and CaO_2@M (CM) were prepared using the identical procedure.

Preparation of CaO_2@Fe-ZIF-8@liposome (CFZL NPs)

CFZL NPs were prepared by the standard extrusion method[3]. A total of 90.8 mg phospholipid was dissolved in 3 mL absolute ethanol, and 100 μL of the above

phospholipid solution was placed in a vacuum drying oven for 3 h at room temperature. Then, 1 mL of HEPES buffer solution was added and dispersed evenly under ultrasonication. An additional 120 μL CFZ (4.5 mg mL^{-1}, dissolved in buffer solution) was added, and the mixture was sonicated in a circulating water bath (4 °C) for 2 h. The suspension was extruded dozens of times through two stacked polycarbonate membranes (pore size: 100 nm) to obtain CFZL samples.

Protein detection of CFZM NPs

The components and the molecular weight of the proteins can be measured by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), including the samples of M1-polarized macrophage cell membrane (M), Fe-ZIF-8@M (FZM), CaO_2@M (CM), and CaO_2@Fe-ZIF-8@M (CFZM).

Release of Ca^{2+} and Fe^{2+} from CFZ NPs

Briefly, 1 mL of CaO_2@Fe-ZIF-8 (4.5 mg mL^{-1}) was dispersed in dialysis bags (14 kDa), immersed in 15 mL of PBS buffer with different pH values (5.5, 6.5 and 7.4) and shaken at 37 °C for 24 h. At different time points, 1 mL of buffer was removed, and 1 mL of buffer with different pH values was added at the same time. Finally, the concentrations of Ca^{2+} and Fe^{2+} ions at different time points were determined by commercial calcium detection kits and ICP-MS, respectively.

Detection of Ca^{2+}: The standard curve was obtained by titrating the solutions of Ca^{2+} at different concentrations (50 μL each) and mixing with the detection solution (150 μL each) from the commercial kit (Beyotime, China). The mixed solutions were

incubated at room temperature in the dark for 10 minutes, before absorbance measurements at the wavelength of 575 nm by a multimode reader (Synergy H1, USA). The Ca^{2+} concentrations of the samples were determined with the reference of the standard curve

Detection of H_2O_2: The standard curve was obtained by titrating the solutions of H_2O_2 at different concentrations (1 mL each), mixing with the alkaline solution (0.2 mL each) and titanium sulfate solution (0.1 mL) from the commercial kit (Leagene, China). The precipitates were dissolved by 2 mL acidic solution. Then absorbance measurements at the wavelength of 412 nm were performed to provide the standard curve for determination of H_2O_2 concentrations of the samples.

***In vitro* detection of •OH generation**

MB and TMB are commonly used to detect the production of •OH. Briefly, CaO_2@Fe-ZIF-8 (40 μg mL^{-1}) was dispersed in MB solution and TMB solution with different pH values (5.5 and 7.4), and the UV-vis absorption of various samples was recorded using a BioTek Multimode Reader after 30 min of incubation. The increase in •OH production mediated by other groups was investigated using the same method as above. Different concentrations of CaO_2@Fe-ZIF-8 (0, 5, 10, 20, 40 and 80 μg mL^{-1}) were also dispersed in PBS buffer (pH 5.5), and 100 μL of TMB solution was added. The solution was incubated at 37 °C for 30 min. After incubation, the samples were detected by using a BioTek Multimode Reader. TMB was used to study the ability of each group to produce •OH under different pH conditions.

***In vitro* cytotoxicity assay**

The cytotoxicities of FZM, CM, CFZL and CFZM were determined by Celltiter-GlO (CTG) luminescence assays. The cells were seeded in 96-well plates at a density of 8000 cells per well and incubated overnight. Different concentrations of FZM, CM, CFZL and CFZM ([Ca] = 0, 1.67, 3.33, 6.67, 13.34 and 26.68 μg mL^{-1}; [Fe] = 0, 0.58, 1.15, 2.30, 4.60 and 9.20 μg mL^{-1}) in the cell culture medium were added into the wells, and the cells were cultured in an incubator containing 5% CO_2 at 37 ℃ for 24 h or 48 h. Then, the standard CTG assay was used to determine cytotoxicity.

Fluorescent cellular live/dead dual-staining experiments were also performed for cell viability assays using calcein-AM/propidium iodide (PI) dyes. The cells were seeded in laser confocal dishes at a density of 1 × 10^5 cells per dish and incubated overnight. Different concentrations of FZM, CM, CFZL and CFZM ([Ca] = 13.34 μg mL^{-1}; [Fe] = 4.6 μg mL^{-1}) in the cell culture medium were added into the wells, and the cells were cultured overnight in an incubator containing 5% CO_2 at 37 ℃. Then, 5 μM AM (excitation wavelength: 490 nm, emission wavelength: 515 nm) and 10 μM PI (excitation wavelength: 535 nm, emission wavelength: 617 nm) were added, incubated for 30 min, and washed with PBS three times, and fluorescence images were obtained using CLSM.

Cellular uptake *in vitro*

The cellular uptake of the hybrid nanoparticles was examined by CLSM. CT26 cells were seeded in laser confocal petri dishes at a density of 1×10^5 cells per dish and incubated in an incubator at 37 ℃ and 5% CO_2 for 24 h. The cells were treated with

CFZM@DiD and CFZL@DiD ([Ca] = 6.67 μg mL^{-1}) suspended in RPMI medium for 0, 1, 2 and 4 h. Then, the cells were washed with PBS buffer solution 3 times, and 1 mL 4% paraformaldehyde was added to each petri dish to fix the cells for 10 min at room temperature. The cells were washed with PBS again 2 times. The cell nuclei were stained with DAPI, and the cells were imaged with a CLSM imaging system.

The cellular uptake behaviors of the hybrid nanoparticles were further investigated by a calcium colorimetric assay kit using blank cells as the negative control. The CT26 cells were seeded into 6-well plates (2 mL RPMI medium) at a density of 5×10^5 cells per well and cultured overnight in an incubator at 37 ℃ and 5% CO_2. The cells were treated with CFZM and CFZL with a calcium concentration of 6.67 μg mL^{-1} suspended in RPMI medium for 0, 1, 2 and 4 h. The RPMI medium was removed, and the cells were collected after gently washing with PBS buffer 3 times. One hundred microliters of sample lysate was added to lyse the cells, and the content of Ca^{2+} was determined by a calcium colorimetric assay kit.

Intracellular ·OH detection

The intracellular production of ROS was detected by fluorescence using CLSM. The CT26 cells were seeded in laser confocal dishes at a density of 1×10^5 cells per dish and cultured overnight in an incubator at 37 ℃ and 5% CO_2. The RPMI medium was then replaced with fresh medium containing FZM, CM, CFZL and CFZM at pH 7.4 and pH 6.5 ([Ca] = 6.67 μg mL^{-1}; [Fe] = 2.30 μg mL^{-1}), and the cells were cultured for 4 h in medium with different pH values as the control. The cell culture medium was replaced, washed twice with PBS, and the cells were incubated with reactive oxygen

species detection reagent (DCFH-DA, 10 μM) for 20 min. The cells were washed with PBS twice and fixed with 4% paraformaldehyde for 8 min. The cell nuclei were stained with DAPI, and the cells were washed with PBS buffer solution and imaged by CLSM (excitation wavelength: 490 nm, emission wavelength: 520 nm, objective: 20 ×) to detect intracellular •OH production.

In vivo imaging and biodistribution study

Tumor-bearing mice (n = 3) were intravenously injected with 200 μL of CFZL@DiD and CFZM@DiD ([Ca] = 8.58 mg kg^{-1}; [Fe] = 2.96 mg kg^{-1}, dispersed in 200 μL of saline). The enrichment of hybrid nanoparticles in tumor sites was detected by a fluorescence imaging system of small animal PE fluorescence imaging at different time points (0, 1, 2, 4, 6, 12, 24 and 48 h) after injection. To explore the difference in biological distribution between CFZL@DiD and CFZM@DiD, the mice were sacrificed at the specified time point, and the main organs were collected for fluorescence imaging and fluorescence intensity quantitative statistical analysis of the hearts, livers, spleens, lungs, kidneys and tumors.

In vivo antitumor efficacy evaluation

For *in vivo* antitumor studies, BALB/c mice carrying CT26 cells were randomly divided into 5 groups (n = 5): PBS, FZM, CM, CFZL and CFZM. During the treatment, three injections were given once every four days, and the first injection was taken as day 0. When the tumor grew to 90 mm^3, the mice in each group were intravenously injected with saline (200 μL) as a control, FZM, CM, CFZL or CFZM ([Ca] = 8.58 mg

kg^{-1}, [Fe] = 2.96 mg kg^{-1}, dispersed in 200 μL saline) intravenously on days 0, 4 and 8, respectively. In each treatment, the concentrations of Fe and Ca were injected at the same dose. The tumor volume (V) was monitored every other day and calculated according to the following equation: V = tumor length × $(width)^2/2$. The mouse weight was recorded using an electronic balance. Two weeks later, all of the mice were sacrificed, and the major organs (heart, liver, spleen, lung and kidney) and tumors were harvested. After weighing and photographing the tumors of mice in different treatment groups, the main organs and tumors were fixed in 4% formalin solution, and the sections were stained with H&E and Ki67, respectively.

Routine blood tests and blood biochemical analysis

Routine blood tests and blood biochemical analysis were performed on the mice after tumor treatment, and blood samples were collected for biochemical and hematological analysis. Briefly, blood samples were obtained by removing the mouse eyeball after the mice were sacrificed at 14 days postinjection and then analyzed to determine the hematological index. Serum samples were prepared by centrifuging the blood samples at 2000 rpm for 20 min and were stored at -80 ℃ before measurements to investigate the liver function (alanine transaminase (ALT), aspartate transaminase (AST)) and kidney function (urea nitrogen (UREA), serum creatinine (CREA)) indices, with the service provided by the shared medical facilities and platforms at Soochow University.

Statistical analysis

Experimental data in the figures were formatted as the mean ± standard deviation. A single factor analysis of variance (ANOVA) analysis was performed to compare the significant differences between the data in different groups, with statistical significance defined as ***$p \leq 0.001$, **$p \leq 0.01$, and *$p \leq 0.05$.

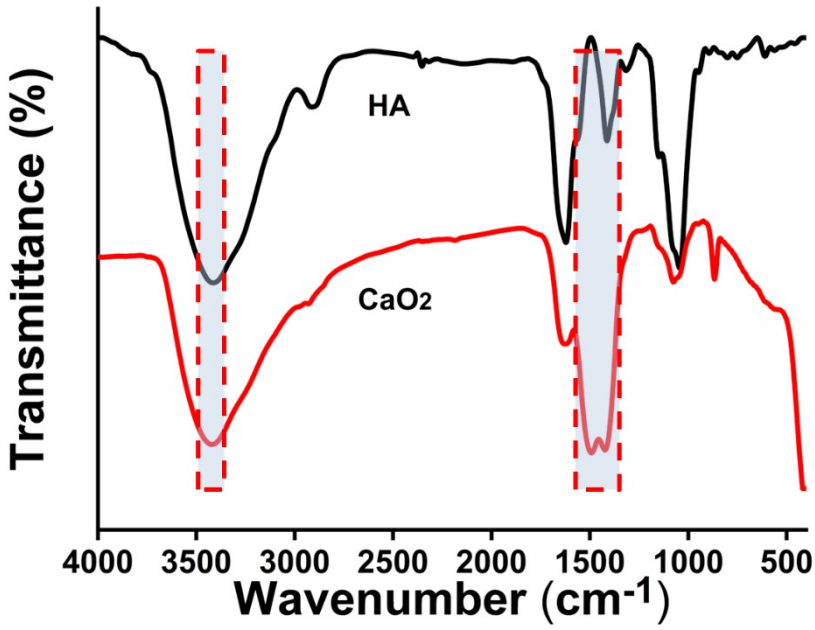

Fig. S1. The Fourier transform infrared spectroscopy (FTIR) spectra of HA and CaO_2 NPs ligated with HA, respectively.

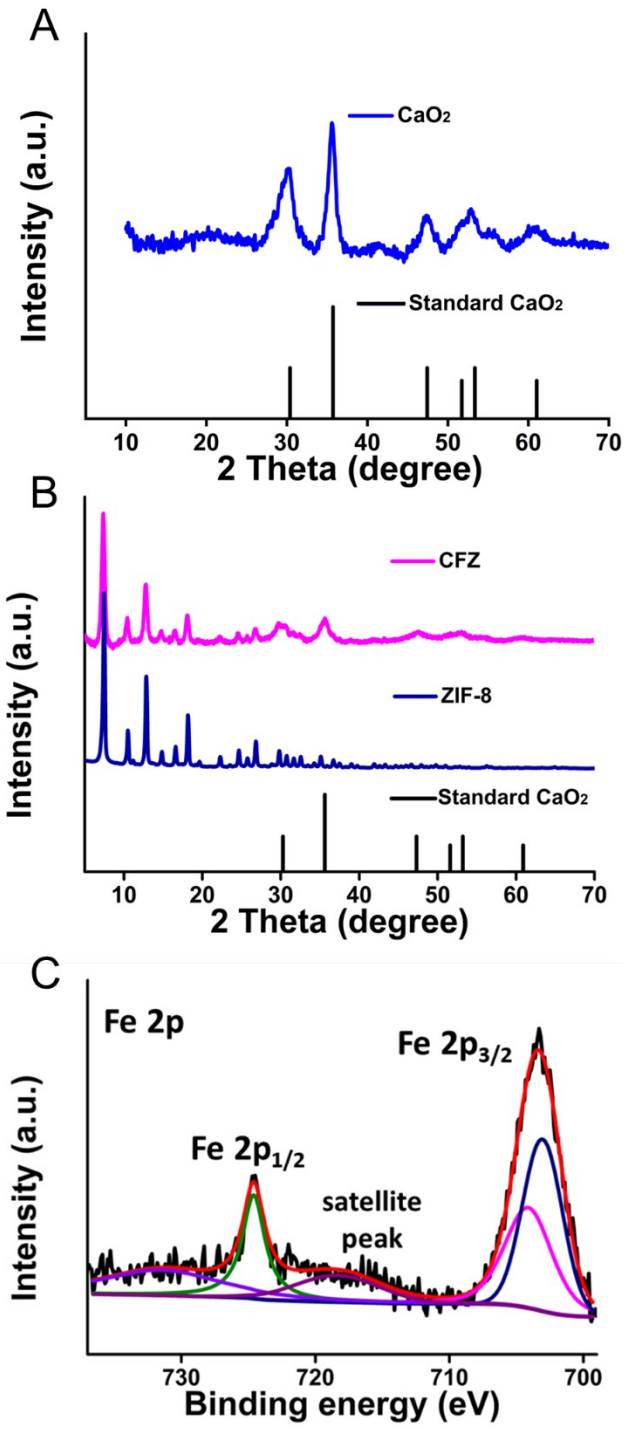

Fig. S2. (A) The X-ray diffraction (XRD) spectra of CaO_2 NPs, and standard CaO_2. (B) The XRD spectra of CFZ NPs, ZIF-8, and standard CaO_2. (C) The High-resolution Fe 2p X-ray photoelectron spectroscopy (XPS) spectra of CFZ NPs.

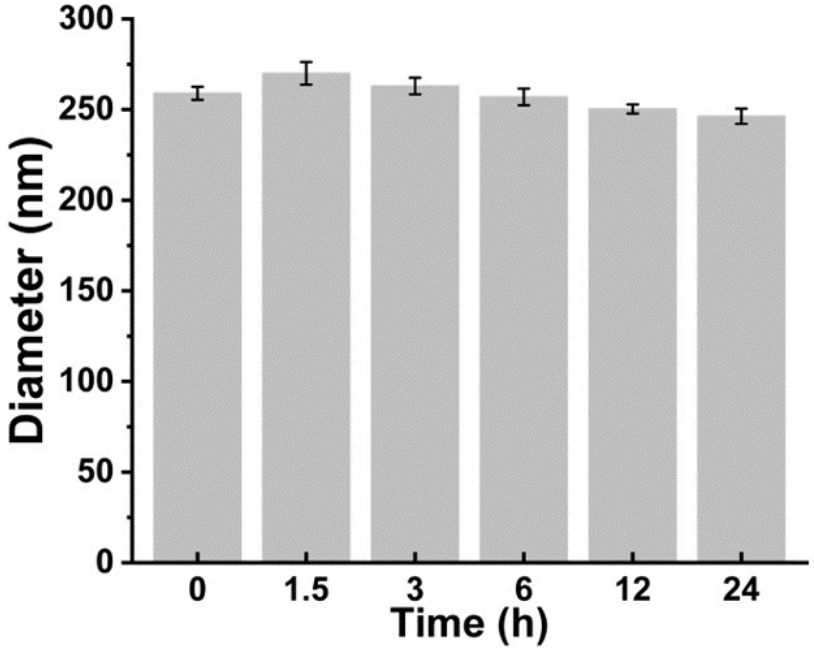

Fig. S3. Stability of CFZM NPs in PBS (pH = 7.4) within 24 h (n = 3).

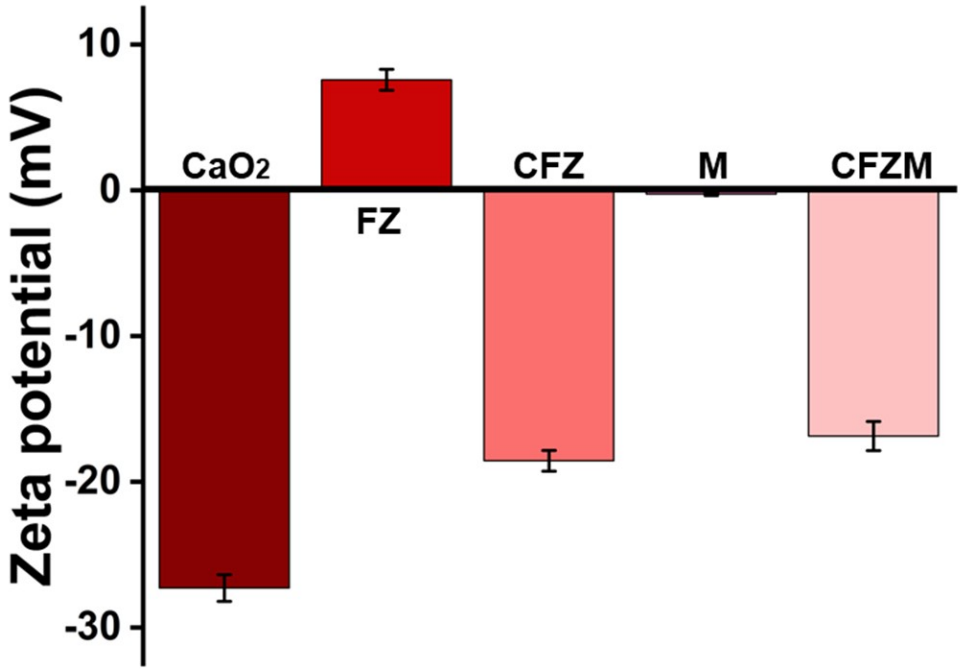

Fig. S4. Surface zeta potentials of CaO_2 NPs, FZ NPs, CFZ NPs, M1 macrophage membrane and CFZM NPs. Error bar: standard deviation (n = 3).

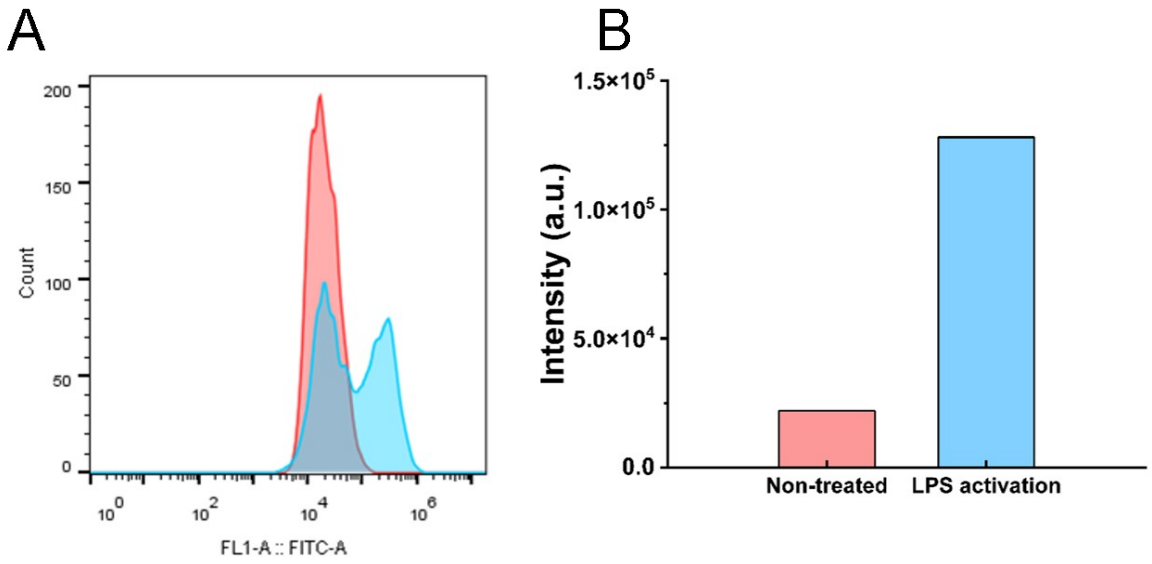

Fig. S5. The verification the M1-type polarization of macrophages. (A) Flow cytometry data of macrophages with or without the stimulation of LPS (250 ng mL^{-1}) respectively. (B) Semiquantitative analysis of the fluorescence intensities of iNOS expression in the macrophages with or without the stimulation of LPS. Color coding, blue: LPS activation; pink: no LPS activation.

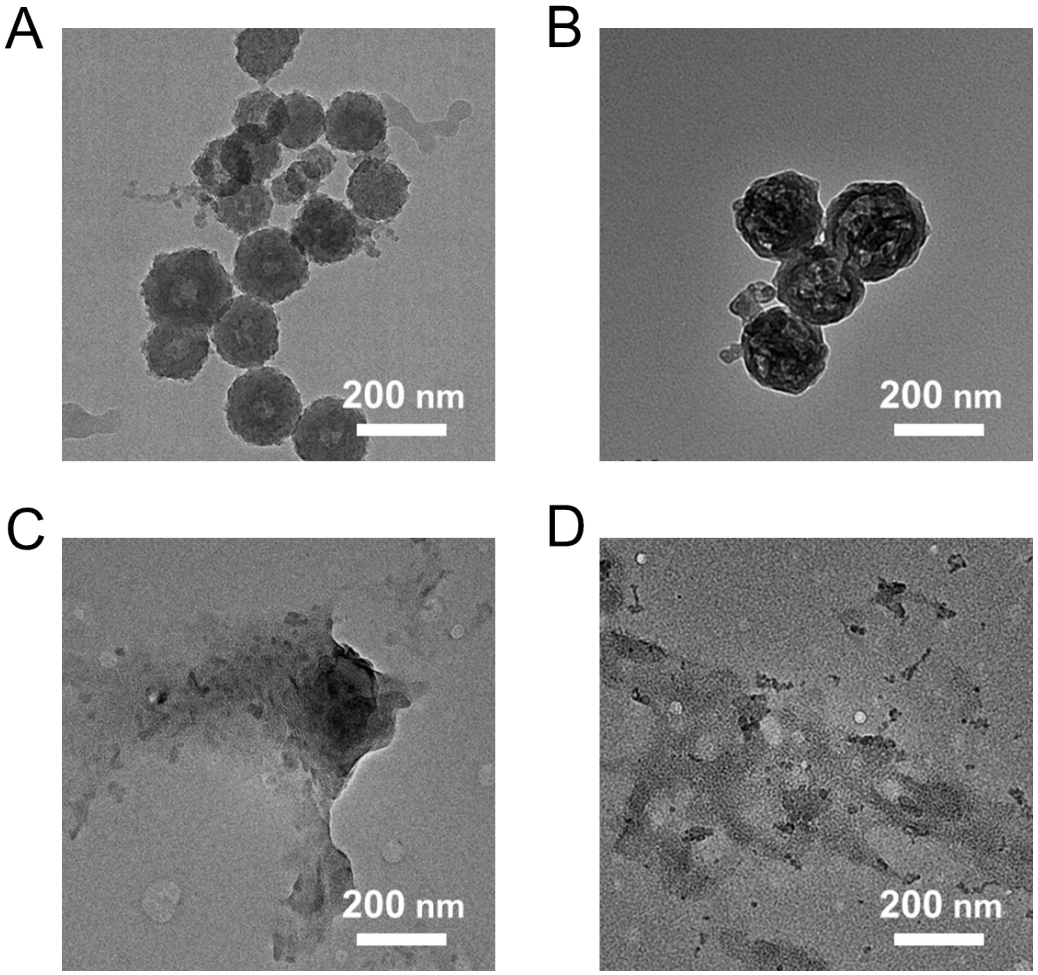

Fig. S6. TEM images of CaO_2 NPs after incubation in different buffers for 24 h. (A) Alcohol, (B) pH 7.4, (C) pH 6.5, and (D) pH 5.0. (Scale bar = 200 nm).

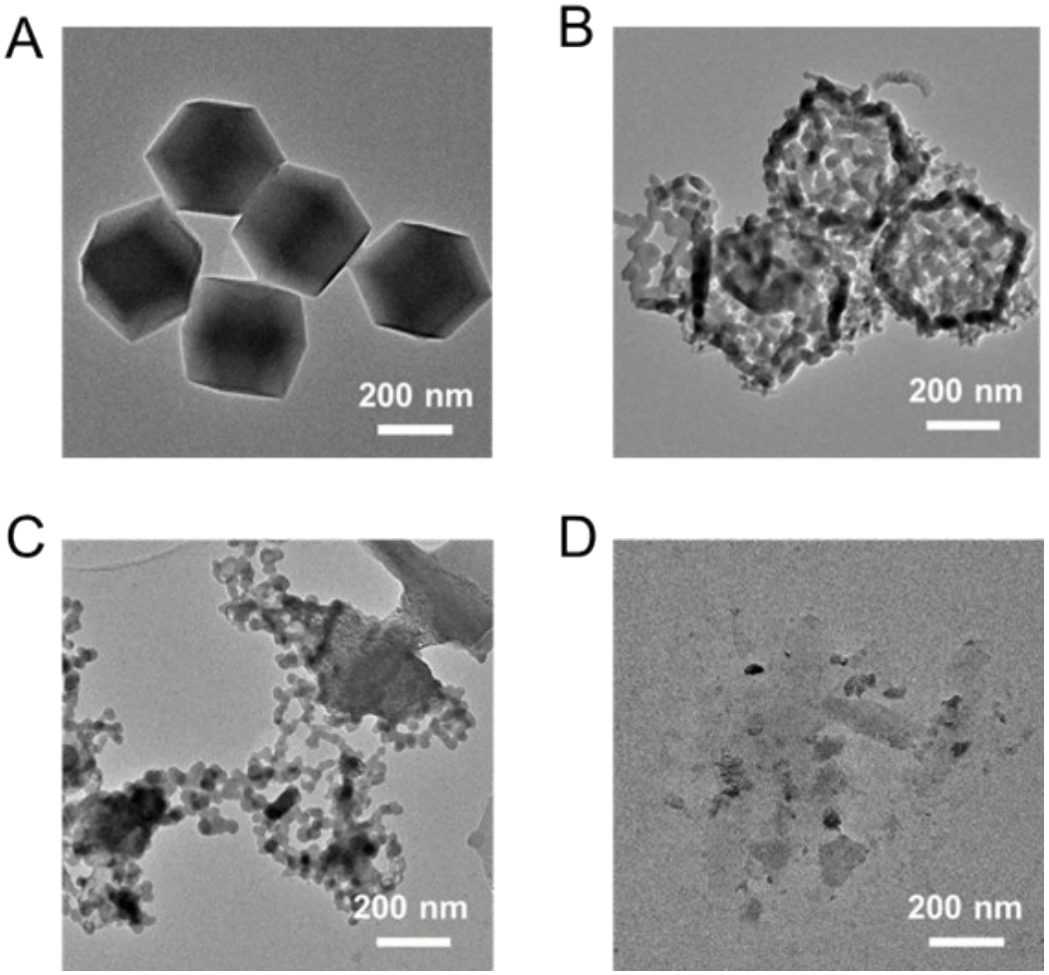

Fig. S7. TEM images of FZ NPs after incubation in different buffers for 24 h. (A) Alcohol, (B) pH 7.4, (C) pH 6.5, and (D) pH 5.0. (Scale bar = 200 nm).

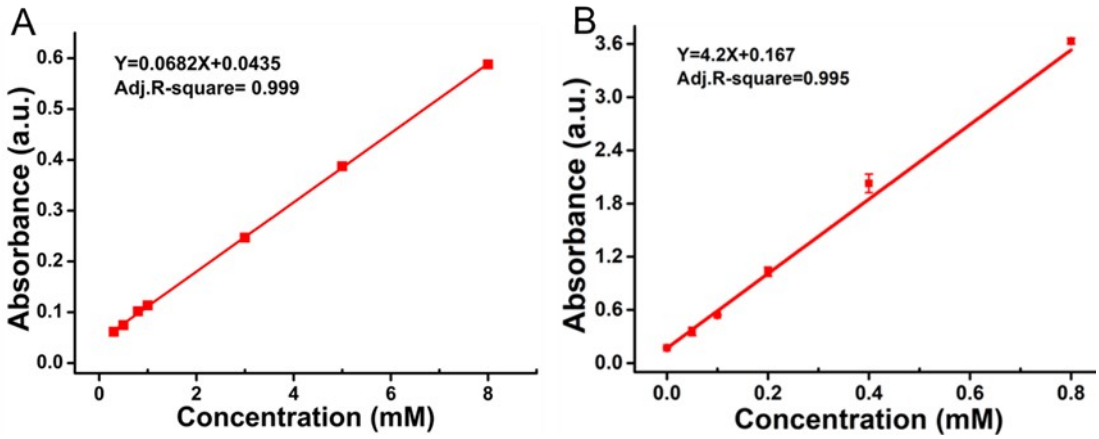

Fig. S8. (A) Standard curve to determine Ca^{2+} release by a commercial kit of Beyotime. Absorbance measured at 575 nm. (B) Standard curve to determine H_2O_2 by a commercial kit of Leagene. Absorbance measured at 412 nm. Error bar: standard deviation (n = 3).

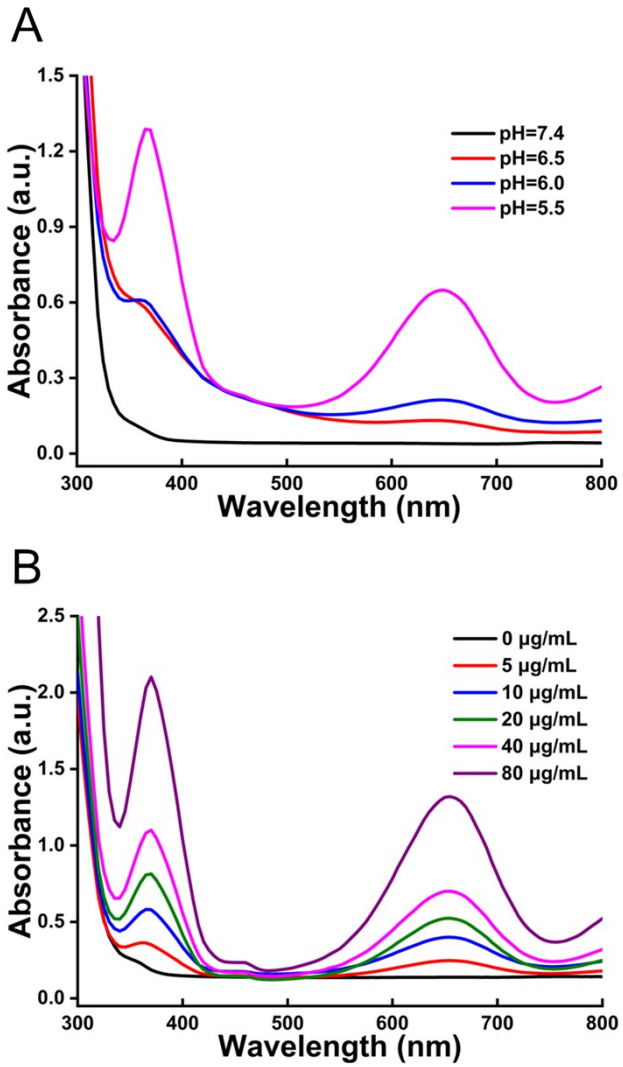

Fig. S9. (A) UV–vis spectra of TMB solution after incubation with 40 μg mL^{-1} CFZ NPs in different pH buffers. (B) UV–vis spectra of TMB solution after incubation with various concentrations of CFZ NPs in pH 5.5 buffer.

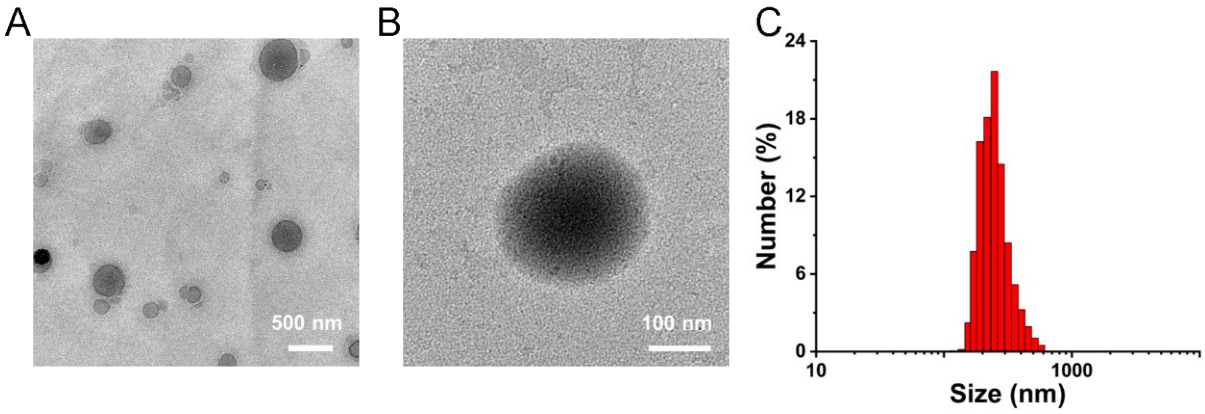

Fig. S10. Characterization of CFZL NPs. (A) TEM image of CFZL NPs. Scale bar: 500 nm (B) TEM image of a single CFZL NPs in a zoomed-in view. Scale bar: 100 nm. (C) DLS characterization of CFZL NPs.

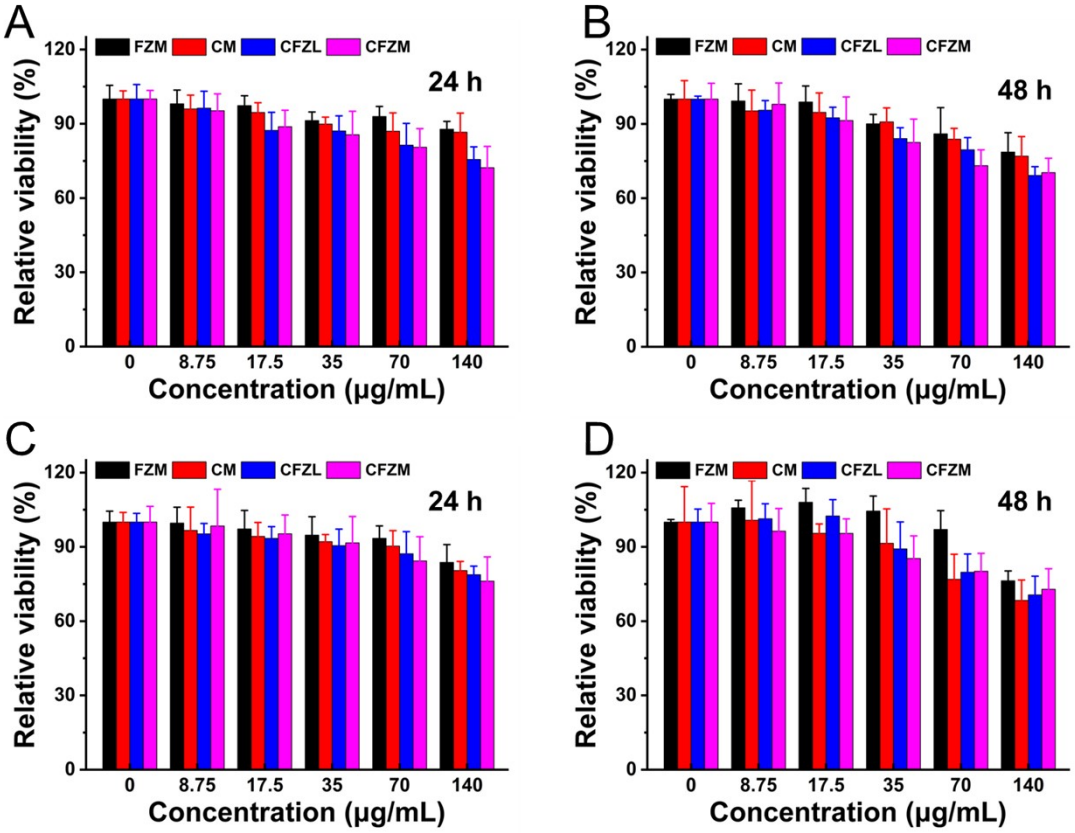

Fig. S11. (A) Cytotoxicity evaluation with different concentrations of FZM NPs, CM NPs, CFZL NPs and CFZM NPs against L929 cells after 24 h incubation (B) Cytotoxicity evaluation with different concentrations of FZM NPs, CM NPs, CFZL NPs and CFZM NPs against L929 cells after 48 h incubation. (C) Cytotoxicity evaluation with different concentrations of FZM NPs, CM NPs, CFZL NPs and CFZM NPs against 293T cells after 24 h incubation. (D) Cytotoxicity evaluation with different concentrations of FZM NPs, CM NPs, CFZL NPs and CFZM NPs against 293T cells after 48 h incubation. Error bar: standard deviation (n = 5).

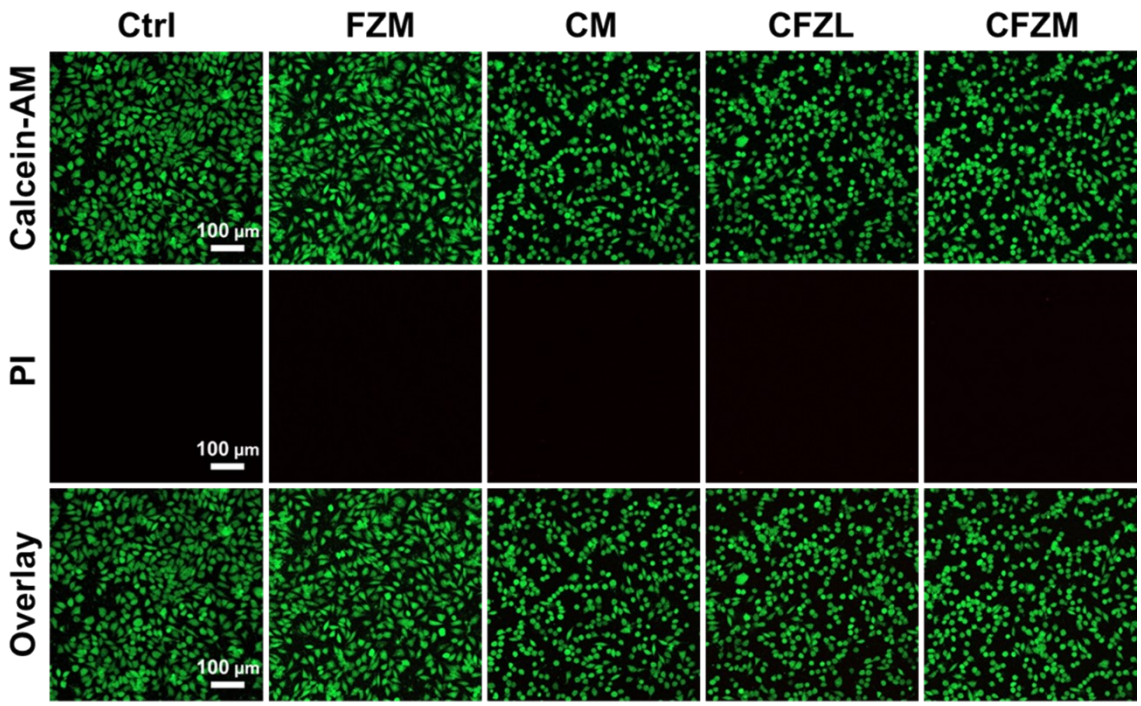

Fig. S12. Fluorescence microscope images of the L929 cells after incubation with dmem medium, FM NPs, CM NPs, CFZL NPs and CFZM NPs for 12 h, with the live cell and dead cell stained by calcein-AM and PI, respectively. Scale bar: 100 μm. Objective 10× .

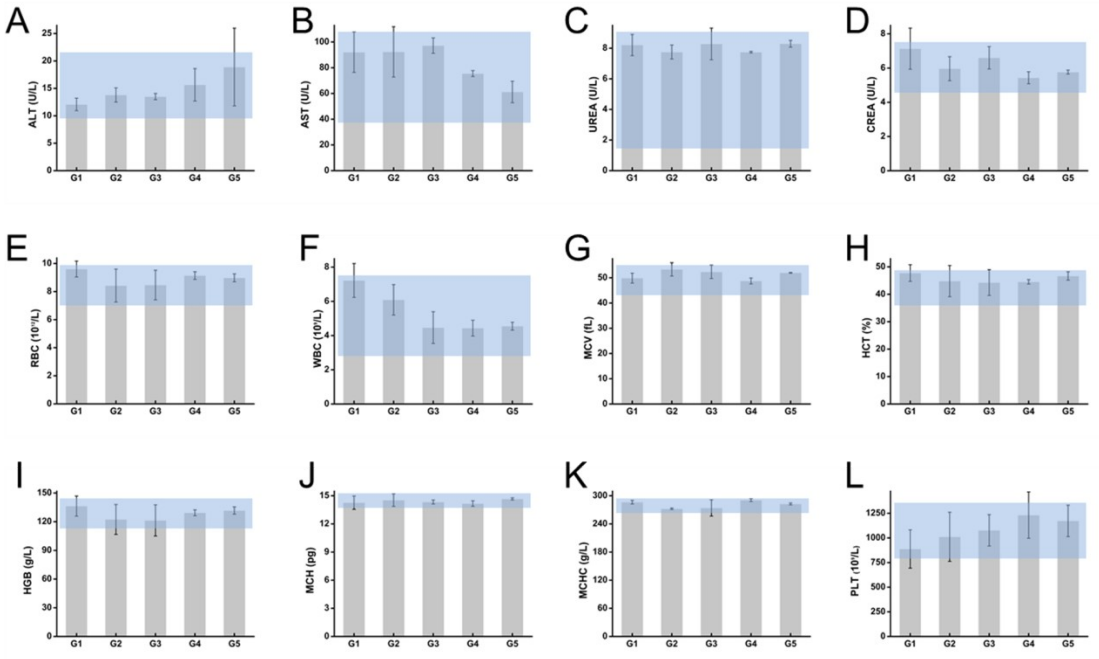

Fig. S13. Blood and biochemical analysis *in vivo* after intravenous injection with different nanoparticles. The examined parameters included (A) alanine aminotransferase (ALT); (B) aspartate aminotransferase (AST); (C) urea nitrogen (UREA); (D) serum creatinine (CREA); (E) red blood cell (RBC); (F) white blood cell (WBC); (G) mean corpuscular volume (MCV); (H) hematocrit (HCT); (I) hemoglobin (HGB); (J) mean corpuscular hemoglobin (MCH); (K) mean corpuscular hemoglobin concentration (MCHC); and (L) blood platelet count (PLT). All hematology data are in reference ranges. Error bar: standard deviation (n = 3). G1: Control, G2: FZM NPs, G3: CM NPs, G4: CFZL NPs, G5: CFZM NPs. Error bar: standard deviation (n = 5).

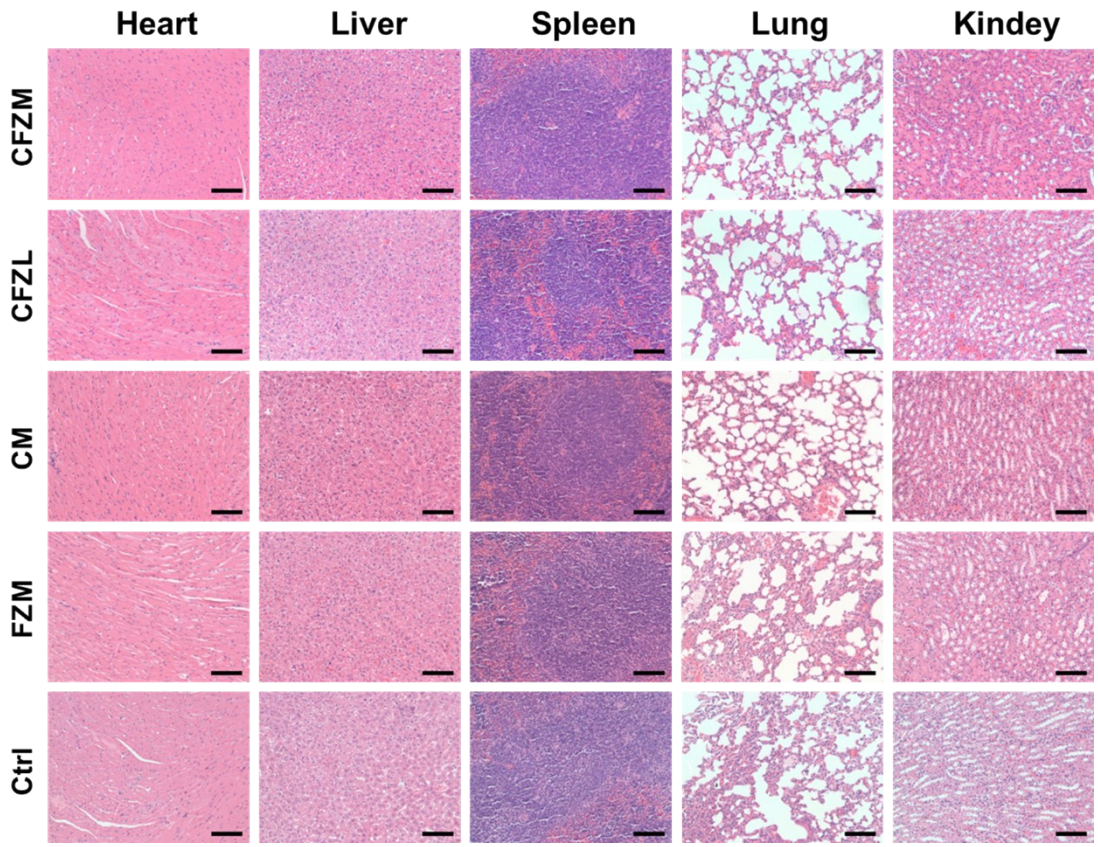

Fig. S14. H&E-stained tissue sections of the main organs in mice after intravenous injection with different nanoparticles. Scale bar = 100 μm.

References

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