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Supporting Information

A pH-Responsive Cascaded Nanoplatform with Circulating Oxygen Supply for Breast Cancer Collaborative Treatment

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Experimental Procedures

2.1 Materials and reagents

Calcium chloride ($CaCl_2$), ammonia ($NH_3•H_2O$), hydrogen peroxide (H_2O_2), N,N-dimethylformamide (DMF), zinc nitrate hexahydrate ($Zn(NO_3)_2•6H_2O$), 2-methylimidazole (2-MIM) and PEG200 were all purchased from Aladdin. Glutathione (GSH), Potassium permanganate ($KMnO_4$), 2',7'-dichlorofluorescein (DCFH) and chlorin e6 (Ce6) were obtained from Sinopharm (Shanghai, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma. Calcein AM/PI double staining kit and reactive oxygen detection kit (2',7,-dichlorofluorescein diacetate, DCFH-DA) were purchased from Beyotime (Nantong, China). Mouse breast cancer cells (4T1) were purchased from Procell Life Science (Wuhan, China). All chemical reagents used in the experiment are of analytical grade and used without any purification. All experimental water is Mill-Q secondary ultrapure water (18.2 MΩ•cm).

2.2 Instrumentation Analysis

Transmission electron microscope (TEM, Hitachi-7700, Japan) was used to characterize the sizes and morphology of nanoparticles. Hitachi SU8010 scanning electron microscope (SEM) was applied for obtained the micrographs of the materials. The fluorescence spectra were obtained on the FLS-980 fluorescence spectrophotometer (Edinburgh). Malvern Zeta Nano Sizing Apparatus (Malvern Instruments) obtained the dynamic light scattering (DLS) sizes of nanoparticles. A Rigaku SmartLab SE X-ray powder diffractometer was used to obtain X-ray powder

diffraction (XRD) patterns. UV-1700-Vis spectrometer was used to obtain UV-Vis absorption spectra. A pH-3c acidity meter was employed to obtain the pH value. Rayto RT-6000 MTT microplate reader was applied to obtain the absorbance of MTT analysis. MCO-15A cell C incubator was applied to culture the cells. A Leica SP8 laser confocal scanning microscope was used to achieve confocal fluorescence images. The Thermo Scientific iCAP 7400 ICP-OES was used to obtain the Mn^{2+} and Ca^{2+} concentrations in the MnCaCZ, and the MnO_2 and CaO_2 loading capacities were obtained by further calculation.

2.3 Synthesis of the MnO_2/CaO_2/Ce6@ZIF-8 NPs

Synthesis of the CaO_2 NPs: 0.1 g of $CaCl_2$ was completely dissolved in 1 mL of water in a round bottom flask under stirring, and then 0.5 mL of ammonia (1 mol/L) and 6 mL of PEG200 was added to keep stirring for 10 min. Subsequently, 0.5 mL of 30% H_2O_2 was added dropwisely at a constant rate of 0.25 mL/min. Finally, the CaO_2 NPs were purified by centrifugation at 13,000 rpm for 10 min, and washed with methanol for three times. The CaO_2 NPs were collected after freeze-drying.

Synthesis of the MnO_2 NPs: Dissolve 5 mg of $KMnO_4$ with 10 mL of water in a round bottom flask, then dropwisely adds 0.2 mL of 30% H_2O_2 in an ice-water bath. The reaction solution was kept stirring continuously during for 10 min, followed by 10 min centrifugation at 13,000 rpm to obtain the final product MnO_2 NPs.

Synthesis of the MnO_2/CaO_2/Ce6@ZIF-8 NPs: Completely dissolve 23.8 mg $Zn(NO_3)_2$•$6H_2O$ with 0.2 mL DMF in a round bottom flask, then 0.8 mg MnO_2 NPs and 0.7 mg CaO_2 NPs were added under ultra-sonication. Subsequently, 443.3 mg 2-

MIM was fully dissolved in 1.8 mL DMF, followed with addition of 0.6 mg Ce6 to keep stirring for 5 min. For the next step, the above two solutions were mixed together and vigorously stirred for 5 min. The product MnCaCZ NPs was purified by 10 min centrifugation (13,000 rpm), and washed triple times with methanol and ultrapure water. Finally, the solution was freeze-dried, and the MnCaCZ NPs product was collected.

When preparing the MnO_2/Ce6@ZIF-8 NPs (MnCZ NPs) and the CaO_2/Ce6@ZIF-8 NPs (CaCZ NPs), CaO_2 and MnO_2 were respectively not added in the synthesis process, and the rest of the steps were same as above. When preparing the Ce6@ZIF-8 NPs (CZ NPs), neither CaO_2 nor MnO_2 were added in the synthesis process, but keeping the rest of the procedures same as above.

2.4 Characterization of the NPs

DLS: Disperse 2 mg of the MnCaCZ NPs in 2 mL PBS buffer solution, and then sonicate in an ultrasonic bath for 10 min. For the next step, use the Malven Zeta Sizer nanometer to measure the DLS sizes of MnCaCZ NPs. For the same sample, measure once a day for seven consecutive days.

TEM: Transmission electron microscope was operated at an accelerating voltage of 120 kV. The NPs samples were prepared by depositing a drop of NPs solution onto the carbon-coated copper grid, followed by ambient evaporation of the solvent.

2.5 Loading efficiencies of Ce6, MnO_2 and CaO_2

Ce6 solutions of different concentrations (0.001, 0.002, 0.005, 0.01, 0.02 and 0.05 mg/mL) were prepared, and the UV-Vis absorption spectra at a wavelength of 642 nm

were measured. When preparing the MnCaCZ NPs, collect the supernatant and all washings solutions after centrifugation and the Ce6 amount can be determined by measuring its UV-Vis absorption intensity at 642 nm. The amount of MnO_2 and CaO_2 can be measured by inductively coupled plasma mass spectrometry (ICP-MS).

$$\text{Loading efficiency} = (M_{\text{initial drug}} - M_{\text{drug in supernatant}}) / M_{\text{initial drug}} \times 100\%$$

2.6 Ce6 release test

The MnCaCZ NPs (1 mg/mL) were suspended in 1 mL PBS buffer with pH = 7.4 and 5.0, respectively. Place the sample in a shaker at a speed of 220 rpm under 37℃. After centrifugation at a predetermined time interval, 500 μL of the solution was collected and then added back to 500 μL of appropriate buffer. The released Ce6 was quantified by the UV-Vis absorbance at 642 nm. All samples were tested for at least three times.

2.7 Evaluation of the MnCaCZ NPs on GSH consumption ability

Mix the MnO_2 NPs (1 mg/mL) with different concentrations of GSH aqueous solution (0，2.5，5 and 10 mM) to observe the color change of the solution. Then, respectively put the MnCaCZ NPs, MnO_2/Ce6@ZIF-8 NPs, CaO_2/Ce6@ZIF-8 NPs, CaO_2/Ce6@ZIF-8 NPs and Ce6@ZIF-8 NPs (1 mg/mL) into multiple groups of PBS (pH = 5.0) solutions containing 1 mM H_2O_2 and 10 mM GSH. Subsequently, the GSH consumption in each group of samples was measured by the GSH test kit at different time intervals (0，5，30，60 and 120 s), and monitored by the absorbance change at 420 nm.

2.8 Detection of the hydroxyl radical

Methylene blue (MB) method was applied to detect the hydroxyl radical (•OH). Since the absorption peak wavelengths of MB and Ce6 are similar, the MnO_2/CaO_2@ZIF-8 NPs, MnO_2@ZIF-8 NPs and CaO_2@ZIF-8 NPs (1 mg/mL) were dispersed in a PBS (pH = 5.0) solution containing 1 mM H_2O_2 and 10 mM GSH, followed by incubation at 37℃ for 2 h. The UV-Vis of MB was measured after centrifugation.

2.9 Detection of the reactive oxygen species

2',7'-dichlorofluorescein (DCFH) was used for the ROS detection. Typically, disperse the MnCaCZ NPs(1 mg/mL) in a PBS (pH = 5.0) solution containing 1 mM H_2O_2 and 10 mM GSH, and incubate at 37℃ for 2 h. DCFH was then added into the solution and followed by sonication (1.0 MHz, 1.0 $W \cdot cm^{-2}$, 3min). After centrifugation, the fluorescence intensities of the solution containing DCFH (λ_{ex} = 488 nm, λ_{em} = 525 nm) were measured with fluorescence spectrophotometer.

2.10 Cell culture

Mouse breast cancer cells (4T1 cells) were cultured in a cell culture dish with a diameter of 10 cm. The culture dish contained RPMI-1640, 10% fetal bovine serum (FBS), 1% penicillin and streptomycin, and was cultivate in a humid atmosphere of 95% air and 5% CO_2.

2.11 Cytotoxicity test

The cytotoxicity was measured by applying the MTT assay. The 4T1 cells were seeded in a 96-well plate (5×10^4 cells/well) and cultured for 24 h (37℃, 95% air and 5% CO_2). Fresh medium containing different concentrations (0, 10, 20, 30, 40, 50 and

60 μg/mL) of the MnCaCZ NPs were then added into each well. After incubating for 24 h under anaerobic conditions, remove the medium and inject 150 μL of the MTT solution (0.5 mg/mL) into each well. After 4 h, the MTT solution was removed, and 150 μL DMSO was added to dissolve the formazan produced. A microplate reader was employed to measure the absorbance of each well at 490 nm, and the survival rate of the cells was calculated based on the recorded data.

2.12 Cell uptake

Place the 4T1 cells in a confocal culture dish and incubate at 37°C for 24 h. The MnCaCZ NPs (50 μg/mL) was added and incubated at pH = 5.0 for 0.5, 1, 2 and 4 h, respectively. The cells were then washed 3 times with PBS, and stained with DAPI for 10 min. The confocal laser scanning microscope was used to observe and image the cells of each group.

2.13 Detection of HIF-1α in cells

Place the 4T1 cells in a confocal culture dish and incubate for 24 h, then respectively inject the MnCaCZ NPs and the Ce6@ZIF-8 NPs (50 μg/mL) into RPMI-1640 medium. The sample was incubated for 4 h under hypoxia, and the expression of hypoxia-inducible factor-1 in cells was detected. The 4T1 cells were fixed with 4% pre-chilled paraformaldehyde fixative solution for 15 min, then treated with primary antibody for 1 h and washed with PBS for three times. Finally, the sample solution was incubated with secondary antibody for 1 h, and being analyzed with confocal laser scanning microscopy (CLSM) after three times PBS washing (λ_{ex} = 488 nm, λ_{em} = 500~550 nm).

2.14 Detection of intracellular ROS

In order to detect the ROS generation of cells, the 4T1 cells were incubated in a confocal culture dish for 24 h, and then respectively incubated with MnCaCZ NPs, MnO2/Ce6@ZIF-8, CaO2/Ce6@ZIF-8, Ce6@ZIF-8 (50 μg/mL) in PBS for 4 h under hypoxia. After removal of the culture medium, each sample was washed with PBS for three times. The cells were then incubated with DCFH-DA for 30 min, and monitored *via* CLSM right after ultrasound irradiation (1.0 MHz, 1.0 $W \cdot cm^{-2}$, 3 min).

2.15 *In vitro* synergistic treatment effect evaluation experiment

MTT method was used to detect the synergistic therapeutic effect of the MnCaCZ NPs on tumor cells. The 4T1 cells (2×10^{-5} cells/well) were firstly incubated in a 96-well plate for 24 h. With the culture medium removal, each RMPI-1640 culture medium (200 μL) containing the MnCaCZ NPs, MnO_2/Ce6@ZIF-8, CaO_2/Ce6@ZIF-8 or Ce6@ZIF-8 (50 μg/mL) was respectively added into each well. After incubating for 4 h under hypoxia, each well was washed with PBS for three times to remove excess nanomaterials. For the next step, the cells were sonicated (1.0 MHz, 1.0 $W \cdot cm^{-2}$, 3 min) and the cell survival rate was calculated after 12 h of continuous culture.

Finally, repeat the above procedure but increase the volume of RMPI-1640 culture medium to 1 mL. After sonication, the cells were divided into two groups: the first group was stained with Calcein-AM and PI, and the cell apoptosis was monitored *via* CLSM; the second group was performed with Annexin V-FITC and stained with PI, followed by flow cytometry analysis.

Supplementary Figures

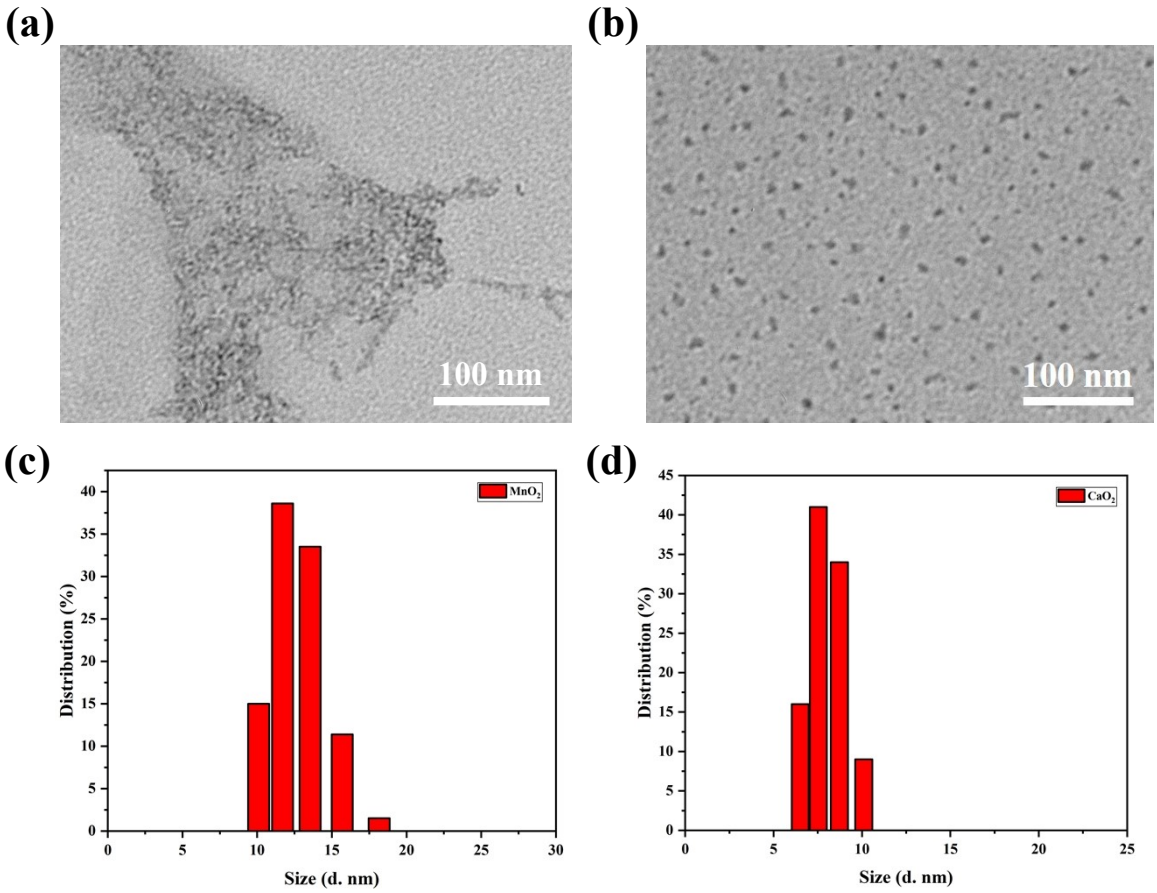

Figure S1. TEM image of (a) the MnO_2 NPs and (b) the CaO_2 NPs; DLS results of (c) the MnO_2 NPs and (d) the CaO_2 NPs.

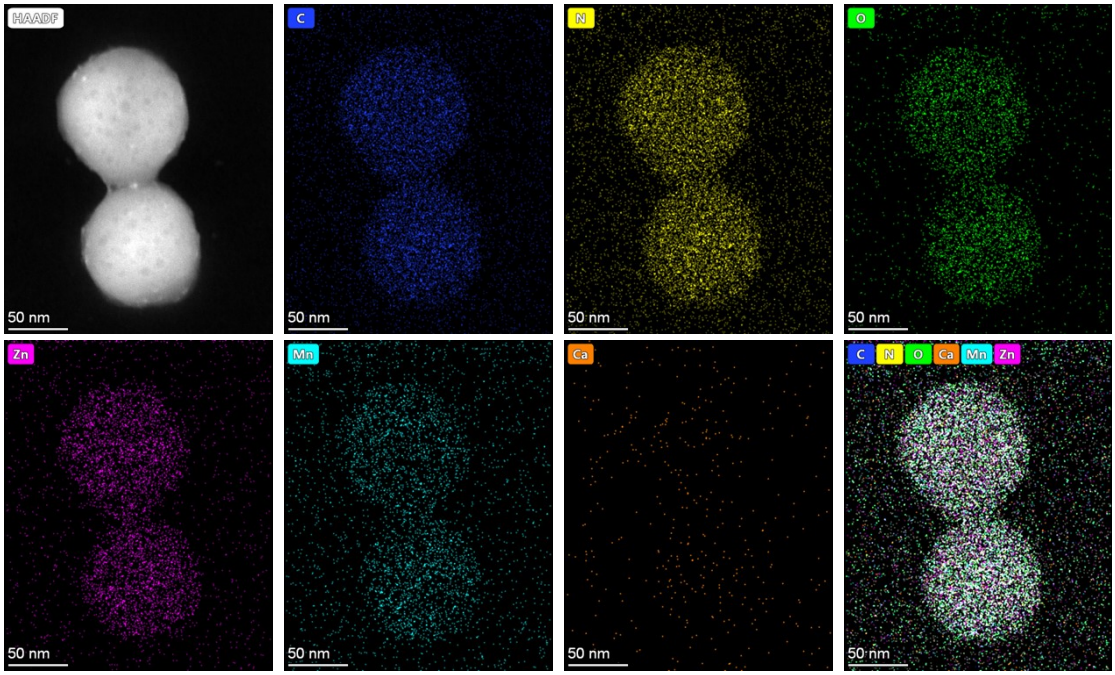

Figure S2. STEM elmentary distribution of the MnCaCZ: The high-angle annular dark-field image (HAADF) of the NPs; elements distribution of C, N, O, Zn, Mn and Ca; The overlap of all elements.

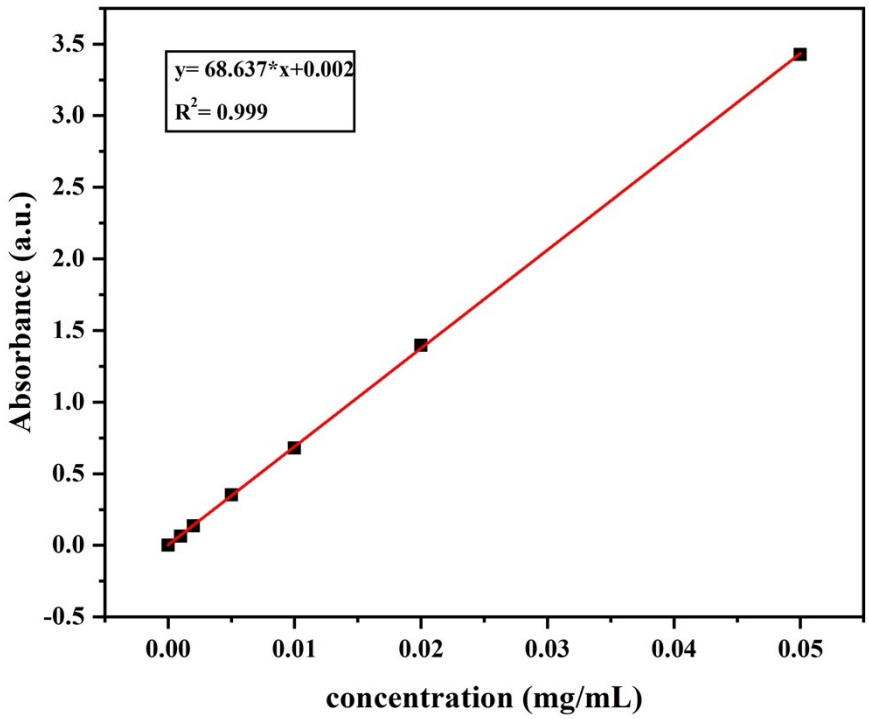

Figure S3. Standard linear calibration curve of Ce6's concentration towards absorption intensities.

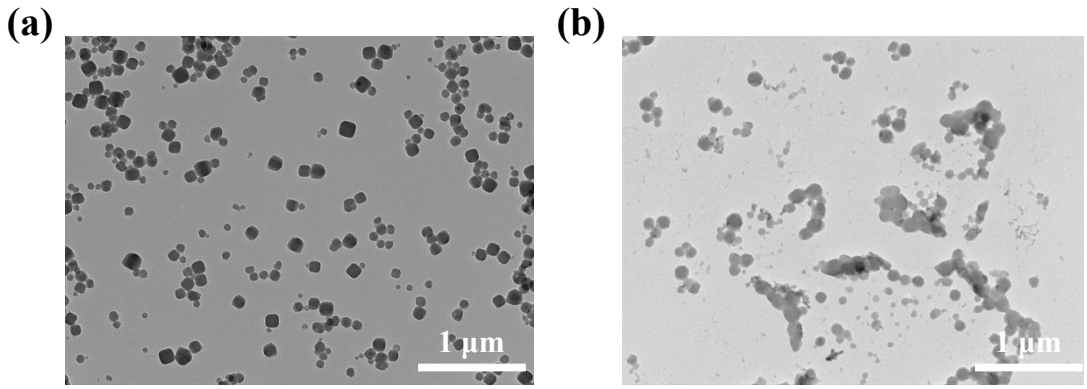

Figure S4. TEM images of the MnCaCZ NPs after immersion in PBS at pH = (a) 7.4 and (b) 5.0.

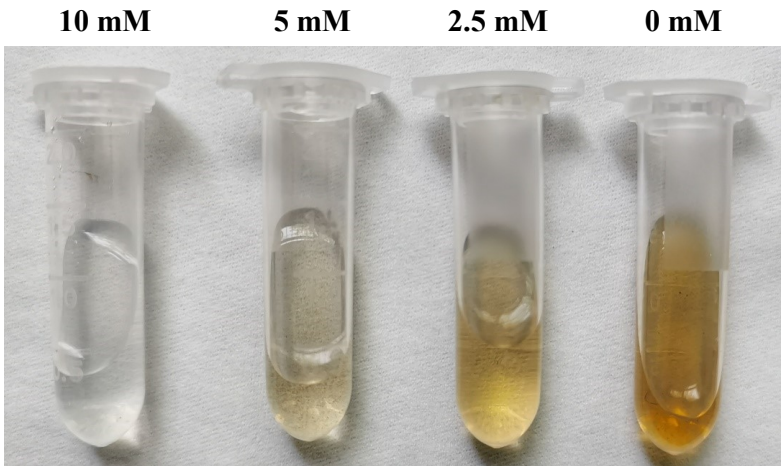

Figure S5. GSH consumption qualitative experiment.

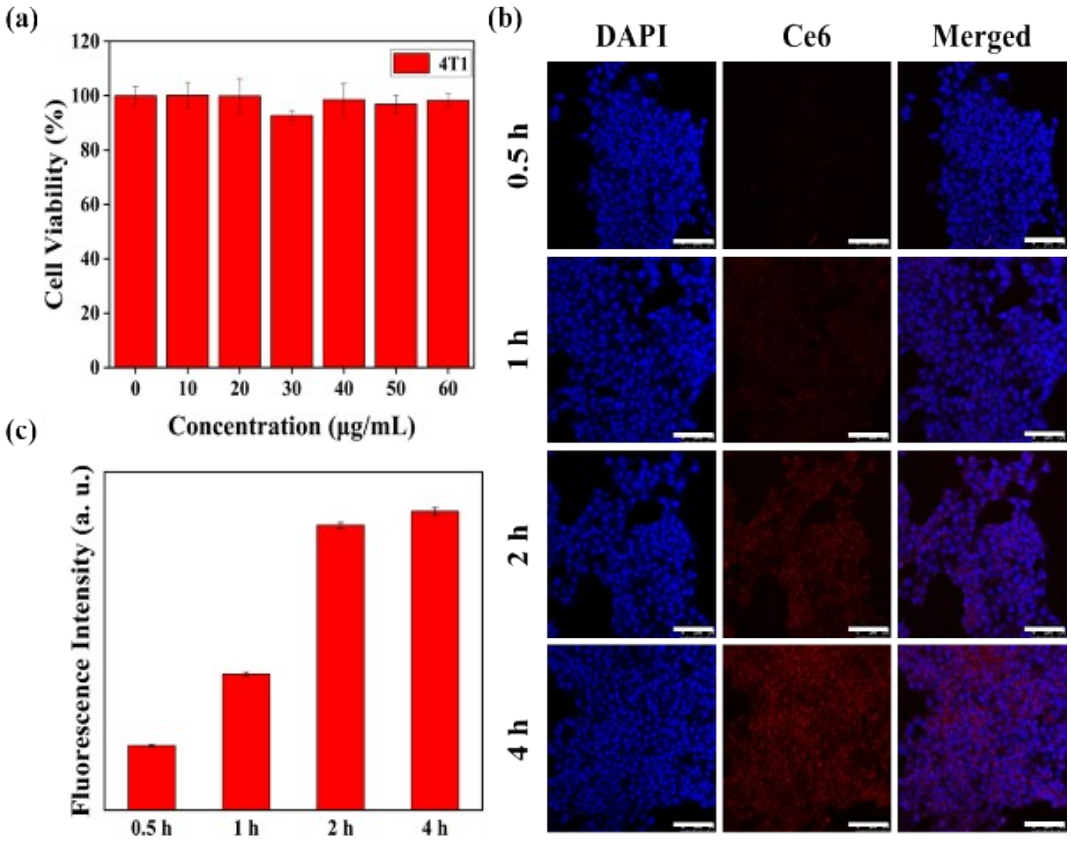

Figure S6. (a) MTT assay of 4T1 cells treated with various concentrations of MnCaCZ NPs for 24 h; (b) CLSM images of 4T1 cells after incubation with MnCaCZ NPs for 0.5, 1, 2, and 4 h; (c) The quantitative results of CLSM corresponding to b. Scale bar: 100 μm

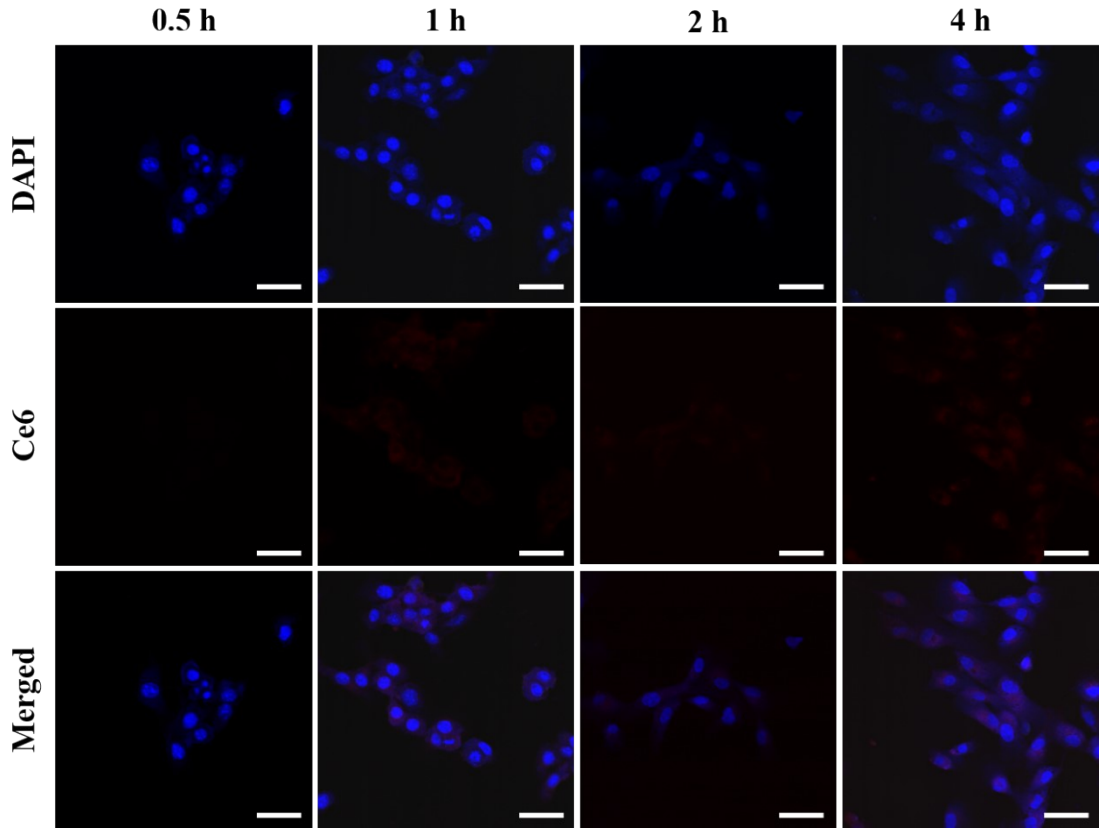

Figure S7. Confocal fluorescence images of the MnCaCZ NPs incubated with MCF-7 cells for different time intervals (scale bar = 50 μm).

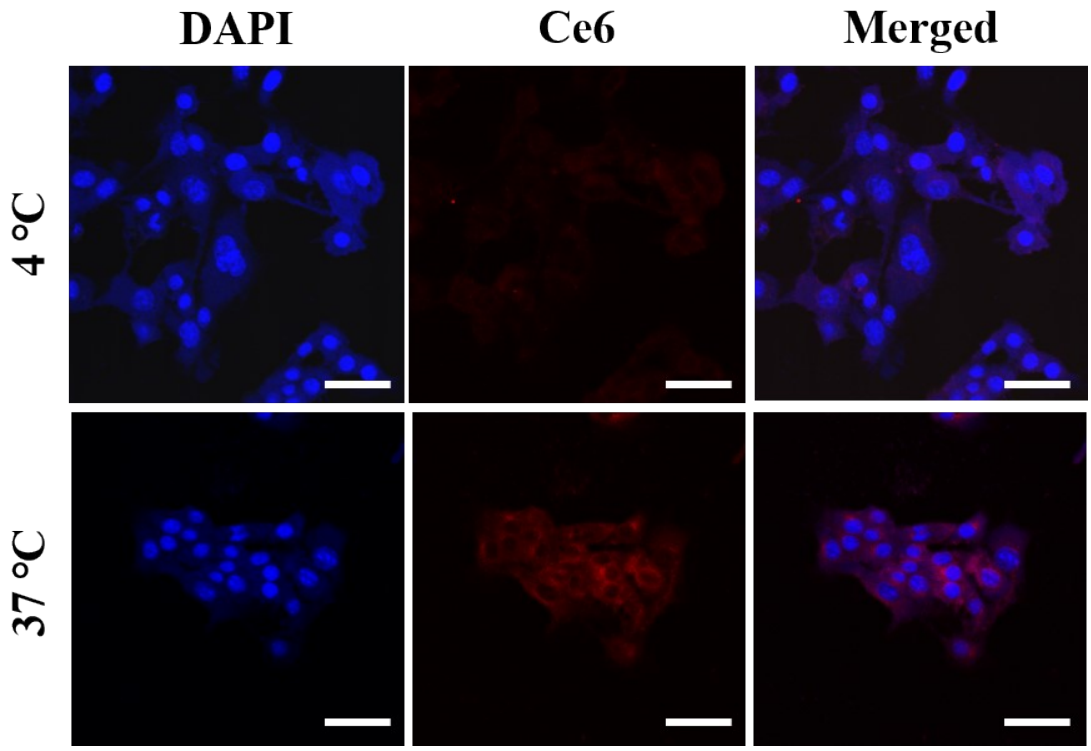

Figure S8. Confocal fluorescence images of the MnCaCZ NPs and 4T1 cells incubated at different temperatures (scale bar = 50 μm)

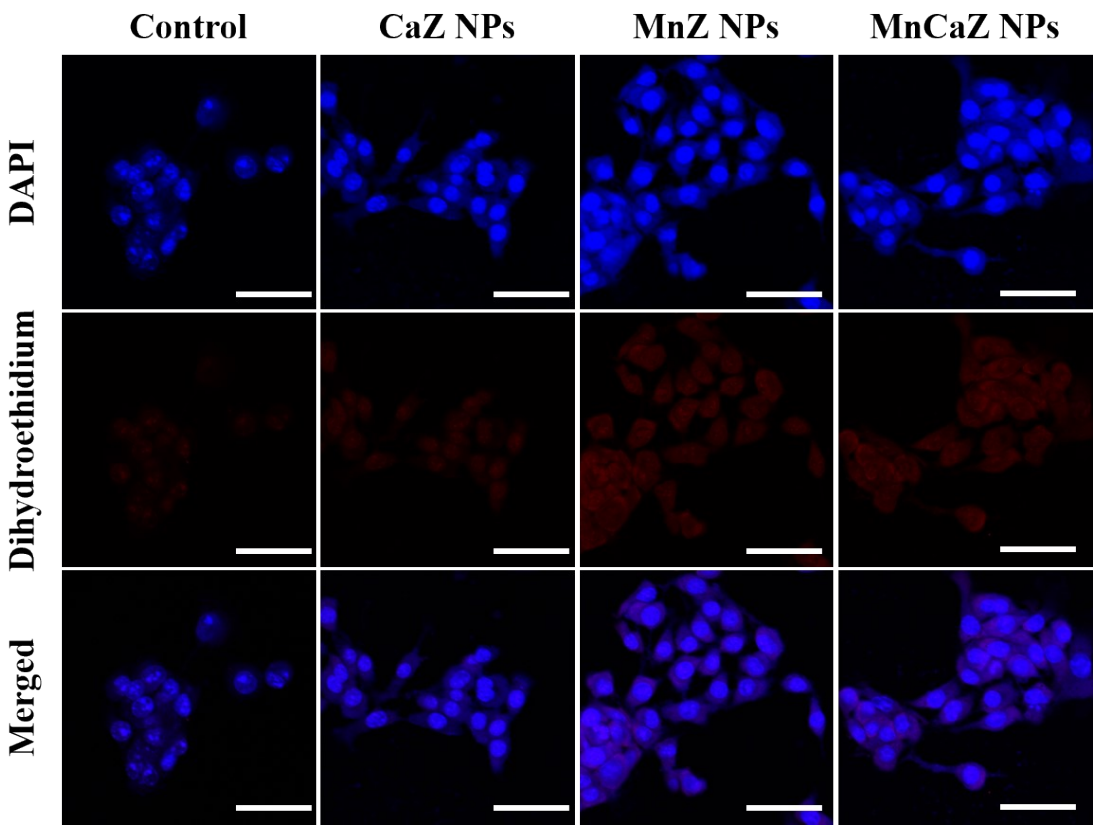

Figure S9. Confocal fluorescence images of dihydroethidium-stained 4T1 cells under different treatment groups (scale bar = 50 μm)

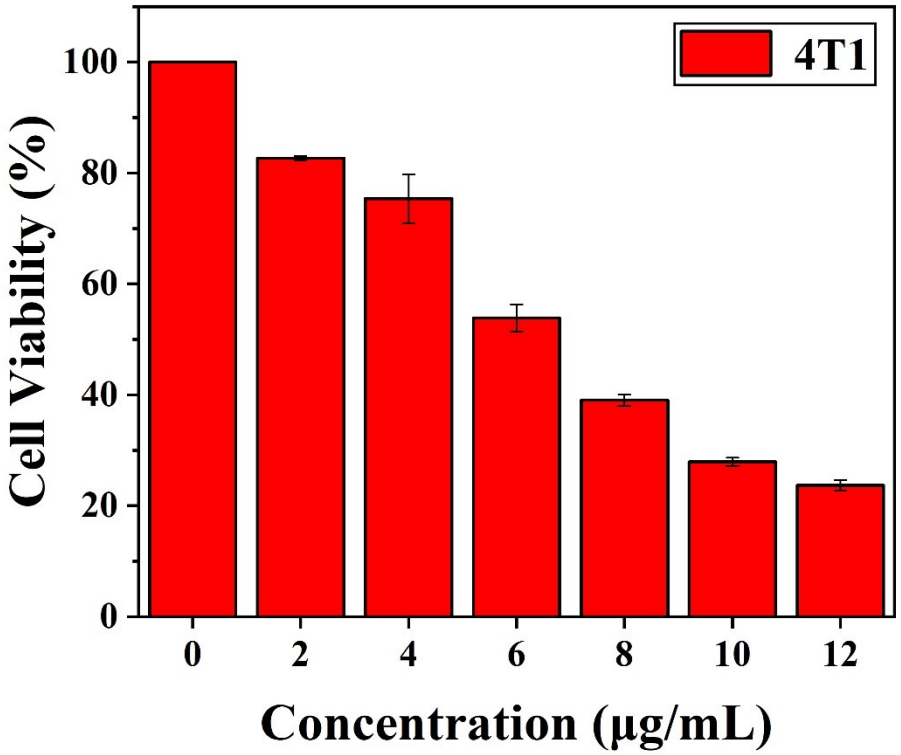

Figure S10. Cell viability of the 4T1 cells incubated and sonicated with different concentrations of the MnCaCZ.

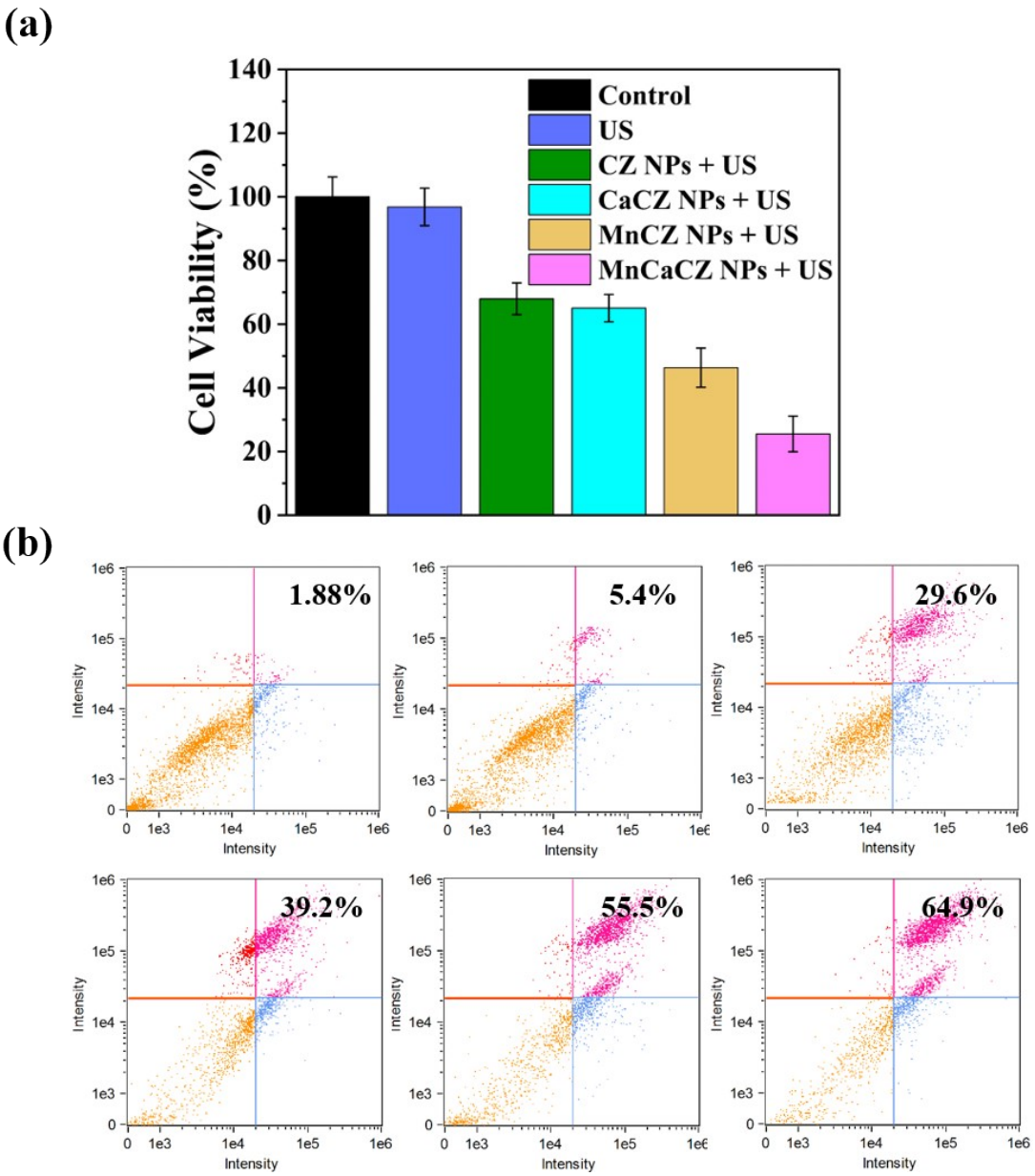

Figure S11. (a) MTT assay of 4T1 cells treated with different nanoreactors under anaerobic conditions; (b) Flow cytometry analysis of cellular apoptosis of 4T1 co-incubated with PBS, US, CZ NPs+US, CaCZ NPs+US, MnCZ NPs+US and MnCaCZ NPs+US. Scale bar: 100 μm.