Supplementary Information

Cerium oxide nanozymes enhance microwaveimmunotherapy by reshaping the tumor microenvironment

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Experimental Section

Materials. All reagents used in this article were analytical grade. Triethylamine (TEA), 2-methylimidazole and 1,2-Bis(trimethylsilyl)ethane (BTEs) were purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. Cetyltrimethylammonium bromide (CTAB), sodium salicylate (Nasal), R837, and Myristyl Alcohol (MA), $Ce(NO_3)_3 \cdot 6H_2O$ were purchased from Shanghai Macklin Biochemical Technology Co., Ltd. Tetraethyl orthosilicate (TEOS) was purchased from Sinopharm Chemical Reagent Co., Ltd. Cell-related reagents and immunohistochemical-related reagents were purchased from Beyotime Biotechnology Co., Ltd.

Synthesis of DCeR nanozymes. Firstly, dendritic mesoporous silica (DS) was synthesized by the template method. 68 mg TEA, 380 mg CTAB, and 168 mg Nasal were dissolved in deionized water (dH_2O). The mixture was stirred at 80 ℃. After 1 h, 3 mL TEOS and 2.4 mL BTEs were added dropwise to the above solution. The reaction was maintained under stirring for 12 h. After that, the resulting products were collected through centrifugation and washed several times with methanol. The template was removed by refluxing the products with HCl/Ethanol (1:9) solution at 60 ℃ for 6 h, followed by vacuum drying overnight. Secondly, DS@CeMOF was prepared. DS was dispersed in a methanol solution containing $Ce(NO_3)_3 \cdot 6H_2O$ (2.5 mmol) using sonication treatment for 30 min to ensure homogeneous absorption of Ce^{3+} ions within the mesopores. Subsequently, 80 ml of methanol solution containing 24 mmol of 2-methylimidazole was added into the mixture and stirred for 12 h at room temperature. The products were collected by centrifugation, washed several times with ethanol and dried. Thirdly, the obtained samples were calcined in air at 500 ℃ for 4 h to obtain $DS@CeO_2$ (DCe). Finally, for drug loading, equal masses of DCe, R837 and MA were dispersed in methanol solution. The mixture was subjected to the negative-pressure pumping to facilitate the entry of R837 and MA into the mesoporous channels.

DCe SOD-like activity. The $\cdot O_2^-$ scavenging activity of the DCe nanozymes was measured using a superoxide anion assay kit. The concentrations of the DCe ranged from 7.25-200 μg mL^{-1}. The corresponding volume of the WST-8/enzyme working solution and the reaction starter working solution were prepared according to the instructions in the kit. The experiment was divided into different groups: the sample group, blank 1 group (without the sample to be tested), blank 2 group (without the reaction starter working solution), and blank 3 group (to exclude sample color

interference). Different concentrations of DCe solutions were reacted with $\cdot O_2^-$ radical test solutions for 30 min at 37 ℃. The scavenging efficiency was calculated based on the absorbance measured at 450 nm.

DCe CAT-like activity. The O_2 production performance of DCe nanozymes was quantitatively measured by an O_2 dissolving instrument. The experiment was divided into four groups: dH_2O, dH_2O+MW, 1 mg/mL DCe, and 1 mg/mL DCe + MW. Total volume of each group was 2 mL, followed by the addition of 10 μL of H_2O_2 (10 mM). Then the mixture was irradiated under microwave (MW) at 0.9 W for 5 min.During the experiment, the O_2 dissolution instrument was used to detect O_2 content in solution in real time, and the DO value was recorded for 20 min. The curve of time-dependent dissolved O_2 content was plotted to quantitatively evaluate the O_2 production performance of DCe.

DCe POD-like activity. The POD-like activity of DCe nanozymes was evaluated by 3,3',5,5'-tetramethylbenzidine (TMB) color reaction in PBS solution. The experiment was divided into four groups: PBS (pH 5.7), PBS (pH 5.7) + MW, 20 μg mL^{-1} of DCe dispersed in PBS (pH 5.7), and 20 μg mL^{-1} of DCe dispersed in PBS (pH 5.7) + MW. 20 μL of TMB (10 mg mL^{-1}) was added to each group, respectively. The MW irradiation condition was 0.9 W for 5 min. The supernatant was collected by centrifugation for UV−Vis spectral analysis. Next, diacetyldichlorofluorescein (DCFH-DA) was further used to measure the total amount of ROS generated. 200 μL of DCFH-DA (0.5 mM) was added to the PBS solution (pH 5.7) of 1 mg mL^{-1} DCe.Then, 10 μL of H_2O_2 (10 mM) was added and the MW irradiation condition was 0.9 W/5 min. All samples were incubated in the dark for 2 h, and then the supernatant was used for dichlorofluorescein (DCF) fluorescence spectrum analysis. Neutral pH conditions (PBS, pH 7.4) were employed to investigate pH-dependent ROS evolution using identical experimental parameters as in the acidic system.

Drug Release of DCe@R837. To study the drug release behavior of DCe@R837 nanozymes, they were divided into four groups: PBS-5.7, PBS-5.7+MW, PBS-7.4, and PBS-7.4+MW. All the groups contained 1% Triton X-100 with three parallel samples. MW irradiation condition was 0.9 W for 5 min. All the test groups were centrifuged. The supernatant was collected for the analysis. The time interval points were 0.5, 1, 2, 4, 6, 8, 12, and 24 h, respectively. The drug release was calculated according to the absorption peak value, and the release rate-time curve was plotted to evaluate the release behavior of DCe@R837.

MW Heating Effect of DCe. To evaluate MW heating effect, we dispersed DCe in 1 mL of saline solution with different concentrations and subjected it to MW irradiation. The concentration range of DCe was 0, 1, 2, 3 mg mL^{-1}. MW irradiation was applied at 0.9 W for 5 min. The temperature change was recorded, and the resulting time−temperature curve was plotted.

Biocompatibility evalution of DCe. Triple negative breast cancer 4T1 cells was used as tumor cell models for cytotoxicity studies. After cells were seeded and incubated for 12 h, DCe was added to the cells at concentrations ranging from 0, 12.5, 25, 50, 100 and 200 μg mL^{-1} for further incubation for 12 or 24 h. Then cytotoxic effect was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. All experimental groups included 6 parallel groups. To evaluate its biocompability *in vivo*, DCes was intravenously injected into mice at 50, 100, 200 mg kg^{-1}. During the treatment period, the behaviors of animals were observed, and their body weights were measured. On day 14, blood was collected for blood analysis. Immediately after that, the mice were sacrificed, and the main tissues were extracted for further histopathological analysis.

Endocytosis of DCe. Cell uptake was analyzed by hyperspectral analysis. A spectral database for DCe was established under dark-field conditions. Cells were seeded into 6-well plates containing coverslips and cultured overnight. Subsequently, DCe were added at a concentration of 50 μg mL^{-1}. Incubation was conducted for 3, 6 and 9 h, coverslips were collected for hyperspectral imaging. The spectral data was compared to the pre-established database of DCe to evaluate its distribution and endocytosis efficiency within the cells.

DCe CAT-like activity at Cell Level. The production of O_2 at the cellular level was investigated by the O_2 fluorescence probe Tris(4,7-diphenyl-1,10-phenanthroline) ruthenium (II) dichloride (RDPP). When the cells were cultured to an appropriate concentration, 100 μL of $CoCl_2$ was added for 12 h to create an oxygen-poor microenvironment at 100 μg mL^{-1}. After that, RDPP at final concentration of 10 μg mL^{-1} and DCe with the concentration range of 0, 25, 50, 100, and 200 μg mL^{-1} were added and cultured for 6 h. 10 μL of H_2O_2 (10 mM) was added for another incubation of 2 h. All groups were washed with PBS. The MW irradiation condition was 0.9 W for 5 min, and fluorescence was observed by hyperspectral microscope.

DCe POD-like activity at Cell Level. The generation of ROS at the cellular level was studied using DCFH-DA probe. DCe at 50 μg mL^{-1} were incubated with 4T1 cells for 4 h, followed by addition of 10 μL of H_2O_2 (10 mM). The culture was continued for 2 h, after that all groups were

washed. DCFH-DA was added for another incubation for 30 min. The cells dispersion was prepared by washing and digesting. The MW irradiation condition was 0.9 W for 5min, and hyperspectral microscopy was used to observe DCF fluorescence intensity. Additionally, flow cytometry was used to quantitatively analyze the generation of ROS at the cell level. 4T1cells were divided into 5 groups: Control, MW + H_2O_2, DCe, DCe + H_2O_2 and DCe + H_2O_2 + MW. After that, all the other procedures were the same as the steps for ROS detection. The final analysis was detected by flow cytometry.

Detection of HMGB1 Expression. The experimental groups were as follows: Control, MW, DCe, DCe + MW, DCeR, DCeR + MW group. MW irradiation was conducted for 0.9 W/5 min and the concentration of DCe and DCeR were 100 μg mL^{-1}. First, 4T1 cells (1×10^5) were incubated with DCe or DCeR for 24 h. Subsequently, MW irradiation was performed for 5 min. After continuing incubation for 24 h, fluorescence staining was conducted with HMGB1 antibody. Finally, the fluorescence level of HMGB1 expression were detected by flow cytometry.

Study on the Immune Mechanism of DCeR nanozymes *In Vivo*. The 4T1 subcutaneous tumor model of Balb/c mice was established. The mice with the volumes of 100 ± 20 mm^3 were randomly divided into 5 groups consisting of 3 mice. The 5 groups were: (1) Control, (2) MW, (3) DCeR, (4) DCe + MW and (5) DCeR + MW. DCe or DCeR was injected intravenous at 50 mg kg^{-1} for 6 h. The mice from the MW groups were subjected to MW irradiation for at 0.9 W for 5 min. After 14 days, the mice were sacrificed, and their tumor and spleen were homogenized, washed, and incubated with antibodies. Flow cytometry was used to analyze the levels of $CD4^+$ and $CD8^+$ T cells in the tumor and spleen, and the levels of IFN-γ in the tumor.

***In Vivo* Antitumor Experiment of DCeR nanozymes.** The 4T1 subcutaneous tumor model with the volume of 100 ± 20 mm^3 was randomly divided into 5 groups, with 4 mice in each group. The treatment strategies were consistent with the protocols described in the aforementioned immune mechanism studies. After 14 days of continuous observation, the mice was weighed, and the tumor volume was measured at interval of 2 d. On day 14, the mice were sacrificed, and tumor were excised and photographed for further analysis.

Cell Line and Mouse Model. The 4T1 cell line was obtained from Wuhan Punosei Life Technology Co., Ltd. Balb/c mice were provided by Beijing Vitonglihua Experimental Animal Technology Co., Ltd. Animal experiments were performed in compliance with the Measures for

the Administration of Experimental Animals of Technical Institute of Physics and Chemistry, Chinese Academy of Sciences, and approved by the Commission for the Administration of Experimental Animals.

Statistical Analysis. To enhance the reliability of experimental results, all data were expressed as mean ± standard deviation. Single factor analysis of variance was used to analyze the data of each group. Differences ($p < 0.05$) were considered statistically significant, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. NS indicated no significant difference.

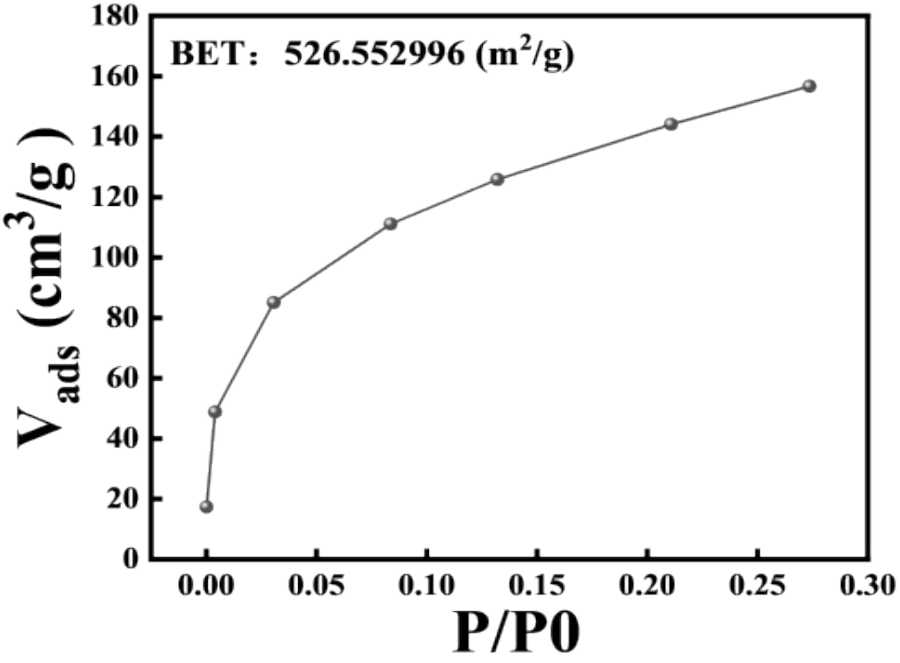

Figure S1. N_2 adsorption curve of DS.

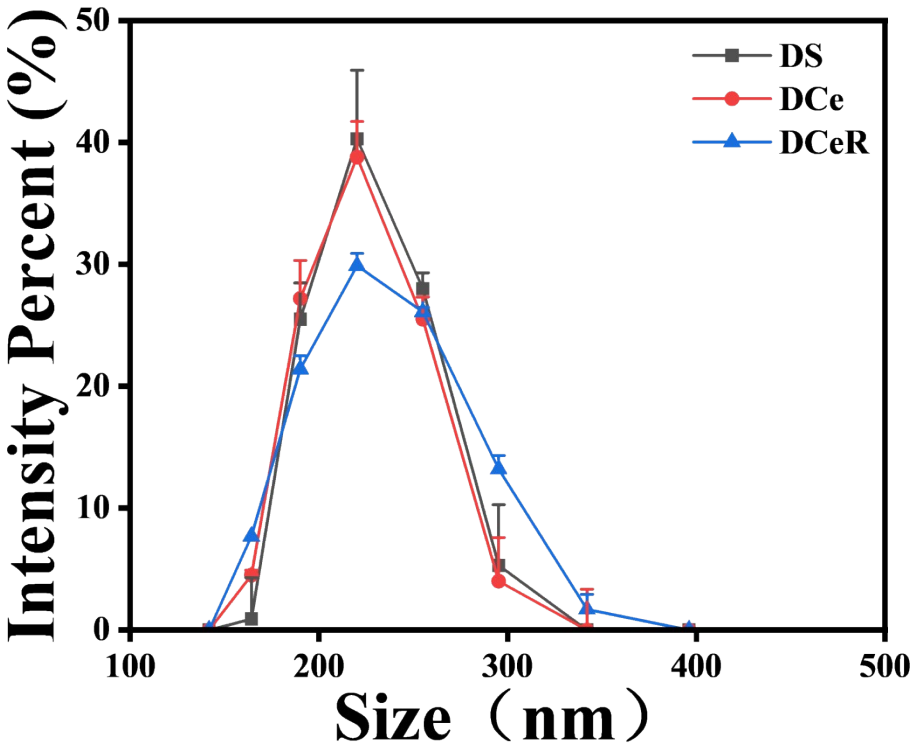

Figure S2. Hydrodynamic diameter of different materials.

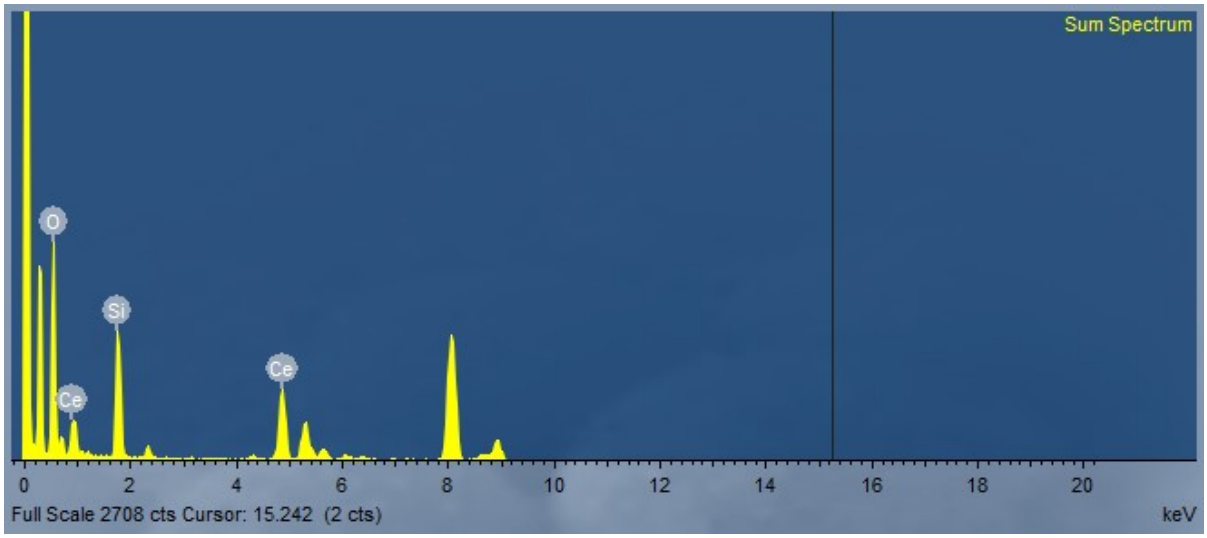

Figure S3. EDS diagram of DCe nanozymes.

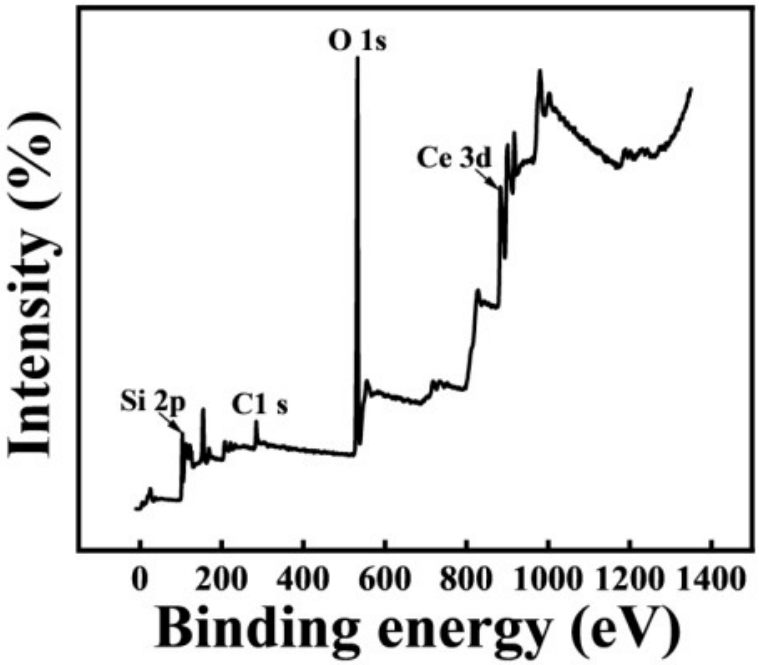

Figure S4. The XPS survey spectrum of DCe.

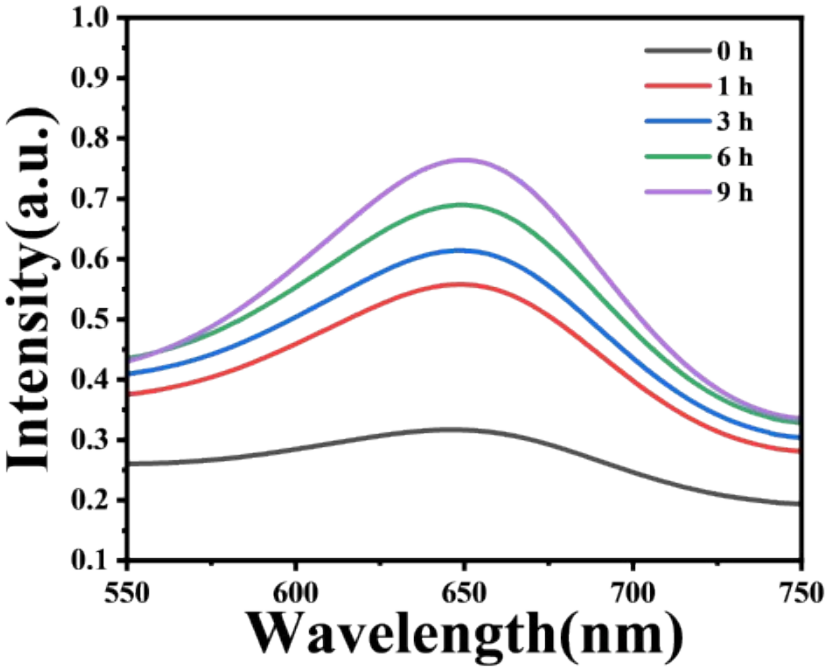

Figure S5. TMB color rendering results of DCe at different times.

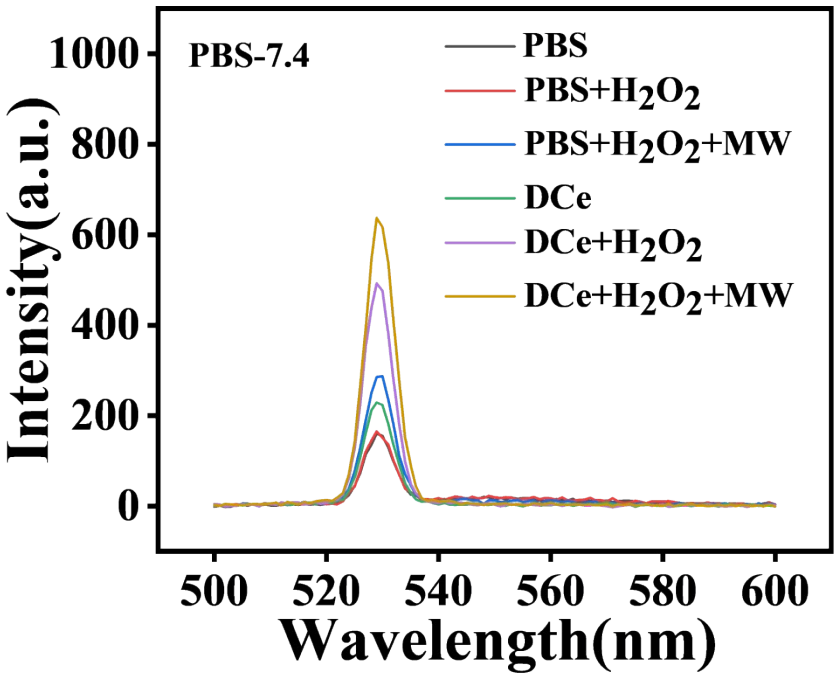

Figure S6. ROS detection results of DCe in PBS at pH 7.4.

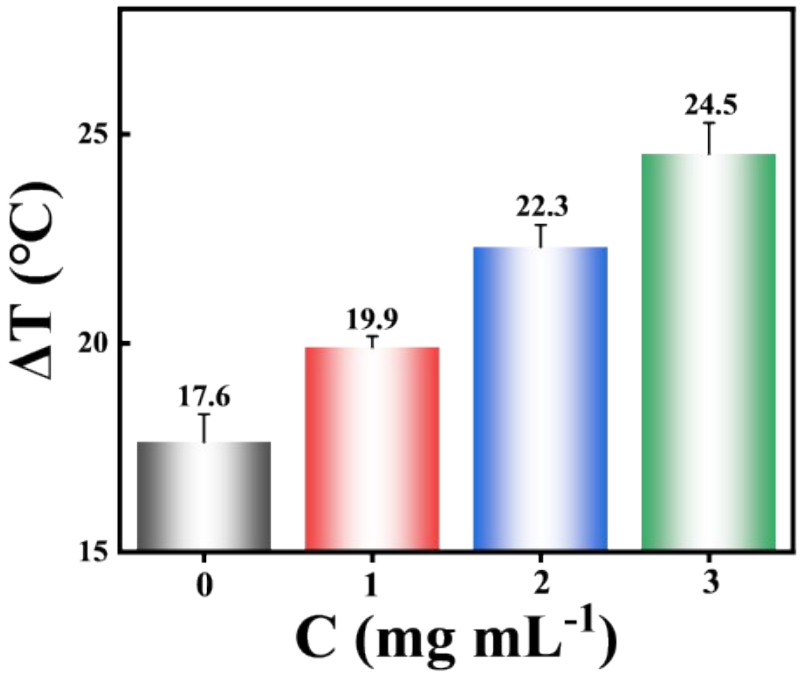

Figure S7. Temperature difference of DCe MW heating.

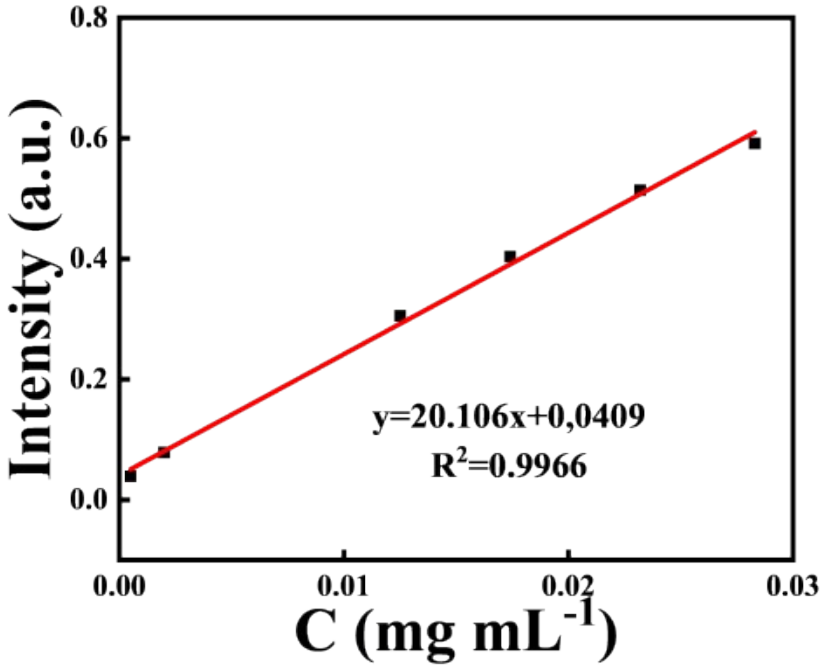

Figure S9. Standard curve of R837 in water containing 1% Triton X-100.

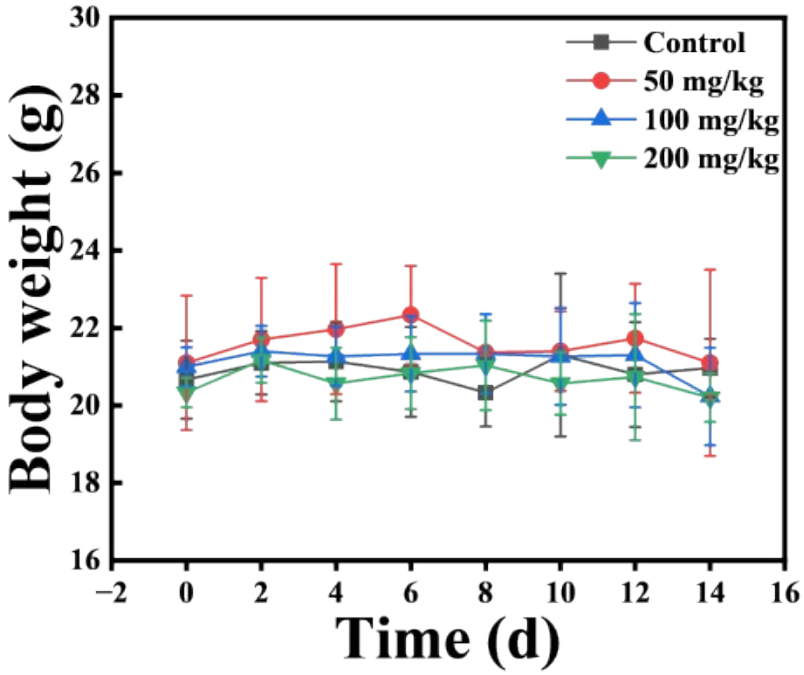

Figure S10. Weight-time curves of mice in different groups.

Figure S11. Blood routine results of mice in different group.

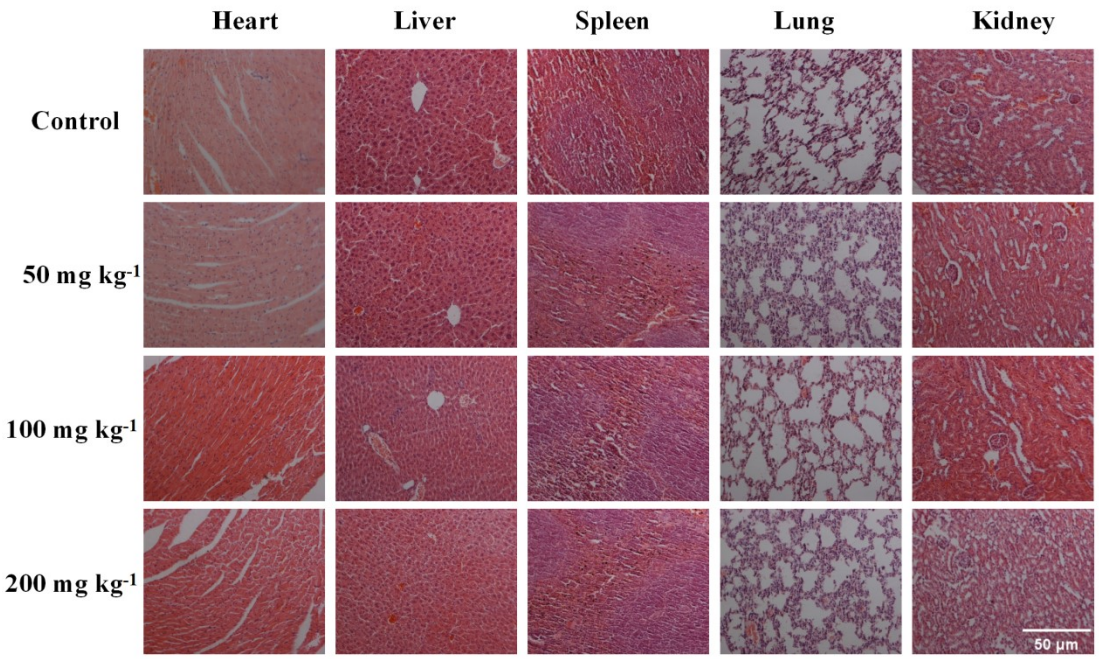

Figure 12. H&E staining images of major tissues of heart, kidney, liver, lung, spleen from the mice treated wih DCe at 50, 100 and 200 mg kg^{-1}.

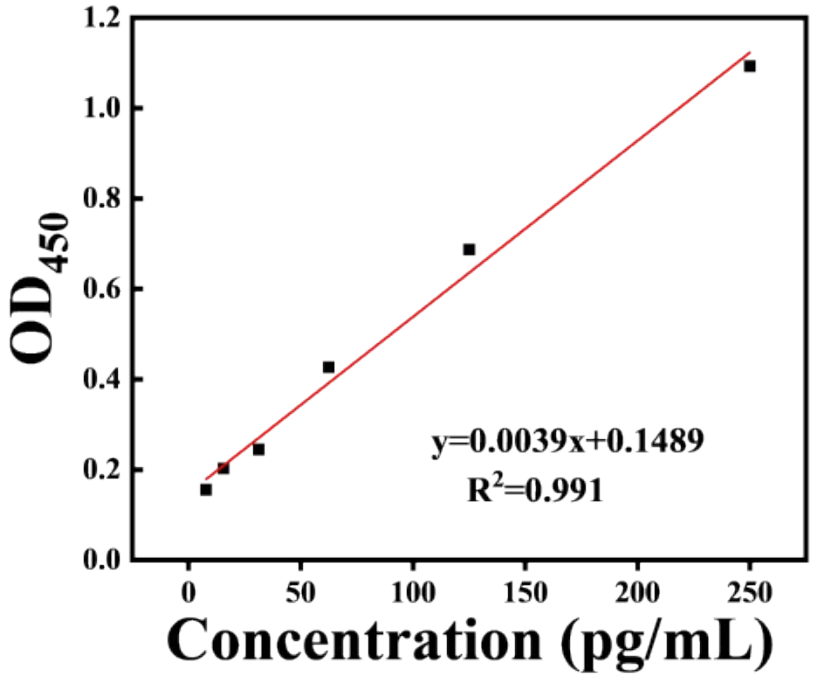

Figure S14. Standard curve of IFN-γ.

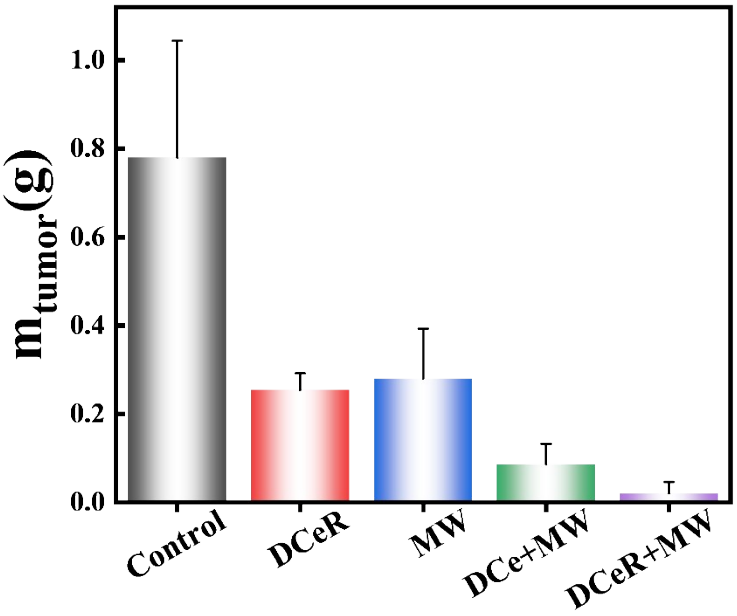

Figure S15. Tumor mass of each experimental group.