Development of a Novel Oxidative Stress Amplifying Nanocomposite Capable of Supplying Intratumoral H_2O_2 and O_2 for Enhanced Chemodynamic Therapy and Radiotherapy in Patient-Derived Xenograft (PDX) Models

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MATERIALS AND METHODS

Materials

$Zn(NO_3)_2 \cdot 6(H_2O)$, 2-methyl imidazole (HmeIM), bovine serum albumin (BSA), $CaCl_2$, $FeCl_3$, and PEG_{200} were purchased from Sigma-Aldrich and used as received. H_2O_2, NaOH, ammonia and PBS were obatained from Thermo-Fisher (Waltham, MA, USA). 2′, 7′-dichlorodihydrofluorescein diacetate (DCFH-DA), ATP assay kit, 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) and Reactive Oxygen Species Assay Kit were purchased from Beyotime Company (China). All of the aqueous solutions were prepared using purified deionized (DI) water purified with a purification system (Direct-Q3, Millipore, USA). The other solvents used in this work were purchased from Sinopharm Chemical Reagent (China) and Aladdin-Reagent (China).

Characterization

Scanning electron microscopy (SEM) images were captured on a Hitachi FE-SEM S-4800 instrument with an acceleration voltage of 3 kV. The samples were prepared by depositing sample dispersion onto a freshly cleaved silicon wafers surface. Transmission electron microscopy (TEM) and energy dispersive spectrometer (EDS) mapping were carried on a JEM-2100F filed emission electron microscope with an acceleration voltage of 200 kV. Powder X-ray diffraction (PXRD) patterns were collected on a PANalytical B.V. Empyrean powder diffractometer, in which data were collected from 5^o to 45^o at a scan rate of 15^o/min.

Synthesis of CaO_2 nanoparticles

2 mL of $CaCl_2$ aqueous solution (0.1g/mL) in an opened grass flask was added 1 mL of ammonia solution (1 M) and 80 mL of PEG_{200}. Then at quick stirring, 1 mL of 30% H_2O_2 was slowly added to the mixture. The mixture was stirred for 6 h at room temperature, and then a clear and colorless solution was obtained. Further, NaOH solution (1 M) was slowly added to the mixture under ultrasound until the pH value of the solution reached 11.5. The clear solution was thus changed to a white color or pale yellow suspension. Then the mixture was centrifuged and a white precipitate was obtained, after washed with NaOH solution (0.1 M), ultrapure water and ethanol, respectively, white CaO_2 nanoparticles were obtained and stored in ethanol at 4 ^{o}C.

Construction of CaO_2 nanodots and Fe^{3+} co-incorporated MOF nanoplatform (CFM) through biomimetic mineralization

Typically, 4 mL aqueous solution containing HMeIM (990 mg, 12 mM), BSA (10 mg) and CaO_2 (10 mg) was mixed with 4 mL of $Zn(NO_3)_2 \cdot 6(H_2O)$ solution (81 mg, 0.175 mM), 400 μL of $FeCl_3$ solution (9.6 mg). The mixture was incubated at room temperature for 5 min, and then the solids were collected and washed with de-ionized water. The yellow solids were subsequently dried at room temperature to obtain the composite CFM. Similarly, FM and CM were constructed as described above except for no addition of $FeCl_3$ or CaO_2. Ca^{2+} and Fe^{3+} release from CFM in different buffer solution were detected by ICP-AES. The obtained CFM was dispersed into 10 mL of

different buffer soulution in branch-necked flask at room temperature. The reaction samples were taken from the flask at predetermined intervals. To visualize the O_2 generation, 10 mg of CFM or CaO_2 solids were added into PBS with different pH value and the O_2 concentration was measured by a dissolved oxygen meter. To verify H_2O_2 generation of CFM under acidic conditions, catalase (CAT) was introduced. 10 mg of CFM solids and CAT were added into PBS with low pH value (5.5) and the O_2 concentration was measured by a dissolved oxygen meter.

Separation of patient-derived cancer cells (PCCs)

Tumor tissues were harvested from bladder cancer patients, cut into ≈1 mm^3 pieces, and then re-suspended in Dulbecco Modified Eagle Medium (DMEM; Gibco, USA) containing 1 mg/mL collagenase IA (Sigma-Aldrich, USA) and DNase I (Roche, Switzerland) for 2 h on a rocking platform at room temperature. Then the resulting cell suspension was treated with BD Pharm Lyse Lysing Buffer and finally passed through a 70 mm cell strainer (Becton Dickinson, USA), resulting in the PCCs.[1] Cell cultures under normoxic conditions (pO_2: 21%) were maintained in a humidified incubator at 37℃ in 5% CO= and 95% air. Hypoxic conditions (pO_2: 2%) were produced by placing cells in a hypoxic incubator (Moriguchi, Japan) in a mixture of 2% O_2, 5% CO_2, and 93% N_2.

Establishment of patient-Derived Xenograft (PDX) Mouse Models

After traditional tumor-reductive surgery, a bladder tumor tissue sample was cut into ≈5 mm^3 pieces and then implanted in BALB/c nude micesubcutaneously. At 1-4 months after implantation, tumors began to appear at the site of implantation. When the tumor volume reached ≈1000 mm^3, the mice were euthanized and the xenografts were then implanted into another group of mice with the same protocol. The passage harboring the patient-derived material was termed P0, with subsequent generations numbered consecutively (P1, P2, P3, and so on). In this work, the third generation of animals (P3) was used for in vivo and in vitro experiments.[2]

Intracellular reactive oxygen species (ROS) generation

For determination of ROS levels via fluorescent imaging, PCCs were incubated for 2 h with 6 different groups: (1) PBS; (2) FM; (3) CM; (4) CFM. The ZIF-8 concentration was 200 μg/mL in group 2, 3 and 4. Then, the fluorescent dye, DCF (10 μM), was added and co-incubated for 20 min at 37 °C. ROS level was determined by confocal laser scanning microscope (CLSM; IX81, Olympus, Japan). The fluorescent intensity of each group was calculated by ImageJ software.

γ-H_2AX immunofluorescence analysis

PCCs were seeded in 24-well plates and then cultured for 24 h at 37 °C under hypoxia condition. The experiment was divided into six groups: (1) PBS; (2) RT; (3) CFMs; (4) FM+RT; (5) CM+RT and (6) CFM+RT. The ZIF-8 concentration was 200 μg/mL in group 3, 4, 5 and 6. Then the cells were immediately packaged with sealing membrane. Next, the cells in group 2, 4, 5 and 6 in sealed 24-well plates were subjected to X-ray irradiation at a dose of 6 Gy. After RT treatment for 2 h, the cells were fixed with 4% paraformaldehyde for 10 min, rinsed with PBS, per- meabilized with methanol for 15 min at −20 °C and then rinsed with PBS again. Then the cells were exposed to a blocking buffer (1% bovine serum albumin (BSA) in PBS solution) for 1 h at room

temperature and further incubated with anti- phospho-histone γ-H_2AX human monoclonal antibody (dilution 1:500) overnight at 4 °C. After washing with PBS, the cells were incubated with Cy5-conjugated sheep secondary antibody (dilution 1:500) for 1 h at room temperature. Excess antibody was removed by rinsing the coverslips in PBS. Cell nuclei were stained by DAPI for 5 min at room temperature. The cells were imaged via confocal fluorescence microscopy (IX81). Quantitative analysis of γ-H_2AX foci density (foci/100μm^2) was performed by automatic counting using the ImageJ software for n = 100 cells in each treatment group.

***In vitro* anti-cancer effect of CFM**

The anti-tumor effect was measured by MTT assay. PCCs were seeded in 96-well plates at a density of 5×10^3 cells per well and incubated for 12 h under normoxia condition. Afterwards, cells were incubated for 6 different groups: (1) PBS; (2) RT; (3) CFMs; (4) FM+RT; (5) CM+RT and (6) CFM+RT. The ZIF-8 concentration was 200 μg/mL in group 3, 4, 5 and 6. Then, cells in group 2, 4, 5 and 6 were exposed to X-ray radiation (4Gy) for 5 min. At the end of the incubation, 5 mg/mL MTT PBS solution was added, and the plate was incubated for another 4 h. Finally, the absorbance values of the cells were determined by using a microplate reader (Emax Precision, USA) at 570 nm. The background absorbance of the well plate was measured and subtracted. The cytotoxicity was calculated by dividing the optical density (OD) values of treated groups (T) by the OD values of the control (C) (T/C × 100%). Anti-tumor test in hypoxia condition are similar to the above.

Extracellular GSH Depletion

A GSH and DTNB solution with the final concentration of 0.1 mM GSH and 0.2 mg/mL DTNB was prepared and maintained at 25 °C. The CM, FM and CFM solutions (equivalent ZIF-8 concentration: 200 μg/mL) were added to the above solution and reacted for 30 min. Every 5 min, the reaction solution was centrifuged at 10000 rpm to precipitate FM, CM and CFM, and UV−vis spectroscopy was applied to detect the absorbance of the supernatant.

ATP level assessment in vitro

To estimate intracellular ATP level after different treatments, PCCs were cultured in a 12-well plate (seeding density: 1×10^5 cells per well) for 8 h. Subsequently, the cells were treated by FM, CM and CFM (equivalent ZIF-8 concentration: 200 μg/mL) for 10 h. After treatments, the cells were lysed and the supernatant was centrifugally collected. Finally, intracellular ATP level was measured by a standard ATP assay kit.

Western blot analysis

Western blotting was performed using standard methods. After being treated with FM, CM, CFM (equivalent ZIF-8 concentration: 200 μg/mL) for 2h. Cell total proteins were extracted 2 hours after radiation and run for western blot. Briefly, cells were collected and lysed in RIPA lysis buffer (Thermo Scientific, Rockford, IL, USA) supplemented with a protease inhibitor cocktail (Roche, Indianapolis, IN, USA) according to the protocol by the manufacturer. Total proteins were extracted and loaded in the wells of 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After the seperation by gel electrophoresis, the proteins were transferred to PVDF membranes (Millipore, Billerica, MA, USA). The membrane was probed with primary antibodies

after being blocked by 5% non-fat milk (Bio-Rad) overnight at 4 °C and washed 3 times with TBS-T buffer, incubated with HRP-conjugated secondary antibody at 1:20000 dilutionsin TBS-T buffer. After washing 3 times with TBS-T buffer, the membrane was developed with ECL substrate (Thermo Scientific) and the signal was detected by a BIO-RAD Fluorescent Imager, following quantification by Image Lab software. The following antibodies were used in this study, all diluted in in TBS-T buffer. Antibodies directed against Cleaved Caspase-3 (ab32042), Cytochrome C (ab13575), Bax (ab32503), Bcl-2 (ab32124) were obtained from Abcam (1:1 000 dilution), β-actin were obtained from Invitrogen (1:5 000 dilution).

PA imaging and immunohistochemistry

The tumors were intratumorally injected with PBS CM or CFM (ZIF-8 concentration 10 mg/kg). 12h later, the PA images of blood saturation were collected. Then the tumors were i.v. injection with pimonidazole hydrochloride (60 mg/kg) (Hypoxyprobe-1 plus kit, Hypoxyprobe Inc) and then surgically excised. Human anti-pimonidazole monoclonal antibody conjugated to FITC (FITC-Mab1) binds to these adducts, allowing their detection by immunochemical means. For immunofluorescence staining, frozen sections of the tumors were prepared with optimum cutting temperature (OCT) compound (Sakura Finetek). For detection of pimonidazole, sections were incubated with FITC-conjugated human anti-pimonidazole antibody (dilution 1:200, Hypoxyprobe Inc.) and Alex 488-conjugated goat anti-human secondary antibody (dilution 1:200, Jackson Inc.), subsequently, following vendors' instructions. Cell nuclei were stained with DAPI (dilution 1:5000, Invitrogen). The stained tumor sections were imaged under the confocal fluorescence microscopy (Leica SP5). Image intensity quantification of tumor hypoxia was performed with ImageJ software. The hypoxic positive area (%) = visible hypoxic marker in tumor tissue section / total area (n = 3/group).[3]

***In vivo* antitumor study**

When tumor size of PDX mice reached approximately 100 mm^3, the mice were divided randomly into 6 groups (each group included 5 mice): (1) PBS; (2) RT; (3) CFM; (4) FM+RT; (5) CM+RT; and (6) CFM+RT. (equivalent ZIF-8 concentration 10 mg/kg).The X-Ray dose in group 2, 4, 5 and 6 was 4Gy and radiotherapy was performed 12h after intravenous injection. The treatment was conducted every 2 days for 14 days. Mice body weight and tumor volume in all groups were monitored every 2 days. A caliper was employed to measure the tumor length and tumor width and the tumor volume was calculated according to following formula. Tumor volume = tumor length × tumor width 2 / 2. After 14 days treatment, all the mice were sacrificed. The blood samples from these mice (≈1 mL) were collected for blood biochemistry analysis. Five main organs (heart, liver, spleen, lung and kidney) and tumors of all mice were harvested, washed with PBS, and fixed with paraformaldehyde for histology analysis. And the tumor tissues were weighed, and fixed in 4% neutral buffered formalin, processed routinely into paraffin, and sectioned at 4 μm. Then the sections were stained with hematoxylin and eosin (H&E) or ki-67 and finally examined by using an optical microscope (BX51, Olympus, Japan).

Statistical analysis

Data analyses were conducted using the GraphPad Prism 5.0 software. Significance between every two groups was calculated by the Student's t-test. *P < 0.01, **P < 0.005, ***P < 0.001.

Figures

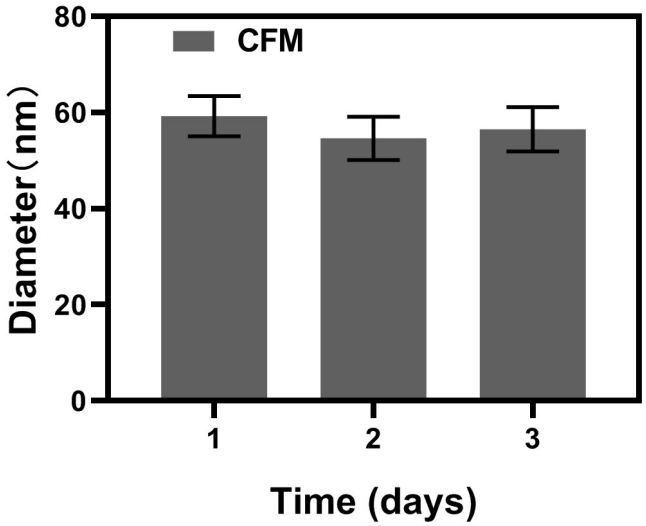

Figure S1. DLS was used to measure the hydrodynamic diameter of CFM.

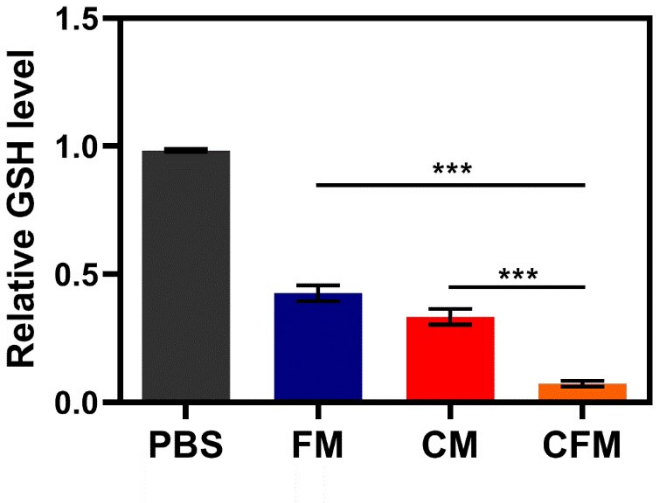

Figure S2. Effects of CFM on the level of intracellular GSH were determined by GSH Assay Kit (n=5).

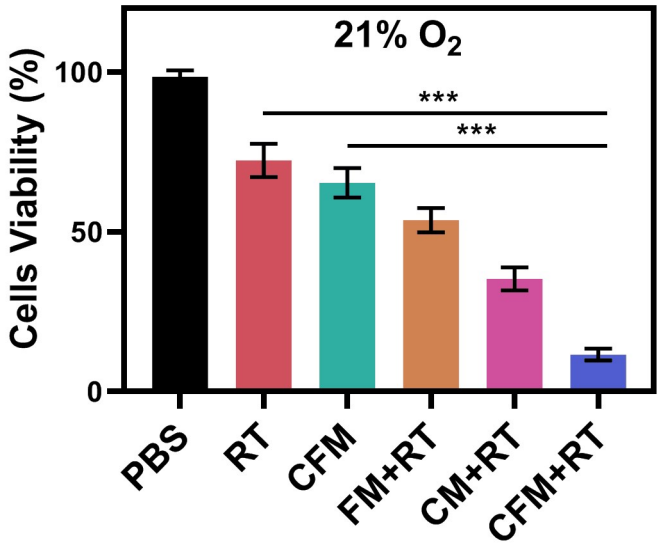

Figure S3. The survival of 4T1 cells with Control, RT, CFM, FM+RT, CM+RT, and CFM+RT detected by MTT assay under 21% O_2 condition (n = 5).

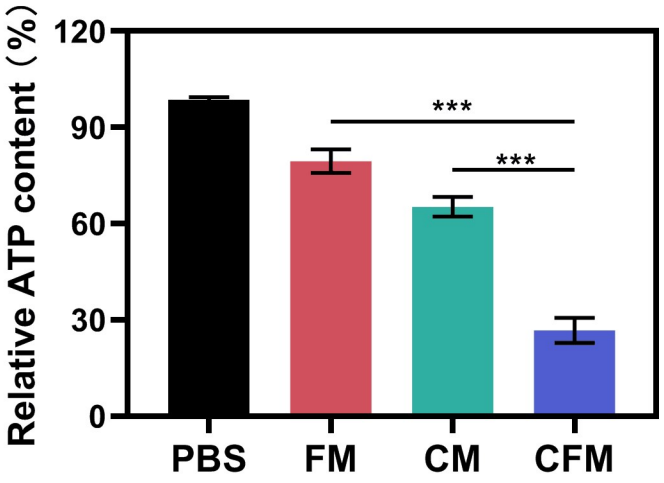

Figure S4. Relative ATP content in the cells after 10 h co-culture with FM or CM or CFM (n = 5).

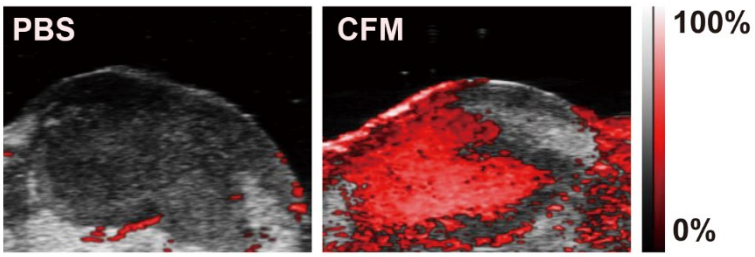

Figure S5. *In vivo* PA images of tumorous sites of mice after different treatment.

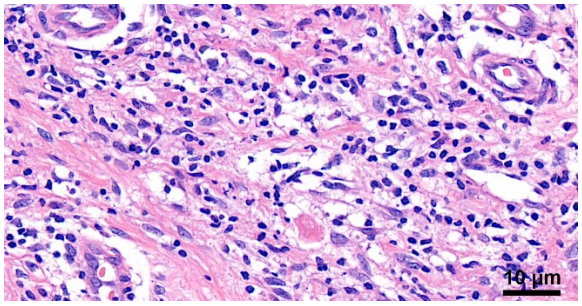

Figure S6. H&E staining on patient-derived sample.

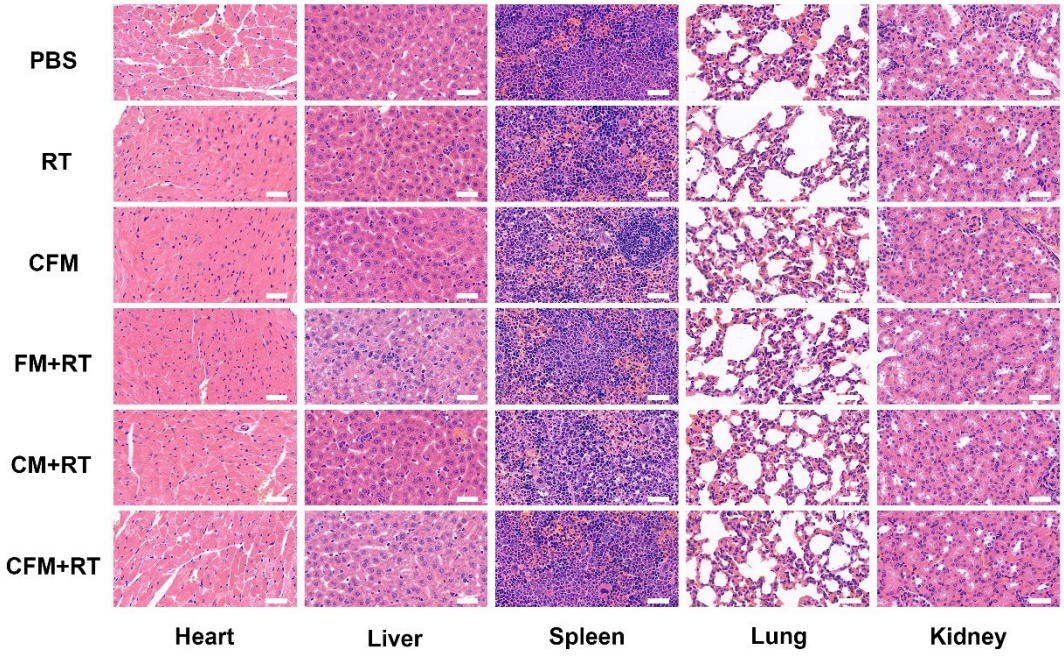

Figure S7. Histopathologic examination of the tissues including heart, liver, spleen, lung, and kidney from BALB/c nude mice after different treatment. Scale bars = 100 μm.

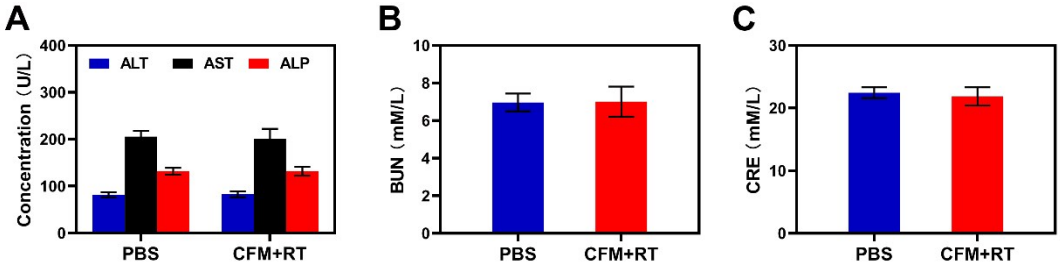

Figure S8. Blood biochemistry data including kidney function markers: (A) liver function markers: ALT, ALP, and AST (B) BUN and (C) CRE, after different treatment.

References

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