Supporting Information

Cationizable Transcytosis Manganese Nano-Oxygenator for Enhanced Chemo-Dynamic Immunotherapy in Deep Tumour Tissue

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Materials

Manganese chloride ($MnCl_2$), Na_2HPO_4, cyclohexane, igepal-co-520, perfluorosulfonic acid (PFSA), ethyl alcohol, chloroform, were purchased from Sigma-Aldrich (Saint Louis, MO). DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine, 18:0 PC), cholesterol, DMG-PEG_{2000} (1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000), D-Lin-MC3-DMA and DOPA (1,2-dioleoyl-sn-glycero-3-phosphate (sodium salt)) were purchased from Avanti Polar Lipids (Birmingham, AL). Water was purified by using a Milli-Q purification system (Millipore, Bedford, MA). Other chemicals or solvents without mention were analytical grade and used as received. Antibodies against cell surface markers for flow cytometry assay were obtained from Biolegend (San Diego, CA). Calcein/PI cell viability/cytotoxicity assay kit was obtained from Beyotime Biotechnology (Shanghai, China).

Characterizations

Mn content was determined on an X Series 2 inductively coupled plasma mass spectroscopy (ICP-OES, Thermo Fisher Scientific, Waltham, MA). Size and zeta potential of nanoparticles measurements were detected using a zeta potential analyzer with dynamic light-scattering (DLS, Malvern Instruments Ltd., UK). Morphology of nanoparticles was examined by JEOL-2010 transmission electron microscopy (TEM, JEOL Co. Ltd., Tokyo, Japan) at an accelerating voltage of 120 kV.

Methods

Preparation and content determination of MnP nanoparticles

The lipid-coated MnP nanoparticles (NPs) were synthesized using a water-in-oil reverse microemulsion method. The lipid composition included DSPC, cholesterol, D-Lin-MC3-DMA, and DMG-PEG_{2000}. The inner layers of the liposome membrane were formed from DOPA and PFSA. In brief, 600 µL of $MnCl_2$ (1.25 M, pH 7.2) was added to 25 mL of mixed oil phase 1 (cyclohexane: Igepal CO-520 = 75:25) in a 100 mL flask, and the solution was stirred at 1000 r.p.m. for 5 minutes to generate a well-dispersed microemulsion phase. For oil phase 2, 100 µL of DOPA lipid (20 mM) and 120 µL of PFSA (20 mM) were added to 25 mL of the same mixed oil phase. A separate microemulsion containing sodium phosphate was prepared by adding 600 µL of Na_2HPO_4 (50 mM, pH 9.0) to 25 mL of mixed oil phase 2, maintaining a calcium (Mn) to phosphate (P) molar ratio of 50:1. The MnP core with a single layer of DOPA and PFSA was formed by mixing oil phase 1 and oil phase 2, followed by stirring at 1000 r.p.m. for 30 minutes.

To collect the lipid-coated MnP cores, 25 mL of ethanol was added to the mixture, which was then centrifuged at 12,000 × g for 15 minutes, followed by three ethanol washes. The collected MnP cores were resuspended in 4 mL of chloroform and centrifuged at 1000 × g for 3 minutes to remove MnP precipitates that lacked lipid coatings. To prepare $MnP^{O_2/MC3}$， MnP with inner layer lipid that resuspending in chloroform was added with 500 uL lipid cocktail (MC3: DSPC: Cholesterol, DMG-PEG_{2000} = 4:4:1:1). After dry chloroform with vacuum, the film was hydrated and sonicated for 5 min at 40 ℃. To determine the Mn content in the nanoparticles, $MnP^{O_2/MC3}$ were dissolved in 1M HNO_3 and determined by ICP-OES.

Oxygen Entrapment and Release and Mn^{2+} release.

Oxygen was loaded into MnP^{MC3} following the method described in the literature. Briefly, MnP^{MC3} was placed in a clean oxygen chamber, and pure O_2 was introduced at a flow rate of 5 L/min for 20 minutes, resulting in oxygen-saturated $MnP^{O_2/MC3}$. To measure oxygen release, a dissolved oxygen meter (Rex, JPF-605B, China) was inserted into 8 mL of deoxygenated water, which was prepared by flowing N_2 for 30 minutes in a hypoxic chamber. Then, 2 mL of the $MnP^{O_2/MC3}$ solution was added, and oxygen concentrations were recorded at specified time (n = 3). The oxygen loading capacity per 1 mL of $MnP^{O_2/MC3}$ was calculated using the following formula. Mn^{2+} release experiment was performed by dialysis against PB buffer with different pH (pH 7.4, pH 6.5, pH 5.0). At determined time points, buffer samples outside dialysis bag were collected for ICP-OES analysis.

Cell culture.

Murine breast cancer 4T1 and luciferase expressed 4T1 cell line were obtained from American Type Culture Collection (ATCC) and cultured in a humidified atmosphere at 37 °C with 5% CO_2. The cells were cultured in DMEM culture medium (Gibco®, Thermo Fisher Scientific, Grand Island, NY), supplemented with 10% fetal bovine serum (FBS, HyClone, Logan, UT) and 1% penicillin/streptomycin (Gibco®, Life Technologies, Grand Island, NY).

***In vitro* ROS production assay of $MnP^{O_2/MC3}$.**

To evaluate the ROS production performance of $MnP^{O_2/MC3}$ at normaxia and hypoxia, 4T1 cells were seeded on 24-well plates with a density of 5 × 10^4 cells per well. The cells were further incubated with $MnP^{O_2/MC3}$ in normoxia and hypoxia (1% O_2, 5% CO_2, 94% N_2) for 10h. After

incubating with DCFH-DA (20 μM) for another 60 min and washed with PBS to remove the free DCFH-DA. The cells were fixed with 4% formaldehyde at room temperature for 15 min and washed for further observation by CLSM.

***In vitro* cytotoxicity assay of $MnP^{O_2/MC3}$ evaluated by dead and live staining.**

To evaluate the photothermal cytotoxicity of $MnP^{O_2/MC3}$ on 4T1 cells, 4T1 cells were seeded on 24-well plates with a density of 5×10^4 cells per well. After incubating with $MnP^{O_2/MC3}$ in normoxia and hypoxia (5% O_2, 5% CO_2, 90% N_2) for 12 h. The cells were washed and incubated with medium for another 8 h and further be stained by Calcein-AM/PI after 4 h (the dilution ratio of Calcein-AM/PI is 1:500).

Colocalization of rab11 and $MnP^{O_2/MC3}$.

The 1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindodicarbocyanine (DiD) dye labelled $MnP^{O_2/MC3}$ were prepared by adding DiD dye into the second layer process in chloroform (2 mg/mL, same volume with cholesterol). 4T1 cells were seeded on coverslips in a 24-well plate overnight (5×10^4 cells per well). Cells were then incubated with fresh medium containing $MnP^{O_2/MC3}$ at 37 °C. After incubation for 4 h, the cells were fixed with 4% formaldehyde at room temperature for 15 min and washed for 3 times. Cells were stained with rab11 rabbit monoclonal antibody (dilution 1:100) (Cell Signaling Technology, MA, USA) at 4 °C for 12 h and followed with goat anti-rabbit FITC conjugated IgG-HRP (Beyotime Biotechnology, Jiangsu, China) for 90 min incubation to visualize the rab11. Cell nuclei were stained with DAPI.

4T1 multicellular spheroids.

5 mL 2% hot agarose solution was coated T25 flask and cooled to room temperature naturally. 4T1 cells (1×10^6) were added to the flask and incubated with 10 mL DMEM medium for 5-7 days to grow into spheroids. The spheroids were transferred to Cellvis 35 mm Glass bottom dish with 20 mm micro-well and incubated at pH 6.7 DMEM medium. Meanwhile, the spheroids were incubated with $MnP^{MC3/DiD}$ and $MnP^{DSPC/DiD}$ (100 μg/mL) for 8 h. After collecting and washing with PBS for 3 times. The spheroids were observed with CLSM Z stack method with 640 nm wavelength channels.

Tumoral Hypoxia Evaluation after $MnP^{O_2/MC3}$ treatment

For hypoxia evaluation, mice bearing 4T1 tumors were intravenously injected with $MnP^{O_2/MC3}$

(20 mg/kg). At predetermined time points (3, 11, and 23 hours), the mice were injected intravenously with pimonidazole hydrochloride (60 mg/kg). One hour post-injection, the mice were sacrificed, and the tumors were excised for frozen sectioning. These tumor sections were then stained with FITC-labeled pimonidazole antibody following the manufacturer's standard protocol. Immunofluorescence images of the tumor sections were captured using the Slideview VS200 automated scanning system with a 20× objective. To assess hypoxia, CaseView software was employed to analyze the intensity of FITC and DAPI staining. Additionally, ImageJ was used to measure the gray values of the FITC signal, ranging from a minimum of 0 to a maximum of 250, to quantify the average optical density.

Tumoral ROS Evaluation after $MnP^{O_2/MC3}$ treatment

For hypoxia evaluation, mice bearing 4T1 tumors were intravenously injected with $MnP^{O_2/MC3}$ (20 mg/kg). Six hours later, the mice were administered DCFH-DA (50 mg/kg) via intravenous injection. After an additional 18 hours, the mice were sacrificed, and the tumors were excised for frozen sectioning. ROS fluorescence (Ex: 488 nm, Em: 520 nm) was observed using the Slideview VS200 system with a 20× objective. ImageJ was then used to measure the gray values of the FITC signal, ranging from a minimum of 0 to a maximum of 250, to quantify the average optical density.

Detection of calreticulin (CALR)

4T1 cells (5×10^4) were seeded in a 24-well plate on coverslips overnight for 12 h, and then incubated with $MnP^{O_2/MC3}$ for 12 h. CRT exposure on the surface of 4T1 cells after different treatments were detected by flow cytometry using FITC conjugated antibody to CALR (1:50, Bioss, Beijing, China).

Detection of high mobility group protein 1 (HMGB1)

4T1 cells (5×10^4) were seeded in a 24-well plate on coverslips overnight and incubated for 12 h, and then treated with $MnP^{O_2/MC3}$ for 24 h. Cells were incubated overnight at 4°C with primary antibodies against HMGB1 (Beyotime Biotechnology, China). After incubation, the cells were washed three times with PBS, followed by labeling of HMGB1 with Alexa Fluor 647-labeled goat anti-mouse IgG (Santa Cruz Biotechnology, TX, USA), actin with phalloidin-FITC (Beyotime Biotechnology, China) and cell nuclei with DAPI. Coverslips were mounted onto glass microscope slides using anti-fade mounting media, and the samples were then examined

using confocal laser scanning microscopy (CLSM).

Detection of ICD *in vivo*

Tumors were excised after 12 h post i.v. injection of $MnP^{O_2/MC3}$. Tumor tissue sections were incubated with individual primary antibodies against CALR (Cell Signaling Technology, MA) for 12 h at 4 ºC and labeled by goat anti-rabbit FITC conjugated IgG (1:500, Santa Cruz Biotechnology, Dallas, TX). For HMGB1 staining, tumors were excised after 24 h post irradiation. Tumor tissue sections were incubated with primary antibodies against HMGB1 (1:200, Beyotime Biotechnology, Shanghai, China) and subsequently labeled with goat anti-rabbit Cy5-conjugated IgG (1:500, Santa Cruz Biotechnology, Dallas, TX). After labeling the cell nuclei with DAPI, the tissues were examined using confocal.

Tumor-bearing animal models

All experiments in live animals are performed according to the relevant laws and institutional guidelines, and approved by animal ethics committee of YunFu People's Hospital.. Balb/C mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. To evaluate $MnP^{O_2/MC3}$ penetration, hypoxia evaluation, ROS detection and tumor suppression experiments, 5×10^5 4T1 cells are injected into subcutaneous right flank in female Balb/c mice.

Tumor penetration of $MnP^{O_2/MC3}$and tumor vessels perfusion

For tumor tissue sections observation, 4T1 tumor bearing nude mice were i.v injected with $MnP^{O_2/MC3/DiD}$ (equivalent dose of 20 mg/kg body weight) and were sacrificed after 12 h for frozen sections. The tumor tissues were labeled with CD-31-FITC mab for tumor vessel visualization by CLSM.

In vivo antitumor efficacy of HTP

4T1 tumor-bearing mice are divided into for four groups (PBS, MC3 liposome, MnP^{MC3}, $MnP^{O_2/MC3}$). When the tumor volume reached 80-100 mm^3, mice were i.v. adminstered with different formulations, respectively (n = 5), every two days for 3 times. Tumor volume and mice weight were monitored every two days after in the next 12 days. The tumor volume was

monitored using calipers and calculated according to the reported equation: Volume = (Tumor Length) × (Tumor Width)^2×0.5.

Immunohistochemical and Immunofluorescence Analyses

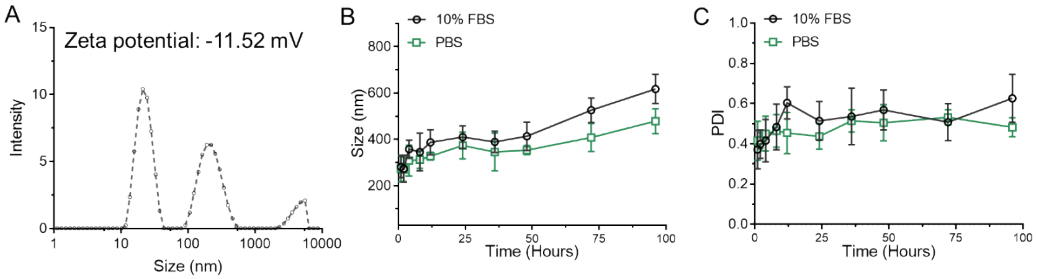
After 12 days of treatment, the tumor tissues and main organs excised from sacrificed mice were fixed in 4% formaldehyde for over 24 h and embedded in paraffin. The tumor tissues slices were further stained by H&E, anti Ki67 mAb (Beyotime, 1:200), anti terminal deoxynucleotidyl transferase dUTP Nick-End Labeling mAb (Beyotime, 1:200), anti Foxp3 mAb (CST, 1:100) and anti CD8 mAb (CST, 1:100) for further visualized analysis.

Flow cytometry assay of cell surface markers

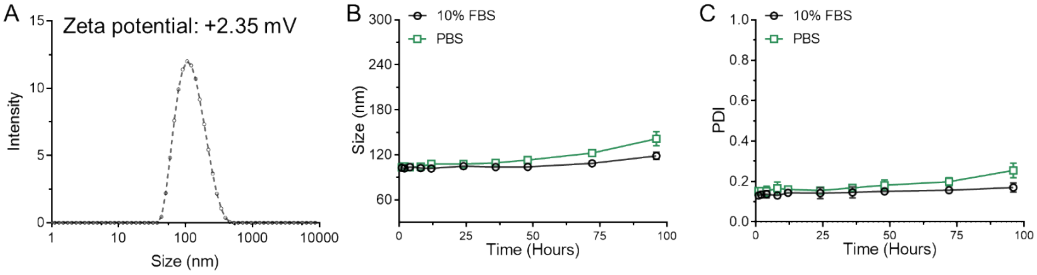
After 12 days from first tumor treatment, the mice were sacrificed and spleen and lymph node were excised. The lymph node and spleen were gently meshed though nylon mesh to obtain single-cell analysis. Cells were stained for 30 min at 4°C with cocktails containing combinations of fluorochrome conjugated monoclonal antibodies for cell surface markers (FITC anti-mouse CD45.2 (1:200, Biolegend), APC-Cy7 anti-mouse CD3 (1:200, Biolegend), APC anti-mouse CD4 (1:200, Biolegend), PE-Cy7 anti-mouse CD8b (1:200, Biolegend), PE anti-mouse CD69 (1:200, Biolegend).

Statistical analysis

The statistical significance of treatment outcomes was assessed using Graphpad prism 7 software (GraphPad, San Diego, CA) with Student's t-test (two-tailed) method. Significant difference among group were assigned as *p<0.05, **p<0.01and ***p<0.001, respectively. *p<0.05 was considered statistically significant in all analyses (95% confidence level).



Revised Figure S1. (A). Size distribution of MnP with single layer lipid coating (DOPA). Time dependent size (B) and PDI (C) development in PBS and 10% FBS.



Revised Figure S2. (A). Size distribution of MnP^{MC3} with double layer lipid coating (1st lipid layer: DOPA, 2nd lipid layer: MC3, Cholesterol, DMG-PEG2000, DSPC). Time dependent size (B) and PDI (C) development in PBS and 10% FBS.

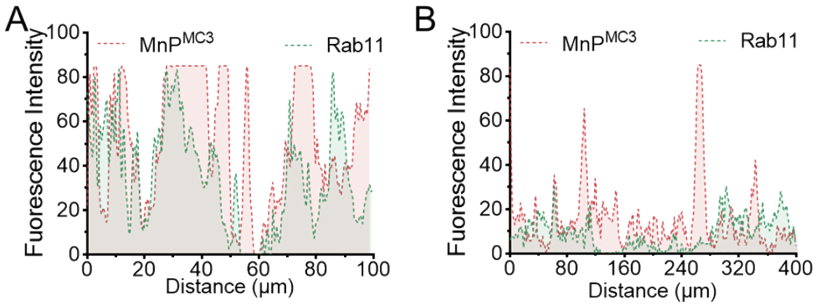


Figure S3. MFI distribution of MnP^{MC3} (A) and MnP^{DSPC} (B) along the dash line in Figure 2J.

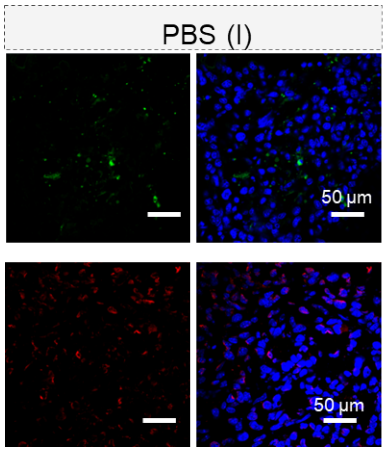


Figure S4. CLSM images of tumor tissues with CALR and HMGB1 staining without treatment as a control related with Figure 3H and 3I.

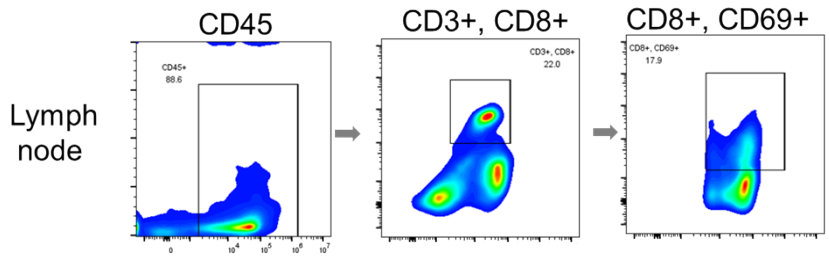


Figure S5. Gating strategy for flow cytometry of total T cells (viable CD45+, CD3+, CD8+ cells) from lymph node single cell suspension.

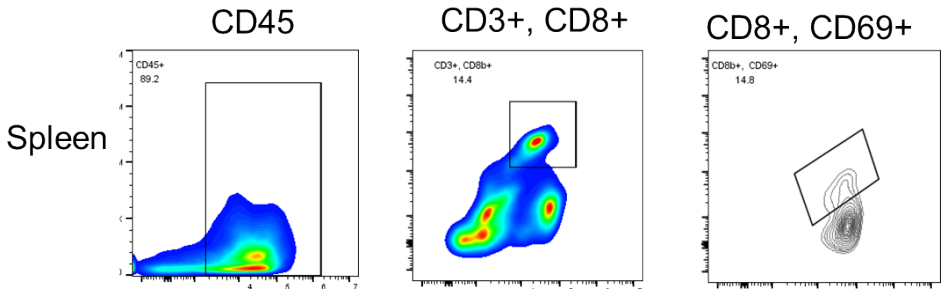


Figure S6. Gating strategy for flow cytometry of total T cells (viable CD45+, CD3+, CD8+ cells) from spleen single cell suspension.