Supplementary Information (SI) for Nanoscale.
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Supporting Information

Enhancing Tumor ROS Generation via Nanozyme-Amplified Photodynamic Therapy with Oxygen-Supplying Bacterial OMVs

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Materials and Methods

Reagents and Chemicals

RPMI 1640 medium and fetal bovine serum (FBS) were purchased from Gibco Life Technologies (Switzerland). Trypsin-EDTA and penicillin-streptomycin were obtained from Corning Life Sciences (China). The Micro-BCA protein assay kit and Annexin V-FITC Apoptosis Detection Kit were from Thermo Fisher Scientific (USA). 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA), methylene blue (MB), and the TUNEL Apoptosis Detection Kit were acquired from Beijing Solarbio Science & Technology Co., Ltd. (China). Hoechst 33342 was obtained from Thermo Fisher Scientific (USA). Fluorescent antibodies (FITC-CD11c, APC-CD80, PE-CD86) were from BioLegend (USA). Potassium permanganate, hydrogen peroxide (H_2O_2), and other analytical-grade chemical reagents were supplied by Aladdin Biochemical Technology (China). *Escherichia coli* BL21 was obtained from the Shanghai Bioresource Collection Center (SHBCC, China). Murine breast cancer 4T1 cell line was provided by Wuhan Pricella Biotechnology Co., Ltd. (China).

Cell Lines and Animals

4T1 cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 1% penicillin, and 1% streptomycin at 37 °C in a humidified incubator containing 5% CO_2. Female BALB/c mice (6-8 weeks old) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (China). All animals were housed under controlled conditions (23 ± 1°C, 55 ± 5% humidity, 12h light/dark cycle).

Preparation of OMVs

Bacterial culture: *Escherichia coli* BL21 was reconstituted from lyophilized stock in sterile saline and streaked on LB agar. After overnight incubation at 37°C, single colonies were inoculated into 5 mL of LB broth and cultured at 37°C, 200 rpm, for 6h. The culture was expanded into fresh LB broth (1% v/v inoculum) until an optical density at 600 nm (OD_{600}) of 0.8-1.2 was reached.

OMVs isolation: cells were removed by centrifugation (8,000 × g, 20 min, 4°C), and the supernatant was filtered through a 0.45 μm membrane. The filtration was concentrated via ultrafiltration, followed by ultracentrifugation (150,000 × g, 3 h, 4°C). The resulting OMVs were resuspended in sterile saline, filtered, and quantified using a BCA protein assay.

Synthesis of OMV@Hb-Ce6-Mn

OMVs and hemoglobin (Hb) were separately treated with tris (2-carboxyethyl) phosphine (TCEP) and glutathione at 37 °C for 30 min. The two mixtures were then combined at a 1: 2 mass ratios (OMVs:Hb), followed by addition of a Ce6 solution in dimethyl sulfoxide (DMSO, pH 7.4). The mixture was stirred and incubated at 37 °C for 2 h. Subsequently, potassium permanganate (100 mmol) was added and stirred for 2 min, followed by sodium hydroxide (1 mmol). The reaction continued at room temperature for 30 min. The resulting product was purified by centrifugal

ultrafiltration (100 kDa MWCO, 12,000 × g, 15 min) to yield OMV@Hb-Ce6-Mn. Final nanoparticles were dispersed in PBS and purged with oxygen (O_2) for 15 min prior to use.

Characterization of OMV@Hb-Ce6-Mn

Morphology and size: samples (0.1 mg/mL) were fixed with 2% (v/v) glutaraldehyde, stained with 2% (w/v) uranyl acetate, and imaged by transmission electron microscopy (TEM, JEM-1200EX, JEOL, Japan). Hydrodynamic diameter and zeta potential were measured using a Zetasizer Nano (ZS90, MalvernPanalytical, UK). Elemental mapping analysis was conducted using scanning electron microscopy with energy-dispersive X-ray spectroscopy (SEM-EDS).

Mn ions release detection: OMV@Hb-Ce6-Mn was incubated in PBS (pH 6.5) with or without 200 μmol/L H_2O_2. After centrifugal ultrafiltration (10 kDa MWCO, 16,000 × g, 30 min), Mn ions in the filtrate were measured by inductively coupled plasma optical emission spectroscopy.

For O_2 detection, samples were sealed with liquid paraffin and purged with N_2 to establish an O_2-free environment. Then, the samples were added to deoxygenated PBS containing 200 μmol/L H_2O_2. Dissolved O_2 concentration was measured using a portable oxygen meter.

ROS detection: hydroxyl radical (·OH) and singlet oxygen ($^1O_2$) generation were verified using electron spin resonance (ESR) spectroscopy (E500, Bruker, Germany). The catalytic activity toward ·OH generation was assessed by monitoring methylene blue (MB) degradation at 664 nm in PBS containing MB (10 μg/mL) and H_2O_2 (200 μmol/L).

To assess the cytotoxicity of OMV@Hb-Ce6-Mn, normal L929 fibroblasts, normal mammary epithelial cells (MCF-10A), and 4T1 breast cancer cells were seeded into 96-well plates at a density of 10^4 cells per well and incubated overnight. The old medium was then replaced with fresh medium containing varying concentrations of OMV@Hb-Ce6-Mn. After an additional 24 hours of culture, cell viability was evaluated using the CCK-8 assay.

***In Vitro* Experiments**

4T1 cells were treated with PBS, OMV@Hb, OMV@Hb-Ce6, OMV@Hb-Mn, or OMV@Hb-Ce6-Mn in the presence of 100 μmol/L H_2O_2 for 12 h at 37 °C. Cells were then exposed to 660 nm laser irradiation (100 mW/cm²). Subsequently, cells were incubated with 10 mmol/L DCFH-DA for 30 min and analyzed for intracellular ROS using confocal laser scanning microscopy (CLSM, Eclipse Ti2, Nikon, Japan) and flow cytometry (CytoFLEX, Beckman Coulter, USA). Apoptosis was evaluated by Annexin V-FITC staining and flow cytometry.

***In Vivo* tumor Accumulation and Therapeutic Efficacy of OMV@Hb-Ce6-Mn**

BALB/c mice were subcutaneously injected with 4T1 cells. On day 14, tumor-bearing mice were randomly divided into two groups and injected with either OMVs or OMV@Hb-Ce6-Mn (OMV: 0.5 mg/kg). On day 15, Cy7-labeled formulations were injected, and biodistribution was monitored over 72 h using an IVIS imaging system (PerkinElmer, USA). In the *ex vivo* imaging experiments, the mice were sacrificed at 12 h post-injection. The major organs (heart, liver, spleen, lung, and kidney) and tumors were collected and imaged immediately. For histological evaluation, 4T1 tumor-bearing mice were injection with OMV@Hb-Ce6-Mn. Dissected major organs and tumors were obtained at 12 h and frozen in OCT tissue. After sectioned into slices and stained with Hoechst 33342, the fluorescence images of all sections were acquired on automatic multispectral imaging system. The fluorescence signals originate from Ce6 inside the formed OMV@Hb-Ce6-Mn.

For therapeutic studies, mice were assigned to five groups (n = 6 per group) and injected with PBS, OMV@Hb, OMV@Hb-Ce6, OMV@Hb-Mn, or OMV@Hb-Ce6-Mn on days 8, 11, 14, 17

and 20 (OMV: 0.5 mg/kg). Tumors were irradiated at 12 h post injection. Tumor volumes were measured every 2 days using the formula: $V = ½ \times L \times W^2$.

Three days after final treatment, tumors were excised for further analysis. Single-cell suspensions were prepared and stained with FITC-CD11c, APC-CD80, and PE-CD86 antibodies to assess dendritic cell maturation by flow cytometry. Tumors were also processed for histological evaluation, including TUNEL staining (10 μm sections) and H&E staining (4 μm sections).

Biosafety Assessment

Healthy BALB/c mice were intravenously injected with PBS or OMV@Hb-Ce6-Mn. Blood samples were collected for biochemical analysis of alanine aminotransferase (ALT), aspartate transaminase (AST), blood urea nitrogen (BUN), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH) using a TBA-40 biochemical analyzer (Toshiba, Japan).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 8.0.1. Two-tailed unpaired Student's *t*-tests were used for comparisons between two groups. One-way ANOVA followed by Tukey's post hoc test was used for multiple comparisons. Statistical significance was defined as: $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***), and $P < 0.0001$ (****), and $P > 0.05$ was considered not significant (ns).

Supporting Figures

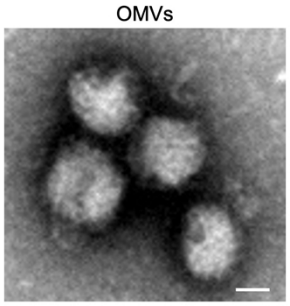

Fig. S1 TEM image of OMVs. Scale bar: 50 nm.

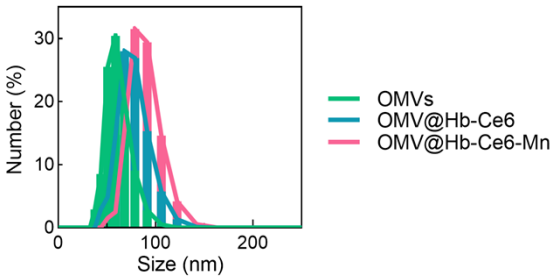

Fig. S2 Size distribution of OMVs, OMV@Hb-Ce6, and OMV@Hb-Ce6-Mn.

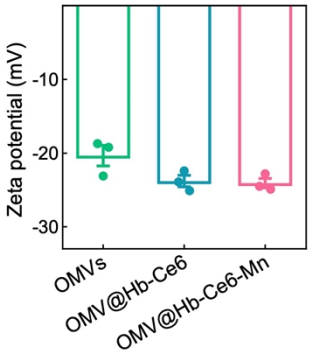

Fig. S3 Zeta potentials of OMVs, OMV@Hb-Ce6, and OMV@Hb-Ce6-Mn. Data are presented as the mean ± SEM (n = 3).

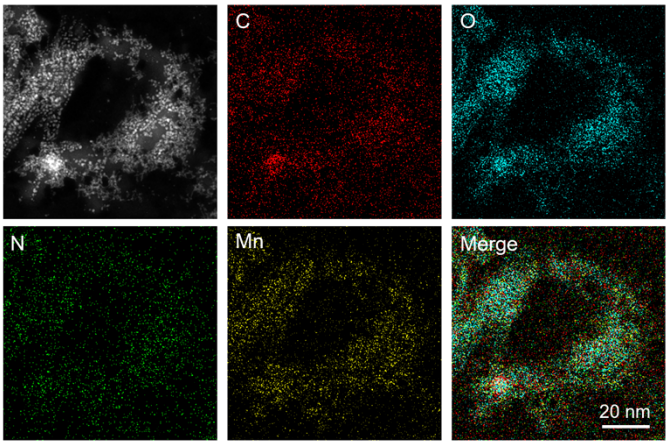

Fig. S4. Element mapping presentation of OMV@Hb-Ce6-Mn.

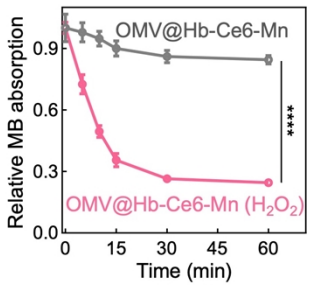

Fig. S5 The relative absorption intensity of MB incubated with OMV@Hb-Ce6-Mn. Data are presented as the mean ± SEM ($n = 3$). Statistical significance was assessed using two-tailed unpaired Student's *t*-test. ****$p < 0.0001$.

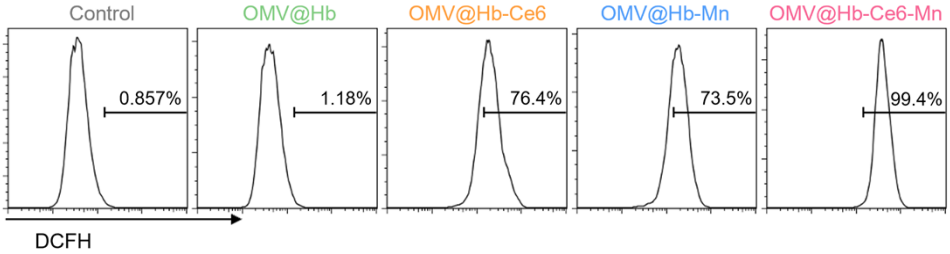

Fig. S6 Flow analysis of ROS production in 4T1 cells under different treatments.

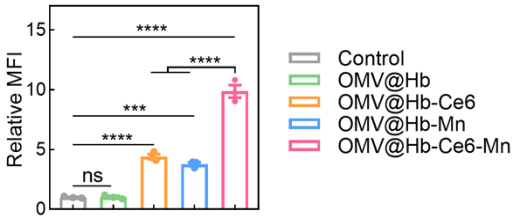

Fig. S7 Relative MFI of CLSM images for ROS production in 4T1 cells under different treatments ($n = 3$). ***$p < 0.001$, ****$p < 0.0001$, *ns*: not significant.

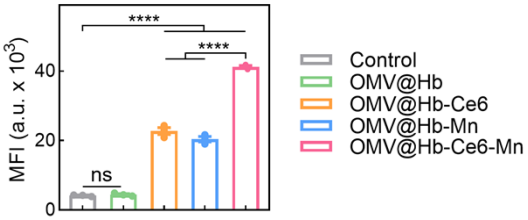

Fig. S8 Flow cytometric quantification of ROS in 4T1 cells under various treatments ($n = 3$). ****$p < 0.0001$, *ns*: not significant.

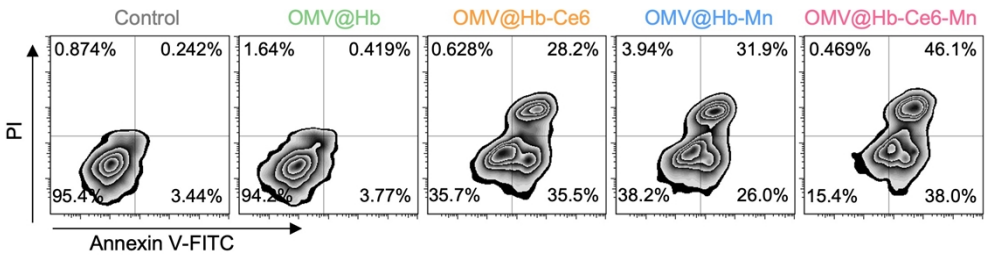

Fig. S9 Representative flow cytometry analysis of 4T1 cell apoptosis under various treatments.

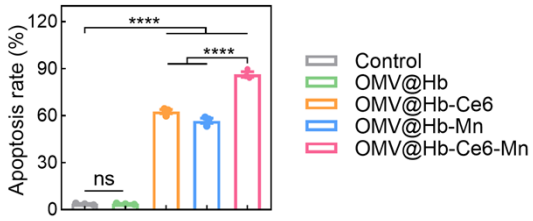

Fig. S10 Quantification of 4T1 apoptosis induced by different treatments ($n = 3$). ****$p < 0.0001$, *ns*: not significant.

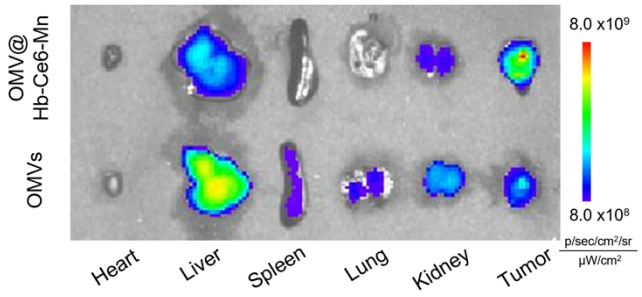

Fig. S11 Representative *ex vivo* images of tumors and other organs at 12 h after different treatments.

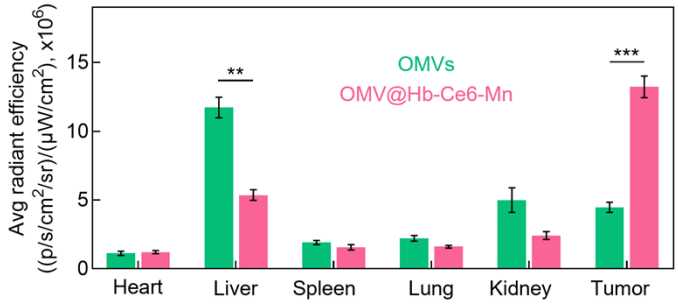

Fig. S12 Biodistribution of OMVs and OMV@Hb-Ce6-Mn in tumors and organs at 12 h post-injection ($n = 3$). **$p < 0.01$, ***$p < 0.001$.

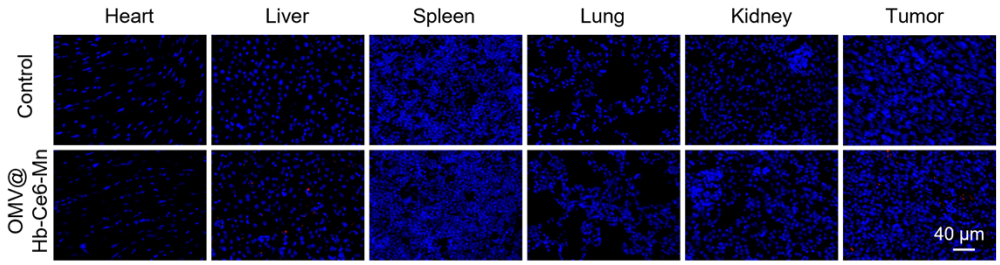

Fig. S13 Representative CLSM images of OMV@Hb-Ce6-Mn in tumors and organs (Red: Ce6 signals originate from the formed OMV@Hb-Ce6-Mn, blue: nuclei).

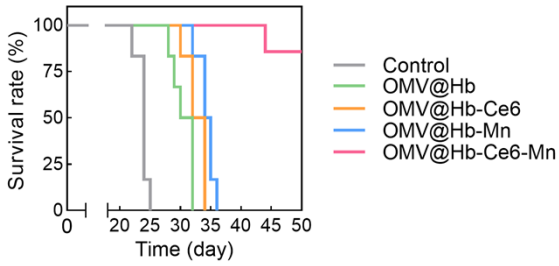

Fig. S14 Survival curves for mice under different treatments.

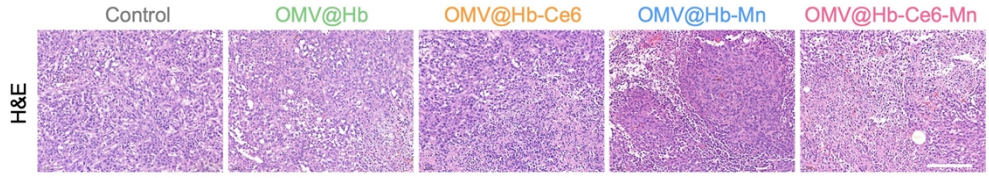

Fig. S15 H&E staining of tumor tissue. Scale bar: 100 μm.

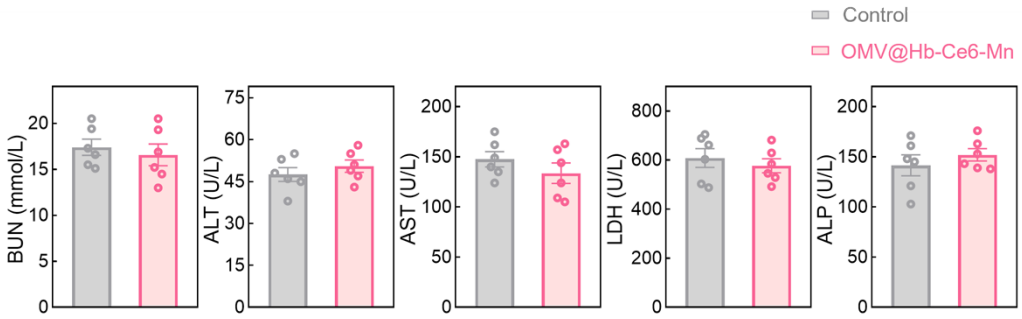

Fig. S16 Serum biochemical profiles following OMV@Hb-Ce6-Mn injection at day 28.

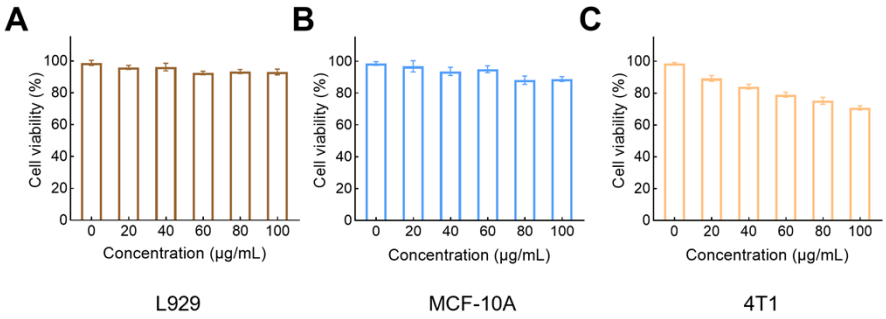

Fig. S17 The cell viability of L929(A), MCF-10A(B) and 4T1(C) cells treated with OMV@Hb-Ce6-Mn. Data are presented as the mean ± SEM (n = 3).