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Supporting Information

Oxygen-defect Zinc oxide nanoparticles as highly efficient and safe sonosensitizers for cancer therapy

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Experimental section

Sample preparation

ZnO precursor was synthesized by a simple precipitation method. 9.5 g $Zn(NO_3)_2 \cdot 6H_2O$, 2 g NaOH and 500 mL deionized water were added into a 1 L beaker in order, kept stirring for 2 h, standing still for 12 h. Then the precipitate was separated by centrifuge and dried at 60 °C for 12 h. The as-obtained ZnO precursor was divided into two parts and annealed in air and N_2 respectively, from room temperature to 500 °C at a rate of 5 °C min^{-1}, then cooled down to room temperature naturally. The one annealed in air was noted as ZnO, and the other one in N_2 was noted as M-ZnO. m-PEG modified M-ZnO or ZnO is synthesized by mixing the M-ZnO or ZnO samples and m-PEG powder at a mass ratio of 1:5 and then grinded in a mortar for 10 min. To prepare M-ZnO/ZnO electrodes for electrochemical tests, 10 mg poly(vinylidene fluoride) (PVDF) powder was dissolved in 6 drops of N-Methyl pyrrolidone (NMP), then 90 mg of M-ZnO/ZnO samples with additional 2 drops of NMP was added into the solution, stirred for 4 h. The obtained dispersion was coated on commercial carbon paper using a film coating machine, then dried in vacuum for 12 h.

Materials characterization

The morphologies, microstructures, and compositions of the M-ZnO and ZnO nanoparticles were characterized by scanning electron microscopy (FE-SEM, JSM-6330F), transmission electron microscopy (TEM, TecnaiG2 F30) coupled with energy dispersive X-ray (EDX) spectroscopy, X-ray diffractometry (XRD, DMAX2200 VPC), and X-ray photoelectron spectroscopy (XPS, ESCA Lab250). Zeta potential was determined on Brookhaven Zetasizer Nanoseries (EliteSizer). Photoluminescence measurements of powder samples was detected by FLS980, EDINBURGH INSTRUMENTS.

Electron spin resonance (ESR) spectrometer (A300, Bruker) was used to quantify the generated $^1O_2$ and •OH. Typically, 0.5 mL M-ZnO (500 μg mL^{-1}) or ZnO (500 μg mL^{-1}) were mixed with 15 μL 2,2,6,6-tetramethylpiperidine (TEMP, for $^1O_2$ detection) or 5,5-dimethyl-pyrroline-N-oxide (DMPO, for ·OH detection). Then the solution was irradiated with US (1 MHz, 1.5 W cm^{-2}) for 1 min and immediately detected by the ESR spectrometer. The LIPUSTIM® Sonodynamic Therapy System (LIPU-STIM330) made by SXULTRASONIC CO., LTD. was used to produce US. The UV-vis spectra were obtained from 750 UV-vis-NIR spectrophotometer (PerkinElmer Lambda) to achieve $^1O_2$ detection. 3 mL of ZnO or M-ZnO solution (500 μg mL^{-1}) was mixed with 10 μL of 1,3-diphenylisobenzofuran (DPBF, 10 mM in DMSO). Then the mixture was irradiated with US at fixed time intervals (1 min) until 10 min.

The US-reacting current measurements were carried out using an electrochemical workstation (CHI 760) with a standard three-electrode electrolytic cell in phosphate buffered solution (PBS) at room temperature. A graphite electrode and a saturated calomel electrode (SCE) were used as the counter electrode and reference electrode, respectively.

In vitro experiments

In Vitro SDT: The killing effect of SDT on cancer cells was evaluated by CCK-8 assay. Typically, MDA-MB-231 cells were seeded into each well of a 96-well plate at 1×10^4 $well^{-1}$ for 12 h to adhere at 37 °C under 5% CO_2. Then PEG-modified M-ZnO of various concentrations (0-100 μg mL^{-1}) were added in to above-mentioned wells preseeded with MDA-MB-231 cells. The cells were exposed to US irradiation (1 MHz, 1.5 W cm^{-2}) for 2 min and continuously incubated for 12 h. After treatment, CCK-8 assay was used to determine the cell viabilities. Afterward, the cytotoxicity of each group under different treatments (including control, US only, M-ZnO only, M-ZnO+US, TPP only, TPP+US, ZnO only and ZnO+US) was tested by the same CCK-8 assay. Briefly, the MDA-MB-231 cells were seeded in 96-well plates at 1×10^4 $well^{-1}$ for 12 h. Then, the cells were treated with control, US only, ZnO only, ZnO+US, TPP only, TPP+US, M-ZnO only and M-ZnO+US, respectively, followed by incubation for 12 h after treating with and without US irradiation. At last, the CCK-8 assay were used to detect the cell viability.

In vitro ROS generation: Cell incubation and US treatment protocols were the same as above. Subsequently, intracellular ROS levels were detected by the ROS assay kit. The intracellular florescence signals were detected by fluorescence Microscope (OLYMPUS IX71).

Live-Dead Cell Staining Assay: The sonodynamic treatment effect of M-ZnO was further verified by the live-dead cell staining assays. Cell incubation and US treatment protocols were the same as above. Then, culture medium containing calcein-AM (2 mM) and PI (1.5 mM) was added to the wells. After being cultured for another 15 min at 37 °C, the living cells display yellow-green fluorescence and dead cells become red under fluorescence microscope.

The Cell Apoptosis Analysis: Typically, MDA-MB-231 cells were seeded into each well of a 6-well plate at 2.5×10^5 $well^{-1}$ for 12 h. The cells were treated in the same way as in the live/dead assay described above. The cells were rinsed gently by PBS for two times to wipe off the redundant M-ZnO or ZnO and detached with trypsin. The AnnexinV-FITC (5 μL) and PI (5 μL) were employed to stain living and dead cells, and the staining was performed at room temperature in darkness for 15 min. Finally, the flow cytometry was carried out to examine the cell apoptosis in different treatment groups.

In vivo experiments

Tumor model: BALB/c female mice (four to five weeks old, 14~15 g) were purchased from Guangdong Animal Experiment Center. Animal care and experiments were performed in compliance with the relevant laws and guidelines from the

Institutional Animal Care and Use Committee of Sun Yat-Sen University Cancer Center, and the committee has approved our experiments (approval number: L102012019220G). 200 μL MDA-MB-231 cells suspension (2×10^7 cells mL^{-1}) was subcutaneously injected into the right axillary of each mouse to build up the tumor model.

In vivo cancer treatment: MDA-MB-231 tumor-bearing mice (~110 mm^3) were randomly divided into four groups (n=5 per group): (1) Control with only 200 μL 0.9 % saline injection; (2) US only (1 MHz, 1.5 W cm^{-2}, 2 min); (3) M-ZnO only (i.v. injection, 0.2 mL, 50 μg mL^{-1}); (4) M-ZnO (i.v. injection, 0.2 mL, 50 μg mL^{-1})+US (1 MHz, 1.5 W cm^{-2}, 2 min); (5) ZnO only (i.v. injection, 0.2 mL, 50μg mL^{-1}); (6) ZnO (i.v. injection, 0.2 mL, 50μg mL^{-1})+US (1 MHz, 1.5 W cm^{-2}, 2 min). The tumor volumes were calculated by the following equation:

$$Volume = \frac{(tumor\ width)^2 \times tumor\ length}{2} \quad (1)$$

At 0.5 h after i.v. injection, the tumors were treated with US irradiation and repeated on the third, seventh and fourteenth day. The tumor sizes and body weights of the mice were measured and recorded every seven days. On day 21, all mice were euthanized via injecting excessive anesthetics. The major organs and the tumors in each group were collected for histological examination.

$$Realative\ tumor\ volume = \frac{tumor\ volume\ on\ day\ X}{tumor\ volume\ on\ day\ 0} \times 100\% \quad (2)$$

Statistical analysis

The results were shown as mean ± SD, and all experiments were carried out at least three times.The two-way ANOVA with Bonferroni posttests was used to calculate the significant difference of different groups. Significance was indicated by $P < 0.05$ (*P < 0.05, **P < 0.01, ***P <0.001). All data were processed via Excel and GraphPad Prism.

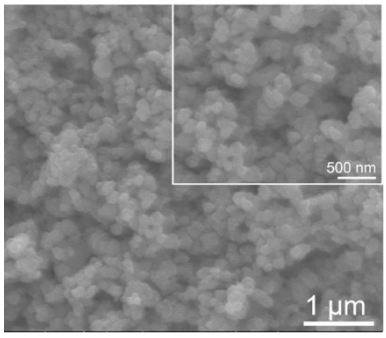

Fig. S1 SEM images of ZnO.

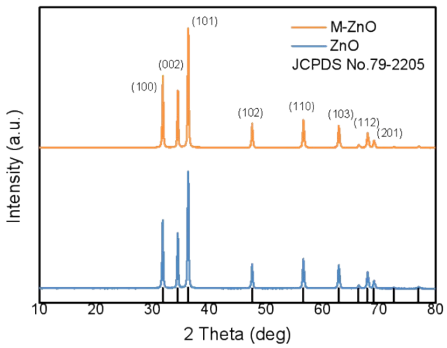

Fig. S2 XRD patterns of M-ZnO and ZnO.

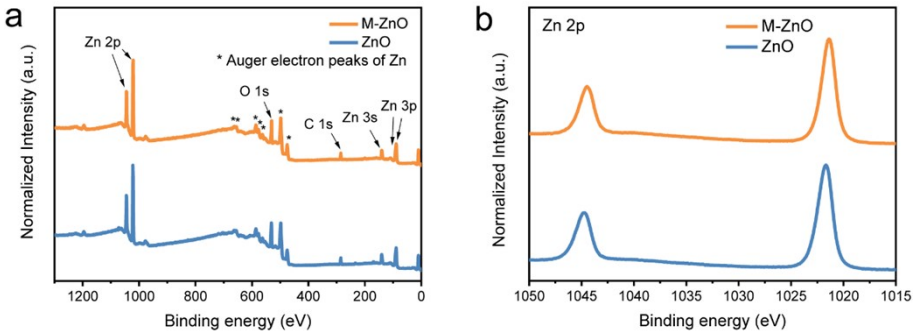

Fig. S3 (a) Survey XPS spectra, (b) Zn 2p XPS spectra of M-ZnO and ZnO.

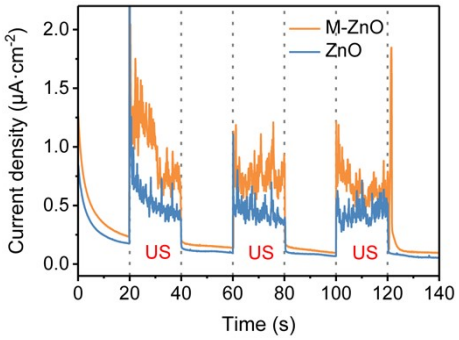

Fig. S4 The US-reacting current curves of M-ZnO and ZnO.

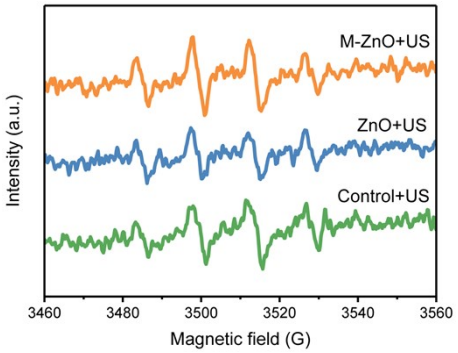

Fig. S5 ·OH detecting ESR spectra of M-ZnO, ZnO and control group.

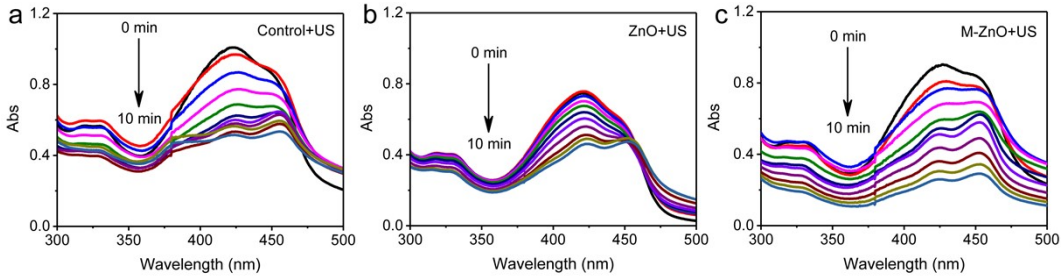

Fig. S6 Time-based DPBF UV-vis absorbance spectra of (a) control+US, (b) ZnO+US and (c) M-ZnO+US group.

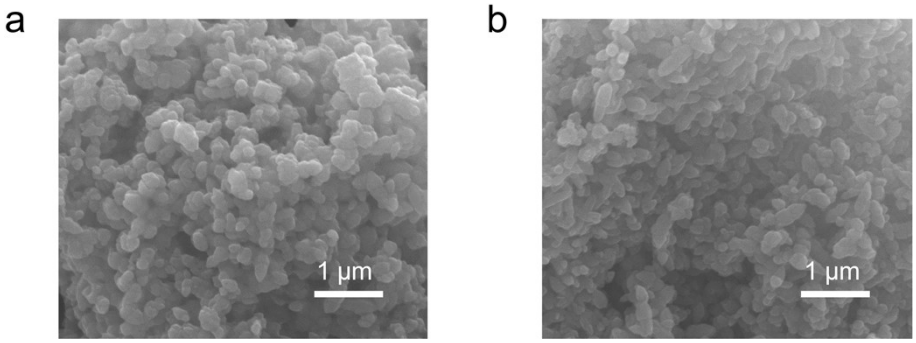

Fig. S7 SEM images of mPEG-DSPE-modified (a) ZnO and (b) M-ZnO.

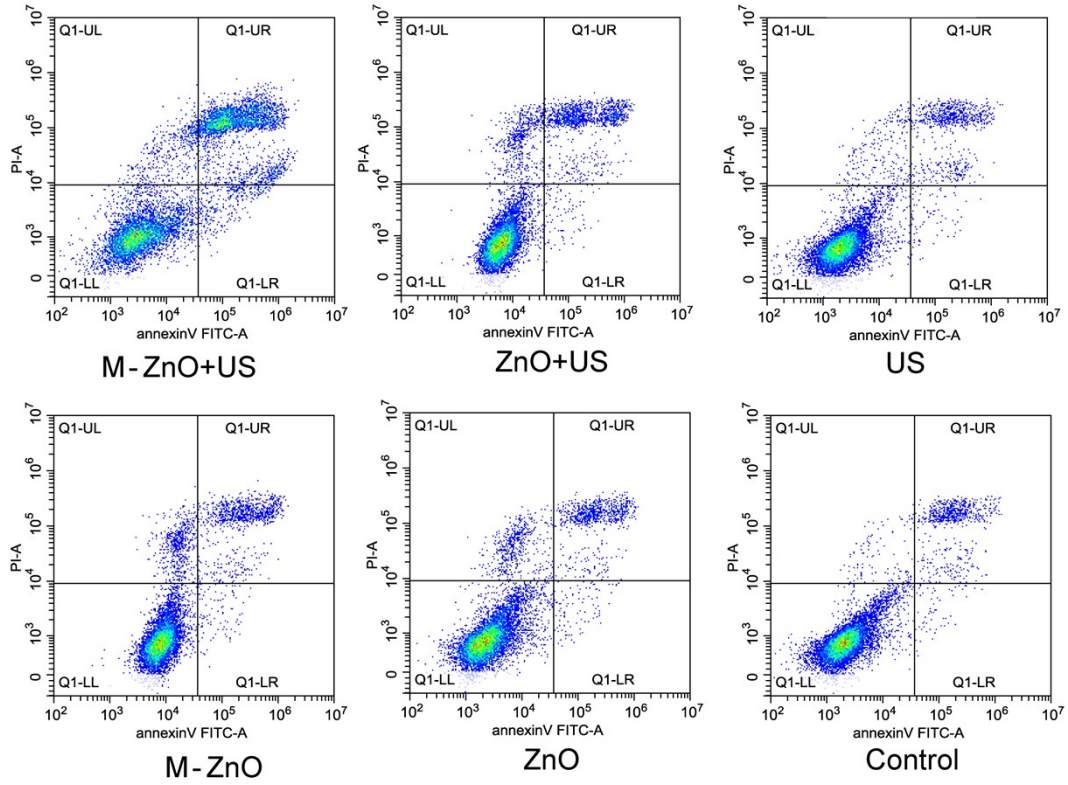

Fig. S8 Flow cytometry analysis of MDA-MB-231 cells apoptosis subjected to different treatments. The similar population of live cells in the control, control + US, ZnO and M-ZnO groups proves that pure M-ZnO barely changes physiological activity. In contrast, only 55% of cells remain alive in the M-ZnO + US group, confirming that the M-ZnO can function as a decent sonosensitizer with excellent cell-killing capability.

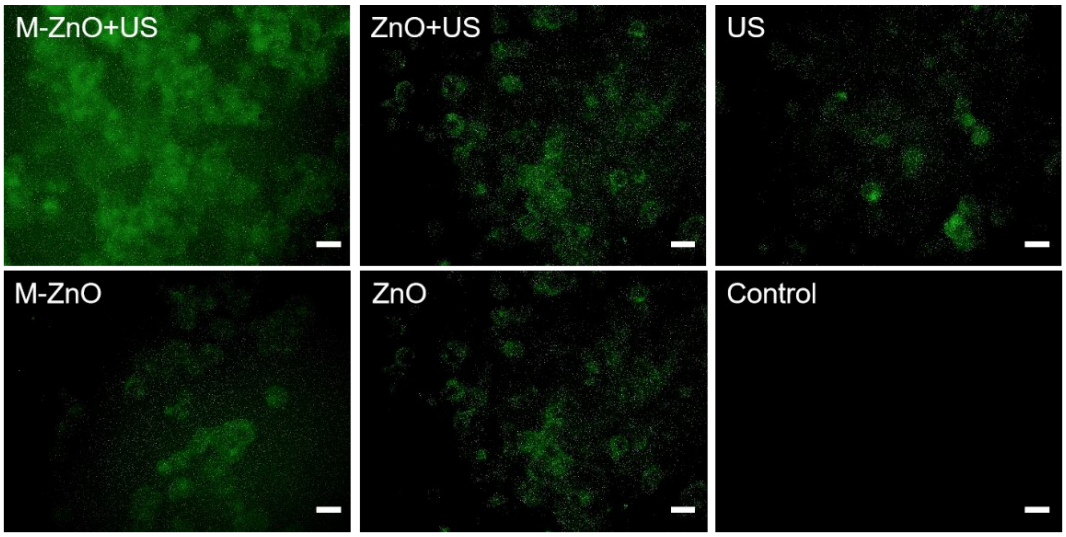

Fig. S9 Fluorescent detection of lipid peroxides in MDA-MB-231 cells after different treatments. Scale bars: 20 μm.

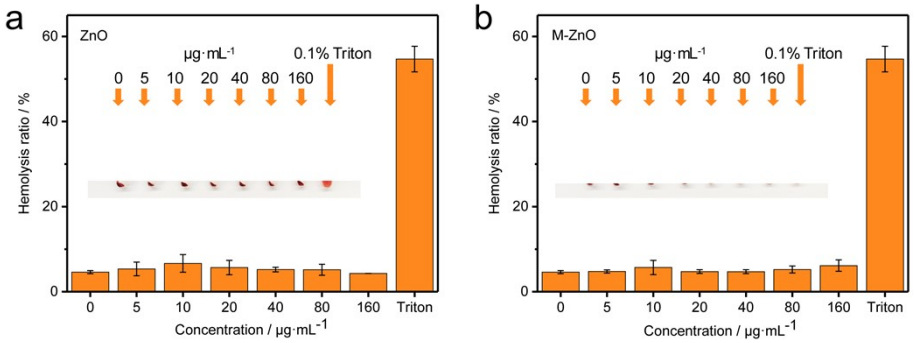

Fig. S10 Digital photograph of hemolysis test and hemolysis rate of (a) ZnO and (b) M-ZnO.

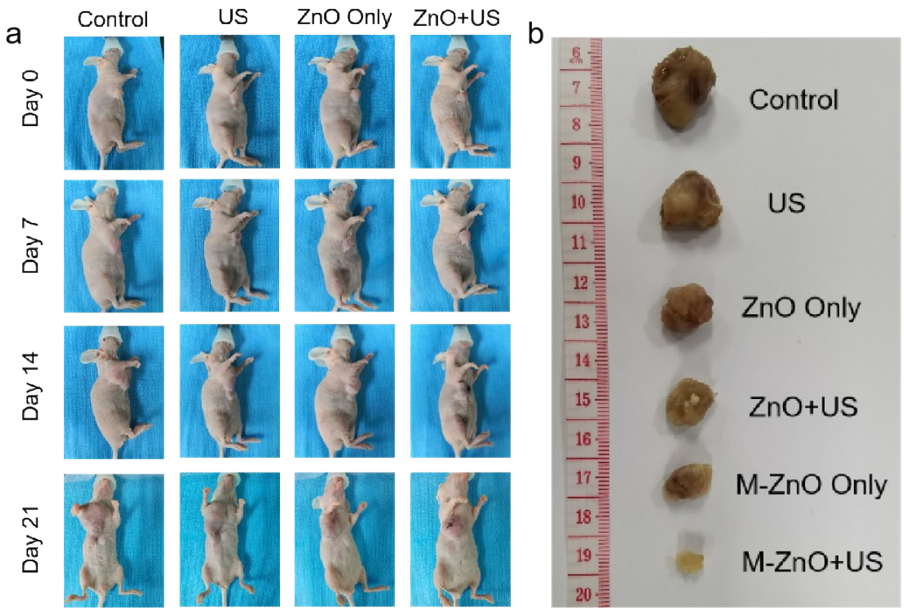

Fig. S11 Photographs of (a) MDA-MB-231 tumor-bearing mice from day 0 to day 21 under different treatments. (b) excised tumors after 21 days of different treatments.

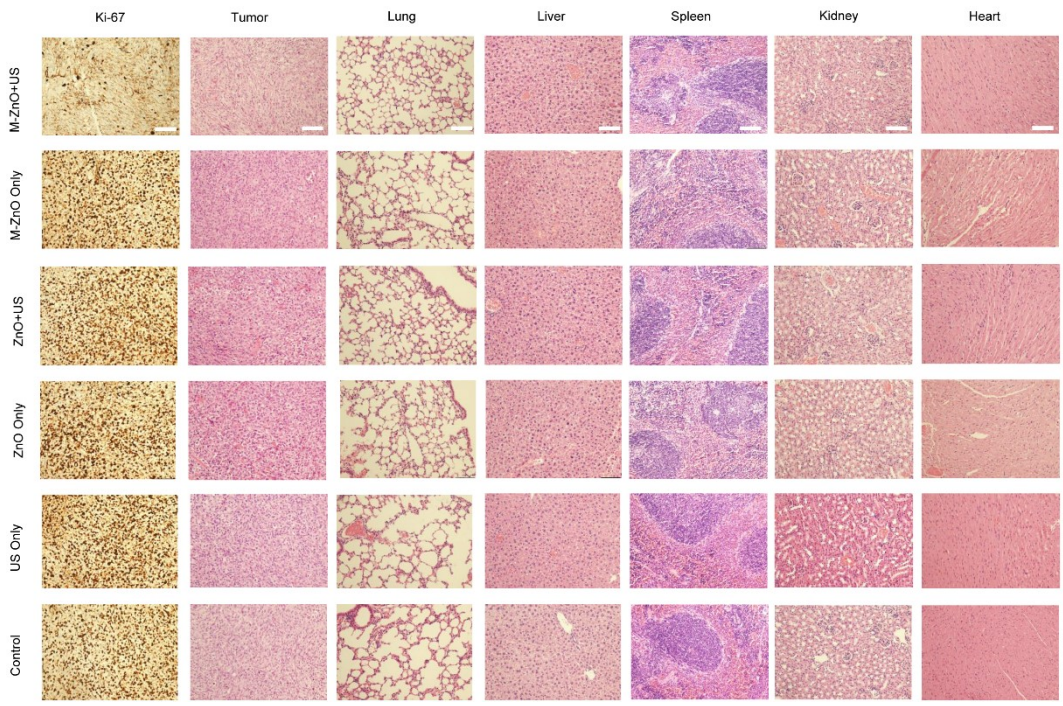

Fig. S12 H&E staining of tumor tissues and the major organs of MDA-MB-231 tumor-bearing mice after different treatments for 21 days. The Ki-67 label index for the M-ZnO + US group is much lower (5%) than that for the M-ZnO group (55%), control + US group (69%) and control group (73%) as the proliferation of tumor cells is largely inhibited.