Supplementary Information

Nanozyme-integrated microneedle patch for enhanced therapy of cutaneous squamous cell carcinoma by breaking the gap between H_2O_2 self-supplying chemodynamic therapy and photothermal therapy

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Experimental section

Materials. Manganese(II) chloride tetrahydrate ($MnCl_2 \cdot 4H_2O$), Copper(II) acetate monohydrate ($Cu(CH_3COO)_2 \cdot H_2O$), methylene blue (MB), and glucose were purchased from Aladdin. Potassium permanganate ($KMnO_4$) was purchased from Delta. The horseradish peroxidase (HRP), sodium diethyldithiocarbamate, sodium hydroxide (NaOH), Methylene Blue trihydrate (MB), polyvinyl pyrrolidone (PVP), and glycerol were purchased from Macklin. 3,3′,5,5′-Tetramethylbenzidine (TMB) was purchased from Sigma–Aldrich. Bovine serum albumin (BSA) was purchased by MRC. Ammonia was purchased by Phygene. Combretastatin A4 phosphate disodium Salt (CA4) was purchased by Meilunbio. 2′,7′-dichlorofluorescein diacetate (DCFH-DA) and mitochondrial membrane potential assay kit were obtained from Beyotime. Calcein-AM/PI double staining kit was purchased from Biosharp. Annexin V-FITC/PI apoptosis detection kit was provided by KeyGen Biotech. Co., Ltd. All the materials were used as received unless otherwise noted.

Instrumentation. Transmission electron microscopy (TEM) images were captured by an FEI Tecnai G20 S-TWIN microscope operating at an accelerating voltage of 120 kV. The elemental binding energy was measured by X-ray photoelectron spectroscopy (ESCALab 250, Thermo Fisher). The hydrodynamic size and zeta potential were evaluated by a particle analyzer (Litesizer 500, Anton Paar). The absorbance spectrum was measured by a UV-vis spectrophotometer (UV 2600, Shimadzu).

Preparation of MnO_2 nanosheets. 15.1 mg $MnCl_2$ and a certain amount of BSA (0.6, 3, 6, 12, 30, and 60 mg) were mixed in 60 mL ddH_2O. After stirring at room temperature for 1 h, NaOH solution was added to adjust the pH to about 10. After stirring overnight, the product was centrifuged and washed 3 times with ddH_2O. Finally, MnO_2 nanosheets were dried and stored in a drying cabinet.

Preparation of MnO_2/Cu_2O nanosheets. 0.1 g MnO_2 nanosheets, 1.0 g mol Cu(CH3-$COO)_2 \cdot H_2O$, and 1.8 g $C_6H_{12}O_6$ were added into 100 mL ddH_2O. After stirring for 12

h at room temperature, the solution was heated to 90 °C and kept for 5 h. The obtained product was centrifuged and washed 3 times with ddH_2O. Finally, MnO_2/Cu_2O nanosheets were dried and stored in a drying cabinet.

Release of Cu from MnO_2/Cu_2O nanosheets. MnO_2@Cu_2O nanosheets (2 mg/mL) were placed in a dialysis bag (MWCO: 14 kDa), which was then immersed in 10 mL of buffers with different pH values (pH 5.6, pH 6.8, and pH 7.4). Each dialysis bag was placed on a horizontal shaker (100 rpm). Then, 300 μL of buffer was withdrawn, and fresh buffer was added at the indicated time points. The withdrawn buffer was added to a 48-well plate, mixed with 20 μL ammonia and 200 μL sodium diethyldithiocarbamate (0.1 g/mL) for 15 min. Finally, the absorption at 452 nm was measured by a microplate reader (Synergy H1, BioTek).

Measurement of glucose oxidase-like activity. Glucose oxidase could catalyze glucose oxidation to produce H_2O_2. The qualitative detection of H_2O_2 was carried out by using the acidic potassium permanganate titration method. First, 1 mL of indicated solution (PBS, 0.01% H_2O_2, 500 μg/mL MnO_2/Cu_2O, 500 μg/mL MnO_2/Cu_2O+100 mM glucose) was mixed with 4 mL acidic potassium permanganate for 10 min. After centrifuging, the absorption peak of the supernatant was measured by UV-vis spectrophotometer. The quantitative determination of the produced H_2O_2 was also performed using TMB chromogenic method as follows. 500 μL of MnO_2 (500 μg/mL) was mixed with 500 μL of 100 mM glucose solution, and 500 μL different pH buffer for the indicated time, which was named solution A. A total of 289 μL of acetate buffer (0.1 M, pH 4.0), 6 μL of TMB (10 mg/mL), and 5 μL of HRP (1 mg/mL) were mixed to generate solution B. Then, 100 μL of solution A was added to solution B for 10 min of reaction. Finally, the absorbance at 650 nm was measured and recorded.

Evaluation of the generated •OH. The produced •OH was detected by a UV–vis spectrophotometer with MB serving as a sensor. In brief, 500 μL of glucose (1 M), 50 μL of MB (0.25 mM), and 100 μL of MnO_2/Cu_2O (2 mg/mL) were mixed with a

solution containing 500 μL of PBS buffer at pH 5.6. Then, ddH_2O was added to the solution to reach a final volume of 1500 μL. After 4 h, the UV–vis absorption of the above solution was measured. This same method was applied to detect •OH under different conditions (without H_2O_2 and glucose, without H_2O_2, without glucose, and with H_2O_2 and glucose).

Photothermal conversion performance. To evaluate the photothermal conversion properties, MnO_2/Cu_2O was dispersed in ddH_2O with concentrations of 0, 0.5, 1, 2, and 4 mg/mL. Then, 500 μL of the above solutions were transferred to a 48-well cell culture plate and irradiated with an 808 nm laser (LSR808H-4W-FC, LASEVER) for 10 min. To study the concentration-dependent photothermal conversion, the above solutions were irradiated with the laser with 1 W/cm^2 for 10 min. To study the power density-dependent photothermal conversion, MnO_2/Cu_2O (2.0 mg/mL) was irradiated with the laser with 0.8, 1, and 1.2 W/cm^2 for 10 min. The photothermal stability was researched for recording the temperature change of MnO_2/Cu_2O (2.0 mg/mL) during the 5 cycles of laser on and laser off (1.2 W/cm^2). The temperature change and the thermal images were recorded with a thermal imager (Hti HT-19, Dongguan Xintai Instrument Co., Ltd).

Preparation of microneedle (MN) patch. The polymer solution of 30 wt.% PVP (24k) and 5 wt.% glycerol was prepared. Then, PBS solution, MnO_2@Cu_2O, CA4, and MnO_2/Cu_2O + CA4 were added, respectively. The above solutions were fully stirred and evenly dispersed. After overnight, adequate hydration was ensured to obtain needle cavity fluid. The microneedle mold was filled with needle cavity liquid, dried by vacuum, and filled repeatedly until no bubble was observed. The microneedle patches were obtained after complete drying and separation from the mold.

Cell culture. A-431 cells (obtained from Procell Life Science and Technology Co. Ltd) were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), streptomycin (100 μg/mL), and penicillin (100 unit/mL) at 37 °C in a humidified

atmosphere with 5% CO_2. HUVEC cells (obtained from Procell Life Science and Technology Co. Ltd) were cultured in DMEM-F12 supplemented with 10% fetal bovine serum (FBS), streptomycin (100 μg/mL) and penicillin (100 unit/mL) at 37 °C in a humidified atmosphere with 5% CO_2.

Scratch assay. A microneedle was immersed in 1ml DMEM-F12 high-glucose medium to obtain different microneedle solutions: control, MN, MN-CA4, MN-MnO_2@Cu_2O, and MN-MnO_2/Cu_2O-CA4. HUVEC cells were seeded in a 6-well plate overnight for attachment. Scratches were made on each well plate with a 200 μL pipetting gun. Scratches were observed at 0 h under a microscope and photographed. Then HUVEC cells were incubated with different microneedle solutions (with or without laser irradiation) for 24 h. Scratches were observed at 24 h under a microscope and photographed.

Cell cytotoxicity assay. The cell cytotoxicity was evaluated using the CCK8 assay. Briefly, HUVEC cells or A431 cells were seeded into 96-well plates overnight. Then, the cells were treated with different formulations and incubated for another 24 h. Subsequently, the CCK8 reagent was added. Four hours later, the absorbance of was measured and recorded using a microplate reader (Synergy H1, BioTek).

Detection of intracellular reactive oxygen species. A431 cells were seeded in 12-well plates overnight. Then, the cells were incubated with different microneedles solution with or without laser irradiation for 4 h, followed by staining with DCFH-DA (10 μM) for 30 min. Finally, the cells were observed by fluorescence microscopy or analyzed via flow cytometry (CytoFLEX, Beckman).

Measurement of mitochondrial membrane potential. A431 cells were inoculated into a 24-well plate overnight. After incubation with different microneedles solution with or without laser irradiation for 4 h. Then, the cells were collected and mixed with 500 μL of JC-1 working solution. The cells were uniformly suspended and incubated

for 15-20 min. Afterward, cells were collected by centrifugation at 2000 rpm for 5 min at room temperature and washed twice with the Incubation Buffer. Finally, the cells were re-suspended with 500 μL of Incubation Buffer and analyzed by flow cytometry (CytoFLEX, Beckman).

Cell apoptosis assessment. A431 or HUVEC cells were seeded in 12-well plates overnight. Then, the cells were incubated with different microneedles solution with or without laser irradiation for 24 h. Next, the cells were stained with annexin V-FITC and PI for 15 min according to the manufacturer's protocol. Finally, the cells were collected and analyzed by flow cytometry (CytoFLEX, Beckman).

In vivo antitumor effect. All animal experiments were conducted according to the protocols of the Institutional Animal Care and Use Committee of the Animal Experiment Center of Sun Yatsen University. Male Balb/c nude mice (obtained from GemPharmatech Co.Ltd) at 6 weeks of age were subcutaneously injected with A431 cells (5×10^6 cells in 0.1 mL). When the tumors reached a volume of about 50 mm^3, the mice were randomly divided into seven groups: (G1) MN with laser, (G2) MN-CA4 with laser, (G3) MN-MnO_2/Cu_2O with laser, (G4) MN-MnO_2/Cu_2O-CA4 with laser, (G5) MnO_2/Cu_2O-CA4 with laser, (G6) MN, and (G7) MN-MnO_2/Cu_2O-CA4, respectively. MN patches were inserted and irradiated by NIR laser for 10 min every two days. The lengths and widths of the tumors were measured every 2 days. Tumor volume was calculated as follows: tumor volume (V) = (tumor length) × (tumor width)^2/2. Tumors from each group were collected and photographed. Additionally, the major organs were collected, fixed in a 4% paraformaldehyde solution, and stained with hematoxylin and eosin (H&E) for histological analysis.

Statistical analysis. All the data are presented as mean ± standard deviation. Statistical analysis was carried out using GraphPad Prism 8.4. The significant differences between sample groups were determined using one-way analysis of variance (ANOVA) and Tukey's test was used for posthoc analysis. The statistical significance was defined as

*p<0.05, **p<0.01, ***p<0.001.

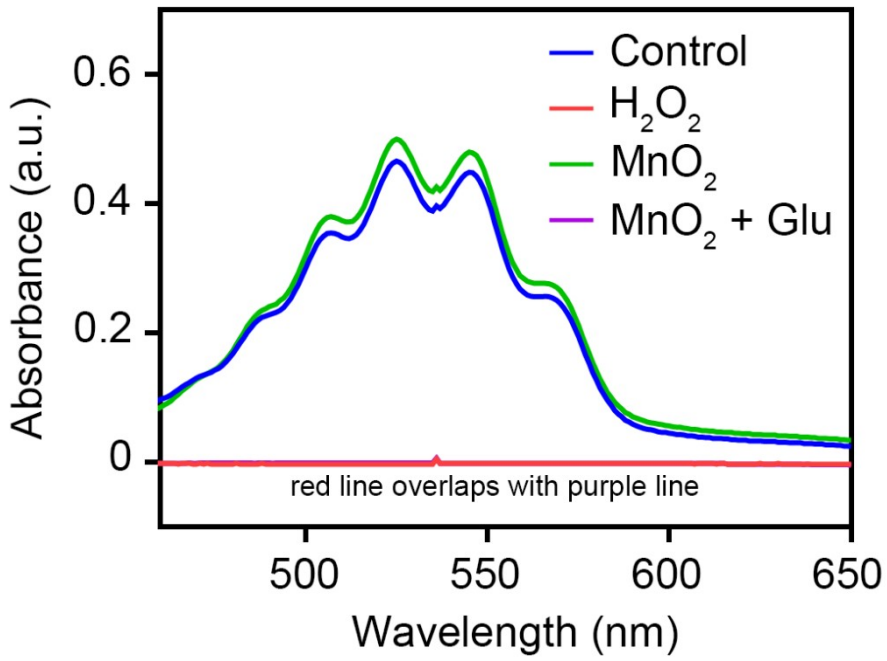

Fig. S1 Absorbance spectrum of MnO_4^- after reaction with indicated formulas. H_2O_2 can reduce MnO_4^- to Mn^{2+}, resulting in the disappearance of pink color.

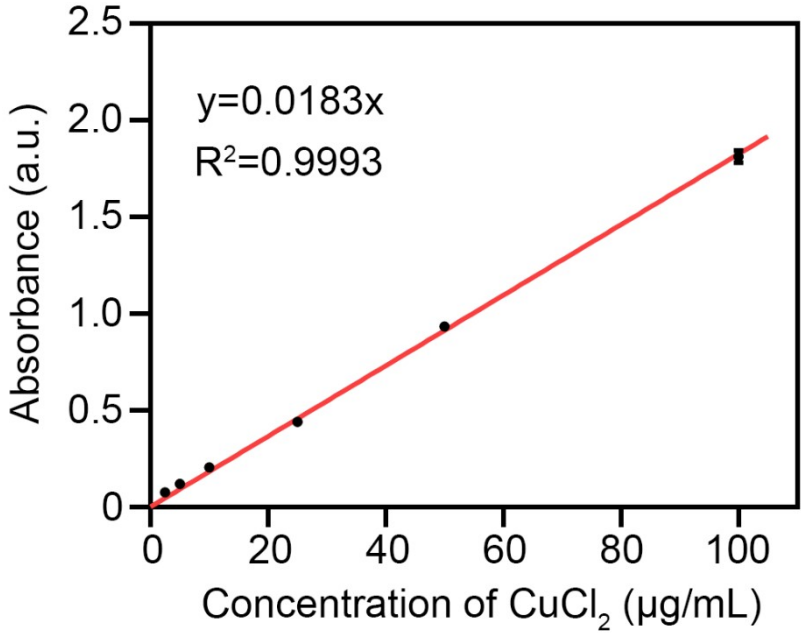

Fig. S2 The standard curve for detecting Cu ions using sodium diethyldithiocarbamate trihydrate.

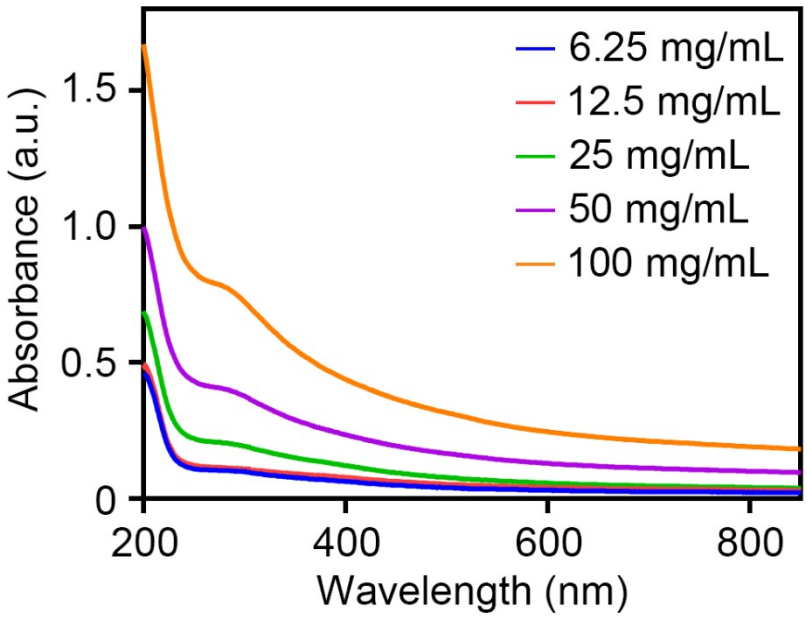

Fig. S3 Absorption spectrum of MnO_2 nanosheet solutions with indicated concentrations.

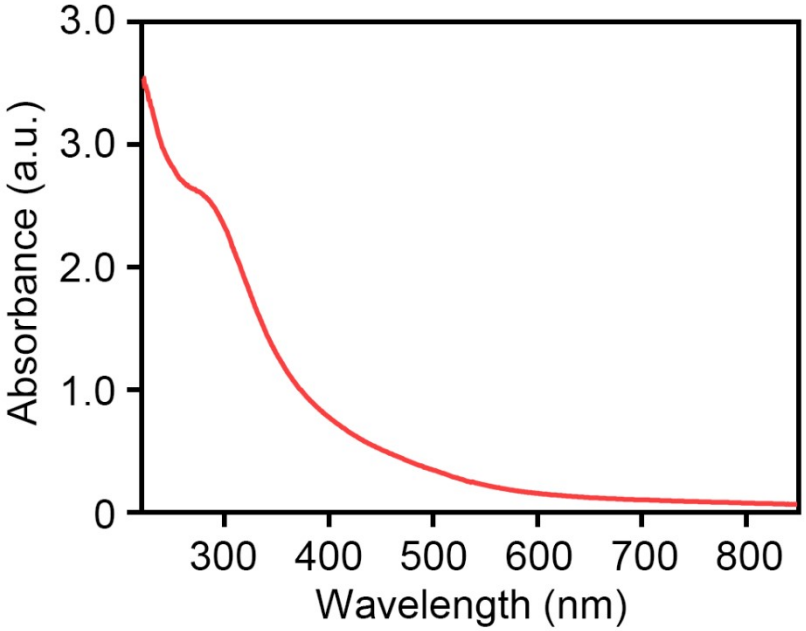

Fig. S4 The absorption spectrum of MnO_2/Cu_2O nanosheet solutions.

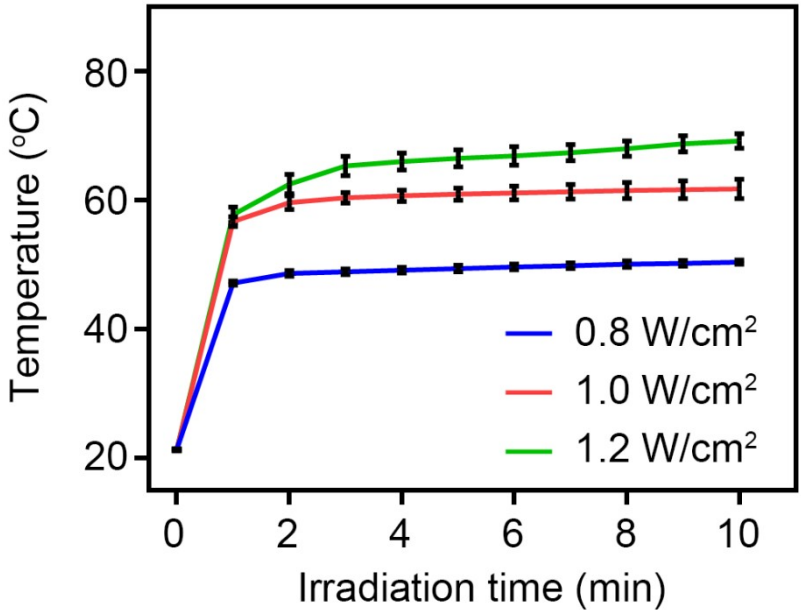

Fig. S5 Temperature changes of MN-MnO_2/Cu_2O with indicated laser powers.

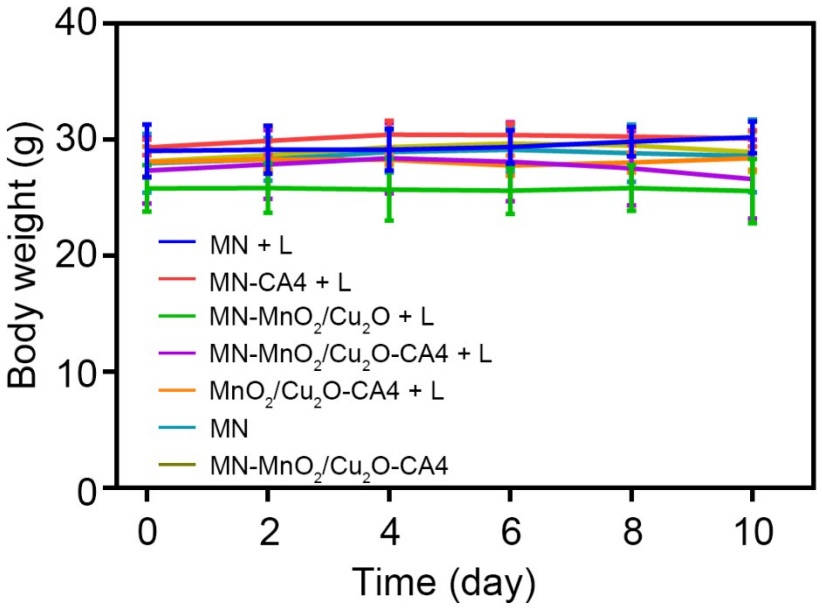

Fig. S6 Body weight of mice measured after indicated treatments every two days.

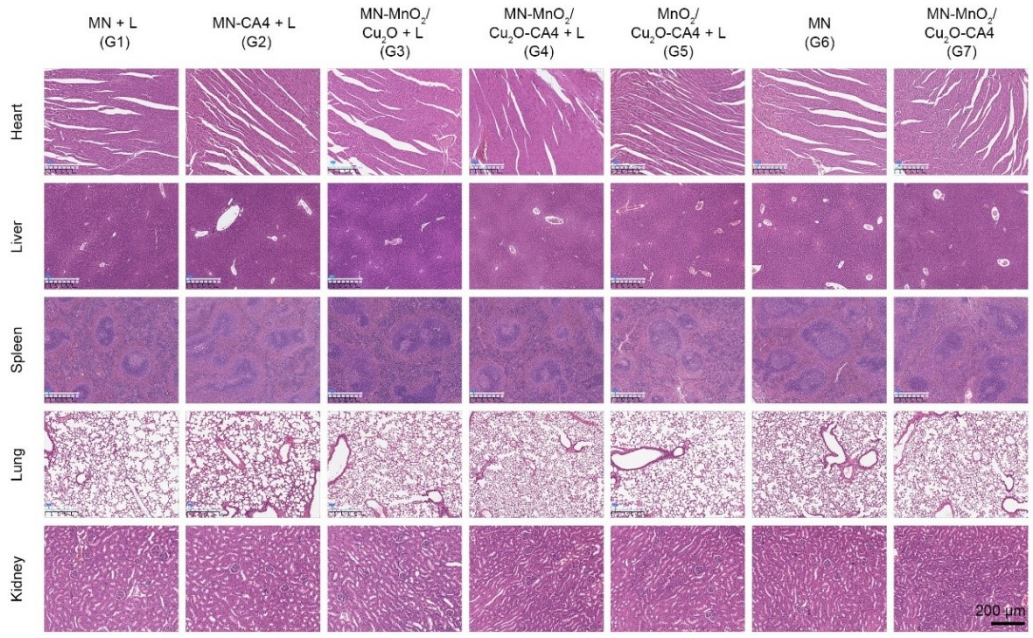

Fig. S7 H&E staining of the major tissues from tumor-bearing mice with indicated treatments.