Supporting Information

H_2O_2-triggered CO release based on porphyrinic covalent organic polymers for photodynamic/gas synergistic therapy

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1. Experimental Section

1.1 Materials

5,10,15,20-Tetrakis(4-aminophenyl)-21H,23H-porphine (TAPP), 2,2'-bipyridine-5,5'-dicarboxylic acid (Bpydc) was purchased from CHEMBEE. N,N-dimethylformamide (DMF) and hydrogen peroxide (H_2O_2)was purchased from Shantou Xilong Chemical Co. Ltd. (Guangdong, China). Calcein acetoxymethyl ester (calcein AM), propidium iodide (PI) were purchased from Beyotime. 2,7-dichlorofluorescein diacetate (DCFH-DA) were purchased from FEIYUBIO. Dulbecco's modified eagle medium (DMEM, PAA Laboratories), fetal bovine serum (FBS, PAA Laboratories), and methylthiazolyldiphenyl-tetrazolium bromide (MTT, Ultra-Pure, Aldrich) were used as received.

1.2 Characterization

Scanning electron microscopy (SEM) images were acquired on a ZEISS Gemini SEM 300. Transmission electron microscopy (TEM) observations were conducted on

a FEI Talox F200X electron microscope at an acceleration voltage of 300 kV. Fourier transform infrared (FT-IR) spectra were recorded on a Nexus 670 FTIR spectrometer. The UV-vis-NIR spectroscopy was recorded by using a Shimadzu UV3600 spectrophotometer. Super-resolution multiphoton confocal lasers canning microscopy (CLSM) was performed on a Leica TCS SP8 STED 3X instrument.

1.3 Synthesis of TAPP(Fe), COPs, COPs@MnCO

Fe-TAPP: In a typical procedure, $FeCl_3$ (50 μmol, 8.1 mg) and TAPP (12.5 μmol, 8.4 mg) was dissolved in a mixture of DMF (5 mL) and chloroform (15 mL). After the two solutions were mixed, then refluxed and stirred at 65 °C for 24 h. When cooling to room temperature naturally, the product was collected by centrifugation at 6000 rpm for 5 min, washed with deionized water 3 times, dried, and stored Fe-TAPP in the dark.

COPs: 0.05 mmol Fe-TAPP and 0.08 mmol Bpydc were dissolved in 20 mL DMF solution. 0.2 mmol N-(3-dimethylaminopropyl)N'-ethylcarbodiimide hydrochloride (EDC) and 0.25 mmol 4-dimethylaminopyridine (DMAP) were added to the solution in turn, and stirred with 500 rpm at 25 ºC for 24 h. The product was collected after high-speed centrifugation, washed with anhydrous ethanol for 3 times, then freeze-dried and stored in the dark.

COPs@MnCO: 30 mg of $MnBr(CO)_5$ was added to 10 mL of COPs (2 mg mL^{-1}) ethanol solution, stirred at room temperature for 12 hours, and then transferred to an oil bath at 75 °C for heating and stirring for 4 hours. After the reaction is over, cool to room temperature naturally, the product was collected by centrifugation at 6000 rpm for 5 min, washed with absolute ethanol 3 times, and stored in freeze-drying and protected from light.

1.4 Measurement of the CO release by a hemoglobin (Hb) method

The released CO in PBS was detected spectrophotometrically by measuring the conversion of hemoglobin (Hb) to carboxyhemoglobin (HbCO). Firstly, hemoglobin from bovine erythrocytes (MP Biomedicals, 4.2 μM final concentration) was dissolved completely in phosphate buffered saline (10 mM pH=7.4 PBS) with different concentrations of H_2O_2, and then was reduced by adding sodium dithionite (SDT, 1.2

mg) under a nitrogen atmosphere. An aqueous solution of COPs@MnCO (100 μg/mL) was deoxygenated by bubbling with nitrogen gas and then added into the above Hb solution. Immediately, the whole 3 mL reaction solution was sealed in a 3.5 mL UV quartz cuvette. The UV adsorption spectra of the solution (350–600 nm) were collected on a Cary 60 UV/Vis spectrophotometer (Agilent Technologies). In order to eliminate influencing factors and enhance the accuracy, two strong adsorption bands at 410 nm and 430 nm, which were attributed to HbCO and Hb respectively, were used to quantify the conversion of Hb to HbCO. The Beer–Lambert law was used to calculate the concentration of released CO (C_{CO}) according to the following Equation (1):

$$C_{\mathrm{CO}} = \frac{528.6 \times I_{410nm} - 304 \times I_{430nm}}{216.5 \times I_{410nm} + 442.4 \times I_{430nm}} C_{Hb}$$

in which C_{CO} and C_{Hb} express the released CO concentration and the initial Hb concentration (4.2 mm), respectively. $I_{410\ nm}$ and $I_{430\ nm}$ express the intensities of the collected spectrum at λ= 410 and 430 nm, respectively.

1.5 O_2 generation

COPs@MnCO (150 μg/mL) were added into different concentrations of H_2O_2 solution (0, 5, 10 mM), which was dissolved in PBS. Dissolved oxygen was detected by a real-time dissolved oxygen instrument.

1.6 Detection of $^1O_2$

The generation of $^1O_2$ was examined by a chemical oxidation method based on DPBF, which could react irreversibly with $^1O_2$ to cause a reduction in the DPBF absorbance. Typically, DPBF ethanol solution (150 μL, 100 μM) was mixed with COPs@MnCO solution (200 μL, 2 mg/mL) and different concentrations of H_2O_2 solution (0, 50, 100 M) and irradiated by a 660 nm laser for different time intervals. The absorbance change at various irradiation times were measured at 410 nm by a UV-vis spectrophotometer.

1.7 In Vitro Cytotoxicity

The cytotoxicity of COPs was detected with HeLa cells by a MTT assay. 1×10^4 cells per well were seeded in 96-well plate (200 μL total volume well^{-1}). Before further treatment, the cells were incubated at 37 °C 5% CO_2 overnight for fully attachment.

Then incubated with different concentrations of COPs, COPs@MnCO, were added for 48 h. The cells incubated with COPs and COPs@MnCO were irradiated with 660 nm laser (1.0 W cm^{-2}, 10 min) and further incubated for another 12 h. To determine cytotoxicity, MTT solution (10 μL, 5 mg mL^{-1}) was added to each well of the microliter plate and the plate was incubated for additional 4 h. Subsequently, the MTT contained culture medium was replaced with 150 μL of DMSO for 10 min. Absorbance values of formazan were determined with a microplate reader at 490 nm.

1.8 Detection of ROS in vitro

The production of intracellular ROS was determined by 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) kit. HeLa cells were seeded into a 6-well plate for 24 h. Then cells were treated with COPs, COPs@MnCO for 6 h. Then cells were incubated with DCFH-DA for 20 min. For irradiated groups, the cells were irradiated with a 660 nm laser at 1.0 W cm^{-2} for 10 min. Finally, the fluorescence of ROS was observed through fluorescence microscopy.

1.9 Imaging of CO in Living Cells

The intracellular release of CO was detected by COP-1 probe (Ex/Em = 475/510), which generates green fluorescence when it reacts with CO. HeLa cells were seeded in a 6-well culture plate for one night before cell imaging experiments. The HeLa cells were treated with PBS, COPs, COPs@MnCO (50 μg mL^{-1}) for 5 h. After that, the medium containing nanoparticles was removed, and the cells were incubated with a mixture of COP-1 probe and $PdCl_2$ (5 μM each) at 37 °C for another 30 min, respectively. Then, the cells were washed twice with PBS and incubated with the fluorescence of COP-1 probe in HeLa cells was observed by fluorescence microscopy.

1.10 Live/Dead Staining Experiment

HeLa cells were seeded in 6-well plates at a density of 3×10^5 cells per well for 24 h. Then, the cells were incubated with COPs, COPs@MnCO respectively for 6 h without laser irradiation or with laser irradiation (660 nm, 1.0 W cm^{-2}, 10 min). After 18 h incubation, the culture medium was discarded, and cells were washed three times with PBS. Cells were washed with PBS and incubated with Calcein AM and PI staining working solution (1 mL per well) contained medium for 30 min. The specific Calcein

AM /PI staining working solution is as follows: 1 μL of Calcein AM solution (1000X) and 1 μL of PI (1000X) were added to 10 mL of Assay Buffer (1X) and mixed well. Then, 1 mL of the staining working solution were added to the 6-well plates and incubated at 37 °C for 30 min. Finally, the cells were washed three times with PBS and imaged by CLSM.

1.11 Mitochondria membrane potential characterization

HeLa cells were seeded in 6-well plate, and after 24 h incubation, treated with PBS (#1), COPs (#2), COPs + Laser (#3), COPs@MnCO (#4), COPs@MnCO + Laser (#5). After 4 h incubated, groups of 3 and 4 were irradiated upon 660 nm laser (1.0 W cm^{-2}) for 10 min, and then incubated for another 4 h. After that, the cells were stained with JC-1 (10 μM) for 20 min. Next, the solution of JC-1 was removed, and washed with PBS for three times. The red and green fluorescence of JC-1 were observed by fluorescence microscope (Beyotime Biotechnology).

1.12 In vivo antitumor efficacy

All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Nantong University and approved by the Animal Ethics Committee of Nantong University. When the tumor volume of HeLa-bearing nude mice reached at about 80 ~ 100 mm^3, all the mice were randomly divided into 6 groups (n = 5) and intravenously injected normal saline, COPs, Laser, COPs + Laser, COPs@MnCO, COPs@MnCO + Laser as a COPs-equivalent dose of 5 mg kg^{-1} every three days (Laser: 660 nm, 10 min, 1.0 W cm^{-2} at 12 h post-injection). The tumor volume and body weight was measured every three days, and the rumor volume was estimated by formula: V (mm^3) = tumor length × (tumor width)^2/2. Relative tumor volumes were calculated as V/V_o (V_o is the tumor volume when the treatment as initiated). The tumor inhibitory rates (TIR) of various treatments are calculated by the equation: TIR (%) = 100 × (mean tumor volume of the PBS group - mean tumor volume of others)/(mean tumor volume of the PBS group). The normal organs and tumor tissues were collected and observed by blood analysis and H&E staining at 20th day.

2. Figures

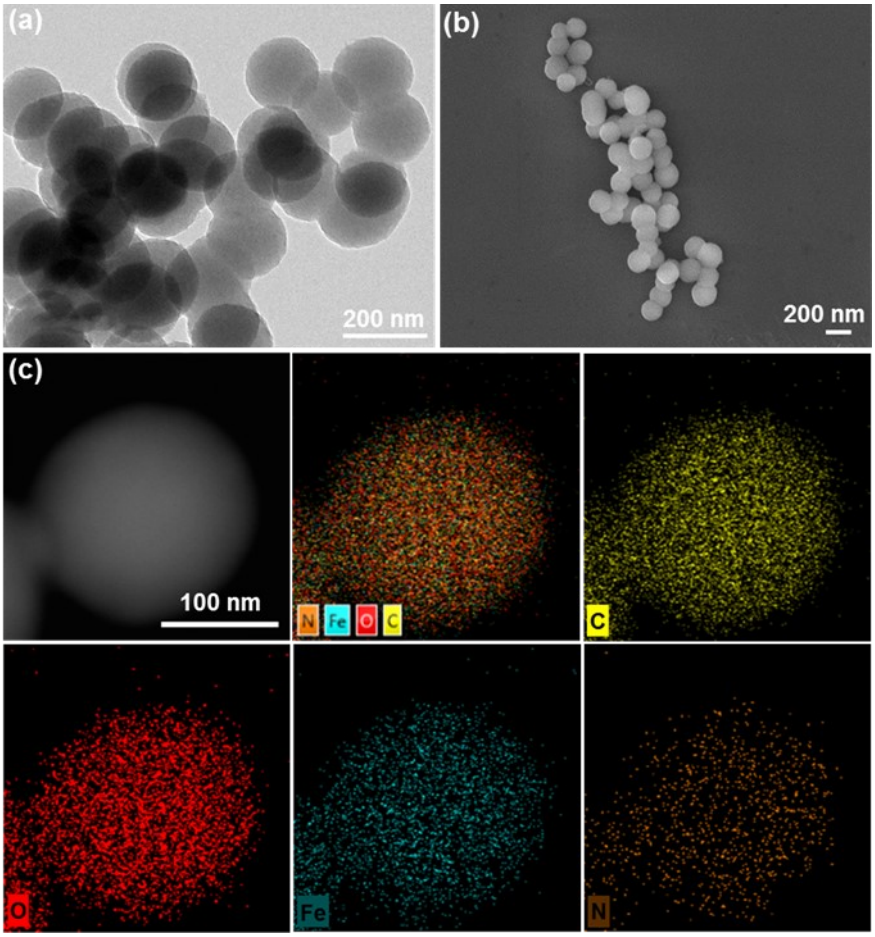

Figure S1. Characterization of COPs nanoparticles. (a) TEM image. (b)SEM image. (c) Element mapping images.

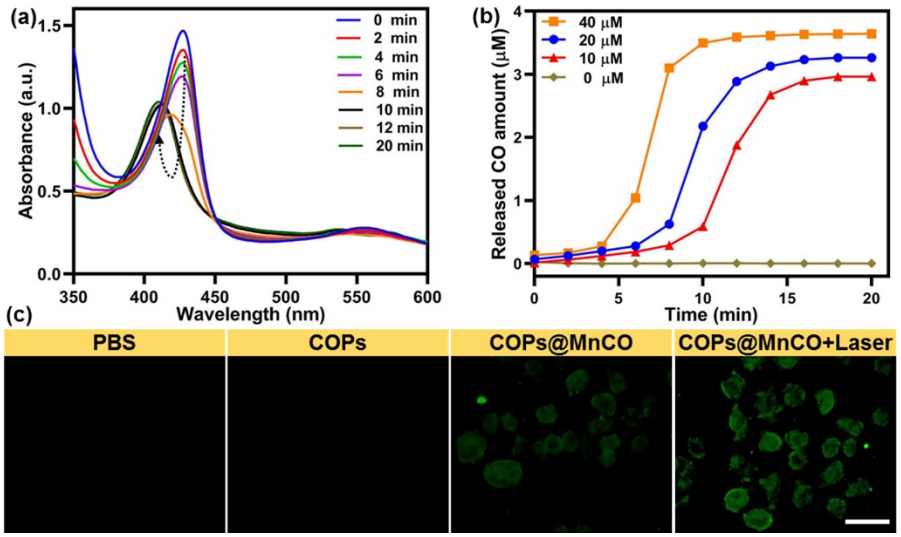

Figure S2. (a) UV–vis absorbance of HbCO indicating CO release from COPs@MnCO

in PBS solution containing H_2O_2 (40 μM). (b) CO release profiles of COPs@MnCO at different concentrations of H_2O_2. (c) Fluorescence images of intracellular CO gas release in different treatments of HeLa cells detected with COP-1 probe. Laser: 660 nm, 1.0 Wcm^{-2}, 10 min. Scale bar: 40 μm.

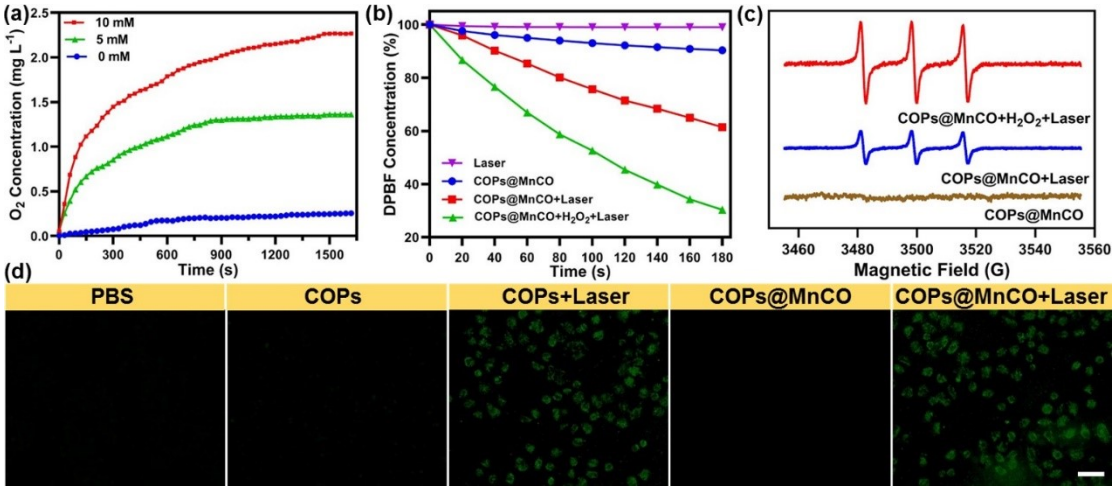

Figure S3. (a) O_2 concentration changes in different concentrations of H_2O_2 solution after adding COPs@MnCO NPs. (b) Time-dependent DPBF degradation curves under different treatments. Laser: 660 nm, 1.0 Wcm^{-2}. (c) ESR spectra of COPs@MnCO under different conditions. TEMP was used as a spin adduct. (d) Fluorescence images of intracellular $^1O_2$ detected with SOSG. Scale bar: 40 μm.

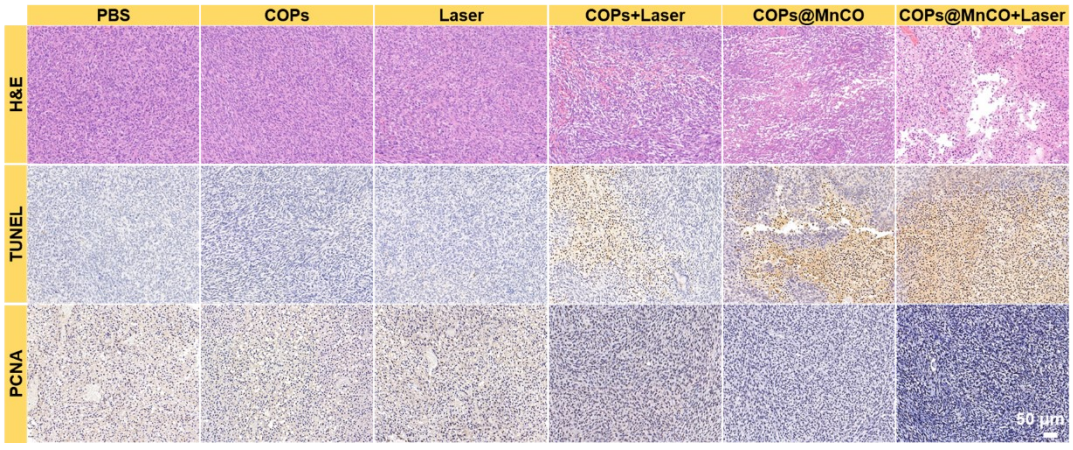

Figure S4. H&E, TUNEL, and PCNA assays of the dissected tumors with various treatments.

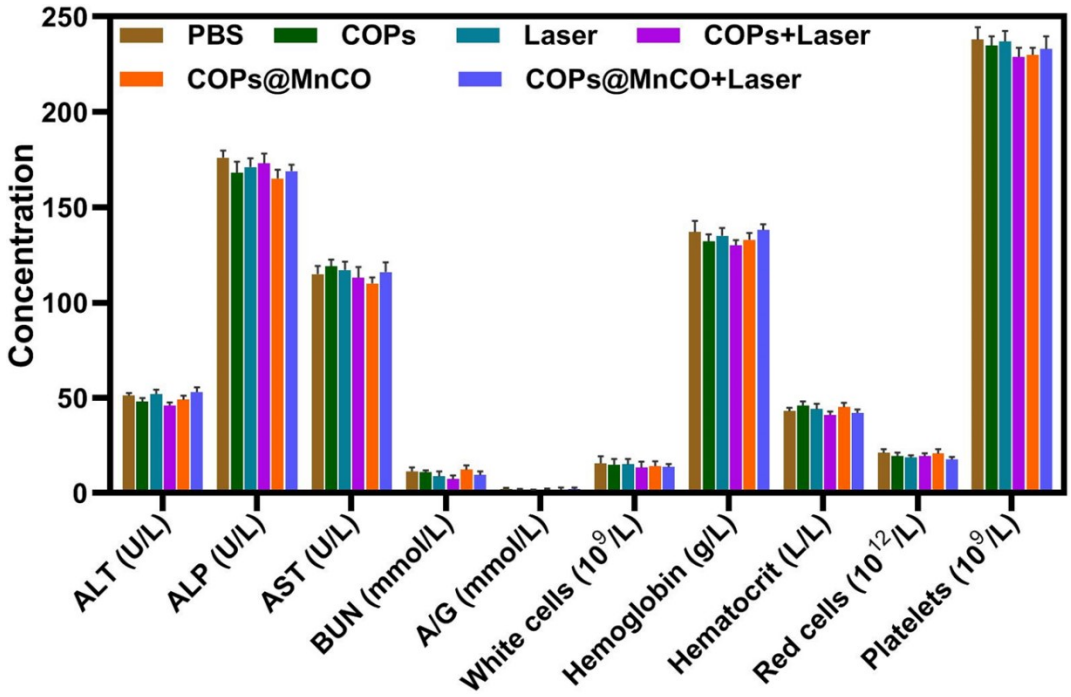

Figure S5. Blood analysis of mice after the different treatments at the end of tumor therapeutic experiments. ALT = alanine aminotransferase; AST = aspartate aminotransferase; ALP = alkaline phosphatase; BUN = blood urea nitrogen.

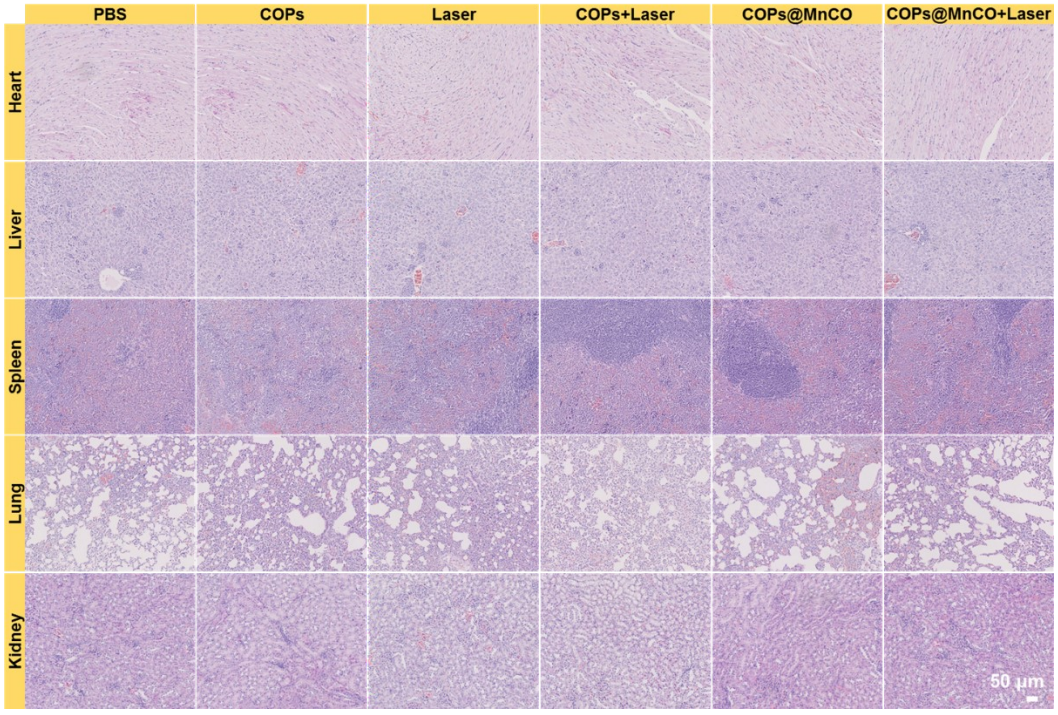

Figure S6. H&E staining assay of mice after the different treatments at the end of tumor therapeutic experiments.