Supporting Information

Low-dose X-ray stimulated NO-releasing nanocomposites for closed-loop dual-mode cancer therapy

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1. Materials and Characterization Technology

1.1 Materials: All of the chemicals have been used without any further purification unless specified. Calcein acetoxymethyl ester/propidium iodide (Calcein-AM/PI), DNA damage detection kit (γ-H2AX immunofluorescence assay), Reactive Oxygen Species detection kit (DCFH-DA), 4',6-diamidino-2-phenylindole (DAPI), the ROS assay kit and 0.25% trypsin-EDTA were obtained from Beyotime Biotechnology. JC-1 probe and MitoTracker Red were purchased from Thermo Fisher Scientific (Shanghai, China). Superoxide anion ($\cdot O_2^-$) fluorescent probe and Peroxynitroso anion ($ONOO^-$) fluorescent probe were purchased from Bestbio. Naphthalene-2,3-dicarboxaldehyde (NDA) was purchased from Shanghai Bihe Biochemical Technology Co., Ltd. Cell Count Kit-8 (CCK-8) was obtained from GlpBio Technology Co., Ltd. Fetal bovine serum (FBS) purchased from Shanghai XP Biomed Ltd.

2. Cell and animal experiments

2.1 In vitro cytotoxicity assay

Cell viability assay. The in vitro cytotoxicity of each nanocomposite was evaluated by CCK-8 assay. In brief, Hela cells (8,000 cells per well) were inoculated into a 96-well plate for 12 hours. The medium was removed, and the medium containing different concentrations of nanocomposites was added, and further incubated for 24 hours. Add 10 μL CCK-8 to each well and incubate for 1.5 hours. The absorbance results were obtained at 450 nm using the Tecan Spark multi-function enzyme labeler. They were divided into 6 groups to test their biocompatibility and cytotoxicity. (1) PBS; (2) ScNP-MS@MC540; (3) ScNP-MS@MC540@NEAA; (4) PBS+X-ray; (5) ScNP-MS@MC540+X-ray; (6) ScNP-MS@MC540@NEAA+X-ray.

Next, we investigated the ScNP-MS@MC540@NEAA nanoparticles' effect on HeLa cell viability under different doses of X-ray radiation: 0, 20, 30, 50, 70, 90, and 100 mGy. The experimental procedure was similar to the one described above, with the ScNP-MS@MC540@NEAA nanoparticles concentration set at 200 μg/mL, and 0 μg/mL serving as the control group. Additionally, to explore the depth of X-ray

penetration, we employed the same treatment method while covering the 96-well plate with pork tissue of varying thickness (0, 1, 2, 3, 4, and 5 cm) during X-ray irradiation. The depth of X-ray penetration was assessed by measuring the survival rate of HeLa cells.

2.2 Intracellular GSH measurement

The amount of intracellular glutathione was measured by using the membrane-permeant naphthalene-2,3-dicarboxaldehyde (NDA), an incredibly sensitive fluorescent probe. NDA can react with GSH to produce strong fluorescence isoindole adducts. HeLa cells were initially inoculated into 6-well plates (2×10^5 cells per well) and cultured at 37 ℃ for 12 h. The cells were then treated with fresh media containing different samples: (1) PBS; (2) ScNP-MS@MC540 (100 μg/mL); (3) ScNP-MS@MC540@NEAA (100 μg/mL); (4) PBS+X-ray; (5) ScNP-MS@MC540 (100 μg/mL) +X-ray; (6) ScNP-MS@MC540@NEAA (100 μg/mL) +X-ray. After removing any remaining nanomaterials, each well received 1 mL of serum-free medium containing 50 μmol NDA. The medium was then incubated for 20 minutes. After washing with PBS for three times, the cells were observed with CLSM.

Intracellular GSH detection: HeLa cells were inoculated on a 6-well plate at a density of 2×10^6 cells per well and incubated in an incubator for 24 h. The cells are then treated with fresh media containing different samples (same as above). After incubating for 4 h, the medium was discarded and 250 μL cell lysis buffer was added to the cells for 30 min. HeLa cells were then collected and washed twice with cold PBS. The triploid cell extraction buffer was re-suspended, the freeze-thaw cycle was repeated 2-3 times (4 ℃, 900 rpm, 10 min), and the supernatant was placed on the ice to be measured. CheKine reduced glutathione colorimetric assay kit was used to measure the absorbance at 412nm with Tecan Spark multi-function enzyme marker, and GSH levels in each group were calculated according to the standard fitting curve.

2.3 Detection of ROS, O_2, NO, and $ONOO^-$ intracellular

HeLa cells were cultured in a 6-well culture plate with a density of 2×10^5 cells. After incubation at 37 ℃ for 12 hours, the cells were treated with fresh media containing different samples (grouped as above). For ROS, O_2, NO, $\cdot O_2^-$ and $ONOO^-$

tests, the probes of DCFH-DA, RDPP, DAF-FM DA, and O58 were added to the corresponding dishes and incubated for 30 minutes according to the manufacturer's instructions. Finally, intracellular ROS, O_2, NO, $\cdot O_2^-$ and $ONOO^-$ were detected using CLSM.

2.4 Mitochondrial Integrity Detection

Mitochondrial membrane potential detection. JC-1 is an ideal fluorescent probe for detecting ΔΨm in cell, tissue or purified mitochondrial membrane potential. JC-1 exists in monomer and polymer form, and their emission spectra are different. In normal cells, the membrane potential of mitochondria is high, and JC-1 exists in the matrix of mitochondria in the form of polymers, producing red fluorescence. The mitochondria are damaged, the mitochondrial membrane potential is reduced, and JC-1 exists as a monomer in the mitochondrial matrix, producing green fluorescence. The change of JC-1 fluorescence from red to green can reflect the decline of mitochondrial membrane potential, so the change of JC-1 fluorescence color can be used as an early detection index of apoptosis. Hela cells were initially inoculated into 6-well plates (2×10^5 cells per well) and cultured at 37 ℃ for 12 hours before being treated with fresh media containing different samples (grouped as above). After removing any remaining nanomaterials, each well received 1mL of serum-free medium containing JC-1 staining solution. The medium was then incubated for 20 minutes. After washing with PBS for three times, the cells were observed with CLSM.

2.5 DNA damage detection

Intracellular DNA damage detection. Intracellular DNA damage was measured using γ-H2AX immunofluorescence. H2AX is one of the variants of histone H2A. When DNA double bond breaks, ATM and ATR in the phosphatidylinositol 3 kinase (PI3K-related kinases (PIKKs) family members phosphorylate serine 139 on H2AX to form acidified H2AX and γ-H2AX. DNA damage is determined by γ-H2AX detection. Repeat the preceding steps.

2.6 Cell viability assay

The effects of nanoparticles on tumor cells were evaluated using living and dead staining methods. The procedure was the same as above. Cells were stained with new medium containing Calcien AM (1 μL) and PI (1 μL) solutions for 30 minutes and

observed under CLSM at 480 nm and 525 nm, respectively.

2.7 In Vivo Animal Experiments

All animal experiments were conducted in accordance with the requirements of the laws of the People's Republic of China (GB14925-2010) and approved by the Shanghai Municipal Science and Technology Commission (SYXK (Shanghai) 2019-0020). Animal testing procedures comply with the guidelines and nursing regulations of the Regional Animal Testing Ethics Committee approved by the Shanghai University Experimental Animal Management Committee. (Shanghai, China). Healthy female Balb/c nude mice (4 weeks old) were purchased from Zhejiang Wetahe Experimental Animal Technology Co., LTD., and 8×10^6 Hela cells were injected into 100 μL DMEM medium and inoculated subcutaneously into the right hind limb of nude mice to establish Hela tumor model. Tumor growth was measured daily. The tumor volume (V) is calculated as $V= (L\times W^2) /2$, where L and W represent the length and width of the tumor, respectively. Relative tumor volume is measured as relative tumor volume =V/V0 (V0: initial volume before treatment). When the tumor volume approached 50-80mm3, nude mice carrying Hela tumors were selected for in vivo experiments.

The tumor-bearing mice were randomly divided into 6 groups (n=3) and given 100 μL of different preparations intravenously: (1) PBS; (2) ScNP-MS@MC540 (100 μg/mL); (3) ScNP-MS@MC540@NEAA (100 μg/mL); (4) PBS+X-ray; (5) ScNP-MS@MC540 (100 μg/mL) +X-ray; (6) ScNP-MS@MC540@NEAA (100 μg/mL) +X-ray. Each group received three injections every three days. The first injection day was called day 0, and the tumor volume was measured every other day after that. At the same time, the weight of each mouse was recorded every other day during treatment. After 14 days of treatment, all mice were euthanized and their tumors were collected for photography and gravimetric analysis. In addition, one tumor and major organ (heart, liver, spleen, lung, and kidney) were randomly selected from each group for hematoxylin and eosin (H&E) and terminal deoxynucleotide transferase (TdT) dUTP notch end labeling (TUNEL) staining.

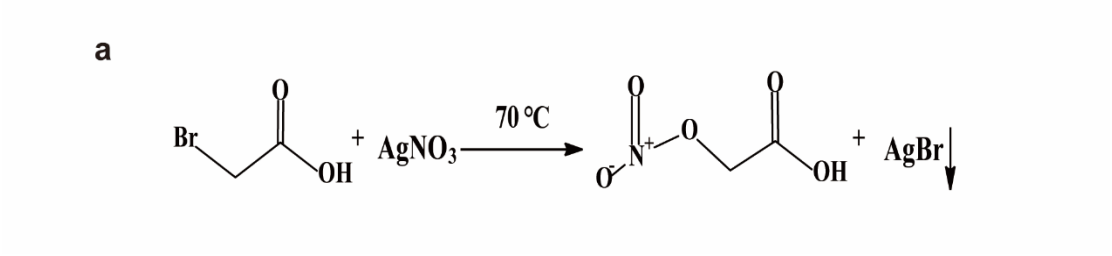

Figure S1. (a) Synthetic route of NEAA.

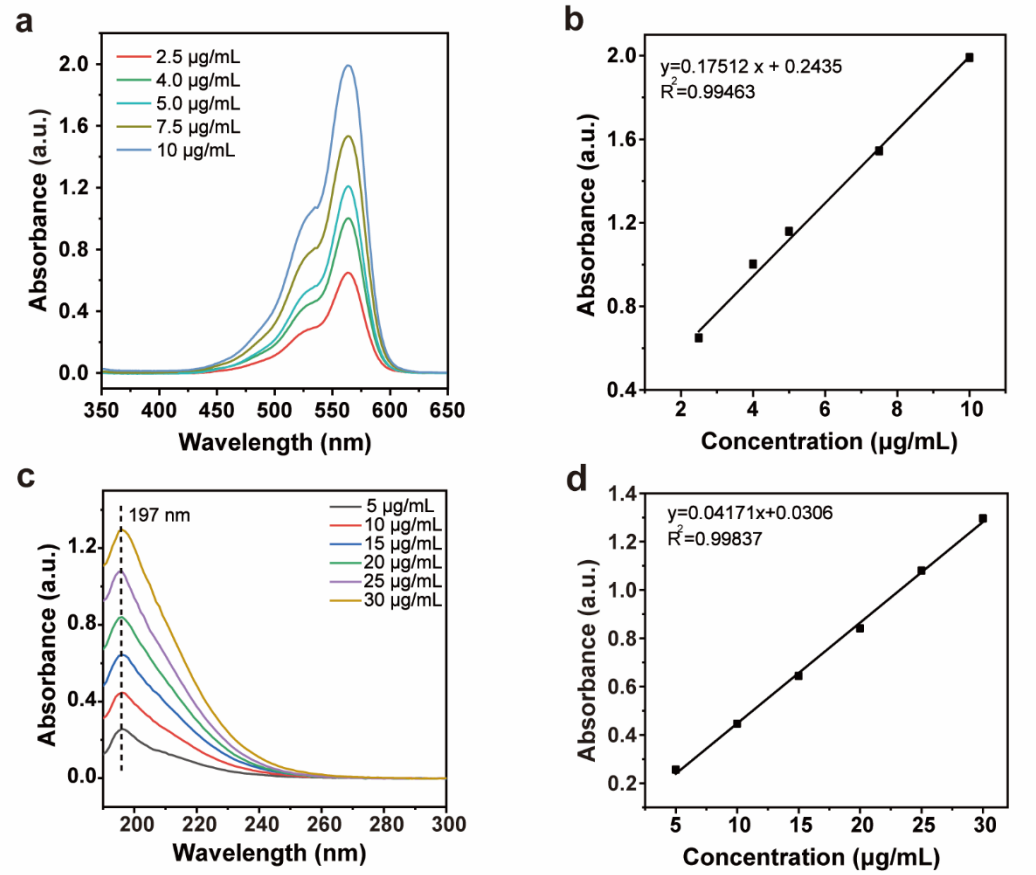

Figure S2. UV-vis absorption curves of MC540 (a) and NEAA (c) at different concentrations. Fitting curve of absorbance at maximum absorption wavelength to different concentrations of MC540 (b) and NEAA (d).

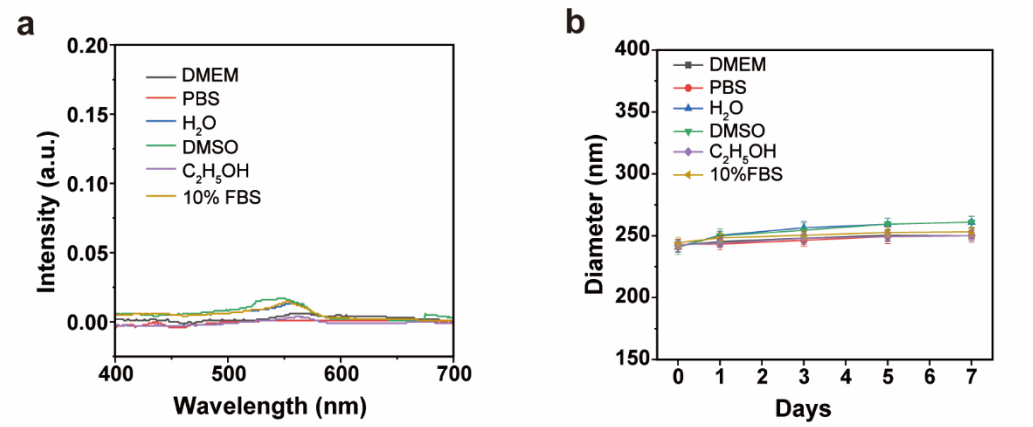

Figure S3. (a) UV-vis absorption spectra of ScNP-MS@MC540@NEAA NPs after 24 h in ultrapure water, DMSO, C_2H_5OH, PBS, 10%FBS and DMEM. (b) Particle size change of ScNP-MS@MC540@NEAA NPs in different solvents for 7 days.

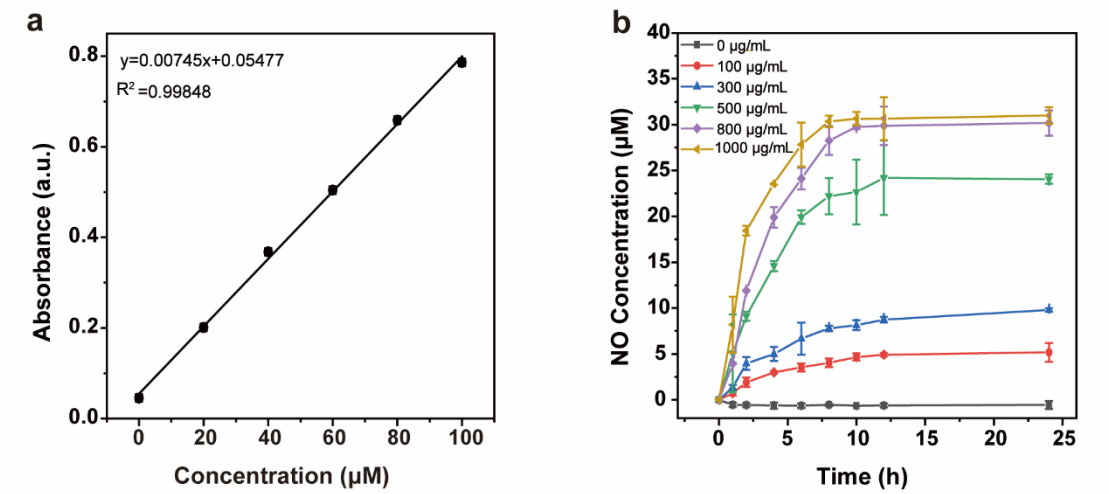

Figure S4. (a) Plot of absorbance versus concentrations of NO. (b) The plot of NO concentrations and reaction time in different concentrations of ScNP-MS@MC540@NEAA.

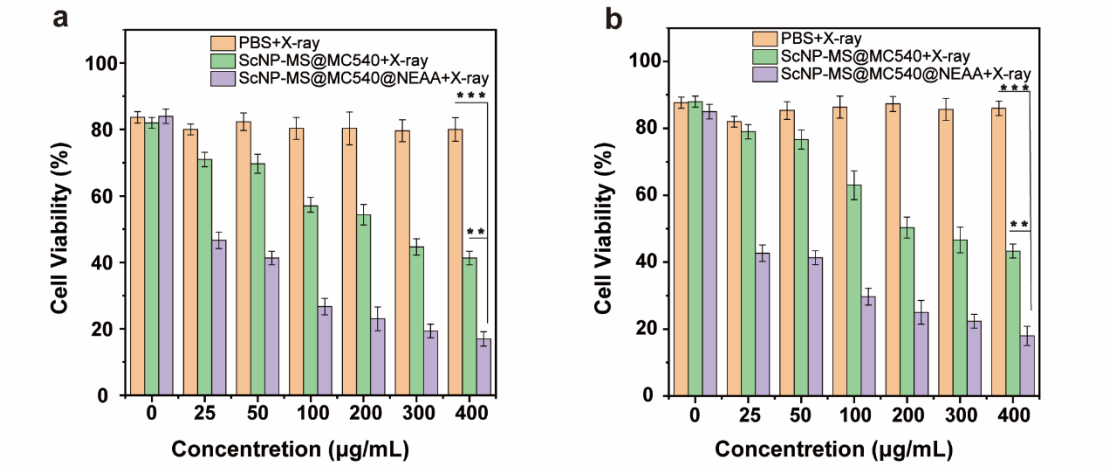

Figure S5. The cell viability of HeLa cells with (a) or without (b) pork tissue (2 cm) cover under different conditions. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

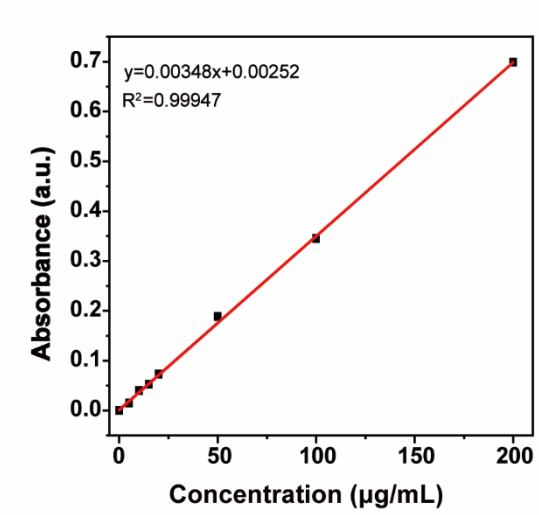

Figure S6. Plot of absorbance versus concentrations of GSH, based on the GSH Kit.

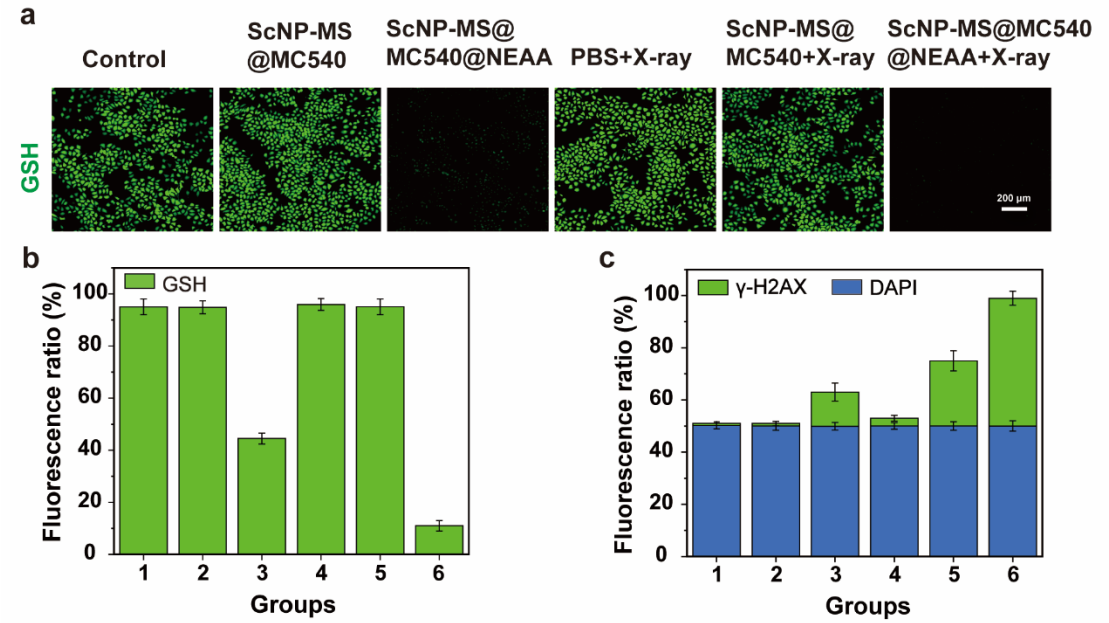

Figure S7. (a) CLSM images and (b) fluorescence quantitative analysis of GSH of HeLa cells with different conditions. (c) Fluorescence quantitative analysis of γ-H2AX of HeLa cells with different conditions.

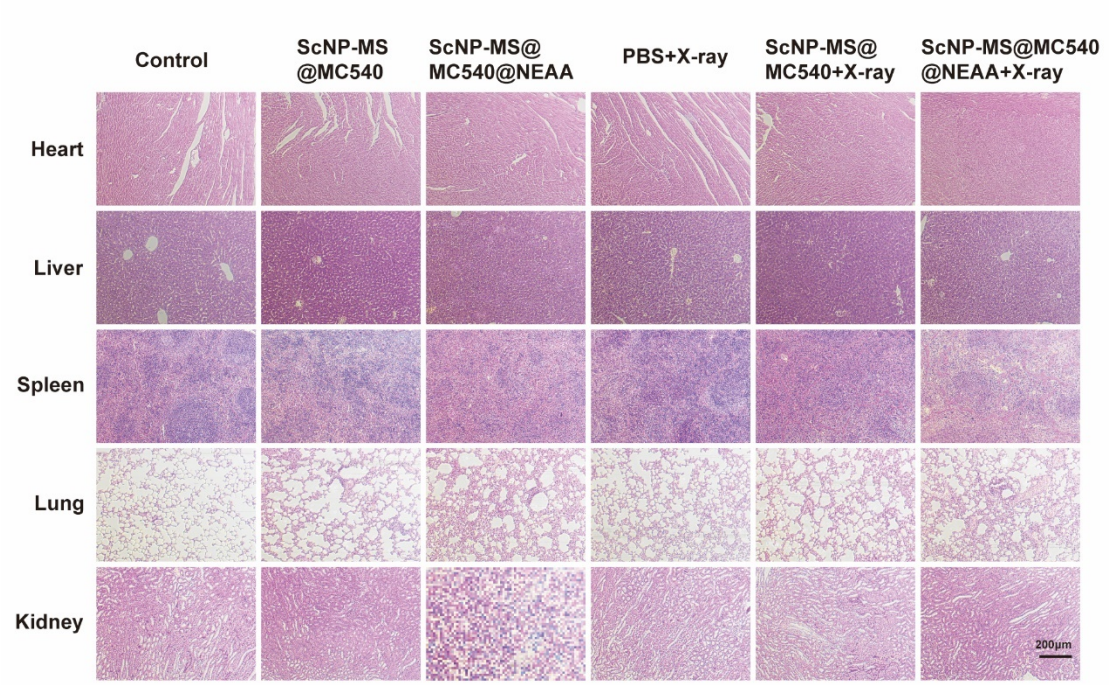

Figure S8. Histological analysis of hematoxylin and eosin (H&E) staining of the major organs (heart, liver, spleen, lung, and kidney).