

Supplementary material

Development of interactive tumor vascular suppression strategy to inhibit multidrug resistance and metastasis using pH/H₂O₂-responsive nanohybrids with oxygen-producing

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S1. Preparation of FA-BSA-MnO₂/DOX/siRNA NPs

FA-BSA-MnO₂/DOX/siRNA NPs were prepared by three separate steps. In the first step, BSA-MnO₂ NPs were synthesized by a typical operation ¹. Briefly, 3 mL BSA solution (2 mg mL⁻¹) and 1.2 mL MnCl₂·4H₂O solution (1 mg mL⁻¹) were added to a beaker and magnetically stirred for 10 min at 37°C. Subsequently, by adding 1M sodium hydroxide, the solution pH was adjusted to 9.0. The reaction was continuously stirred for 2 h to create BSA-MnO₂ NPs.

In the second step, BSA-MnO₂/DOX/siRNA NPs were prepared based on a desolvation and cross-linking technique ². DOX solution (1 mg mL⁻¹, 2 mL) and 0.5 OD siRNA were added into the prepared BSA-MnO₂ solution while stirring, and adjust the pH of the solution to 9.0 by adding 1 M NaOH. Then 1.2 mL absolute alcohol was mixed to the solution, after that stirring for 1 h at room temperature. Next, 50% glutaraldehyde (3 μL) was added to cross-link the amino groups on the protein. This process was stirred in the dark for 12 h. After these steps, we obtained the BSA-MnO₂/DOX/siRNA NPs.

The last step, FA-BSA-MnO₂/DOX/siRNA NPs were prepared by conjugating BSA-MnO₂/DOX/siRNA with FA via amino group ³. NHS and EDC (molar ratio of FA/EDC/NHS: 1/5/5) were dissolved in 3 mL of PBS for activation of FA (0.6 mg) at room temperature, and the

reaction was carried out for 2 h under dark conditions. Subsequently, the activated FA solution was added into BSA-MnO₂/DOX/siRNA solution, and the solution was stirred for 12 h. For the purification of the prepared FA-BSA-MnO₂/DOX/siRNA NPs, ethanol was evaporated and the non-encapsulated two drugs were removed through a 30 kDa Amicon filter (Millipore) and FA-BSA-MnO₂/DOX/siRNA NPs were washed three times with Milli-Q water. At last, the FA-BSA-MnO₂/DOX/siRNA NPs were stored in a refrigerator (4°C) for the subsequent use.

S2. Characterization of nanoparticles

A dynamic light scattering (DLS) instrument was used to characterize particle size and zeta potential. Transmission electron microscopy (TEM) was used to characterize the morphology of nanoparticles. The optical properties of FA, BSA, DOX, BSA-MnO₂ and FA-BSA-MnO₂/DOX/siRNA were detected by UV spectroscopy. X-ray photoelectron spectroscopy (XPS) was employed to analyze the chemical property of Mn in FA-BSA-MnO₂/DOX/siRNA. An oxygen probe (JPBJ-608 portable Dissolved Oxygen Meters) was used for measuring the dissolved O₂ of the different concentration FA-BSA-MnO₂/DOX/siRNA NPs in 100μM H₂O₂ solution⁴. Magnetic resonance imaging (MRI) of NPs was recorded with a 3-T MRI instrument. The agrose gel electrophoresis was used to confirm the stability of siRNA in serum⁵. The content of MnO₂ in the purified nanocomposites was detected by ICP-MS.

S2.1. Characterization of siRNA

Firstly, the prepared carrier BSA-MnO₂ was negatively charged, and then the mixture of DOX and siRNA was added to the above carrier solution. Since the positive charge of DOX is much higher than the negative charge of siRNA, the mixed potential of DOX and siRNA was 8.06 mV (Fig.S1), eliminating the electrostatic repulsion between siRNA and BSA. Secondly, DOX and siRNA were

encapsulated in a carrier by cross-linking. Under the action of a cross-linking agent, DOX and siRNA were encapsulated in the carrier protein. Therefore, siRNA can be successfully packaged.

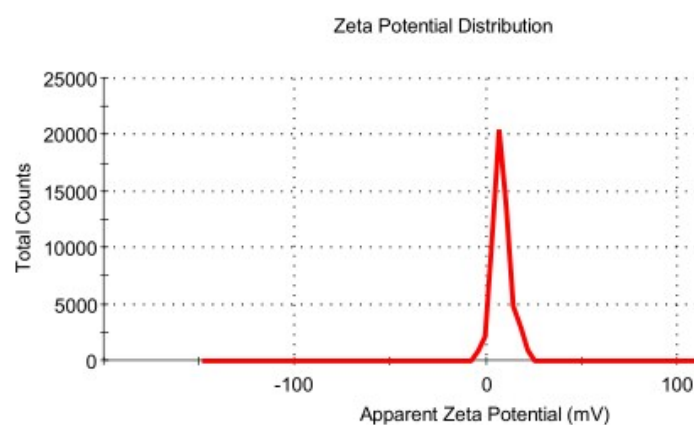


Fig. S1. The mixed potential of DOX and siRNA

The fluorescence intensity of ethidine bromide (EB) increases when it binds to the A/G base of nucleic acid. Firstly, the prepared FA-BSA-MnO₂/DOX/siRNA NPs were ultra-filtered to remove impurities, and then the purified FA-BSA-MnO₂/DOX/siRNA NPs were decomposed with hydrogen peroxide to release DOX and siRNA. The fluorescence spectra of the EB solution, EB and the lysed FA-BSA-MnO₂/DOX/siRNA were measured.

As shown in **Fig. S2**, the fluorescence intensity of EB increases with the amount of siRNA in the solution (EB concentration was 5 $\mu\text{g mL}^{-1}$). The results showed that siRNA was successfully encapsulated in FA-BSA-MnO₂/DOX/siRNA NPs.

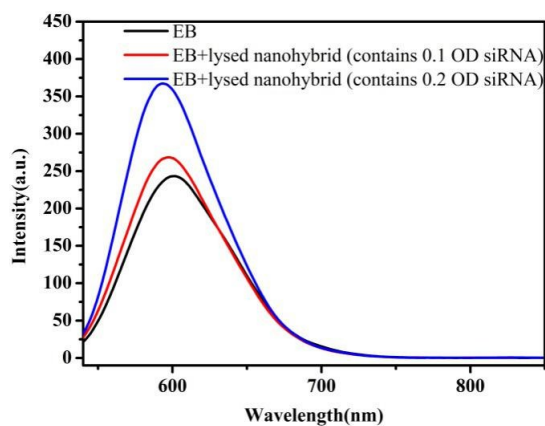


Fig. S2. The fluorescence spectra of different solutions

Gel retardation assay: To explore the proper weight ratio of carrier to the siRNA, agarose gel retardation assays were carried out. We synthesized FA-BSA-MnO₂/DOX/siRNA NPs containing different siRNA contents and removed unreacted substances and siRNA by ultrafiltration centrifugation (molecular weight of 30 KD). FA-BSA-MnO₂/DOX/siRNA NPs fabricated at different molar ratios (siRNA to carrier: 1:12, 1:24, 1:36, 1:48) were mixed with an appropriate amount of loading buffer and electrophoresed on 1% (w/v) agarose gel at 90 V for 20 min. After electrophoresis, the gel block was stained with ethidium bromide for 8 minutes. The gel was visualized using a multifunctional imaging instrument (Ultra-Violet, USA).

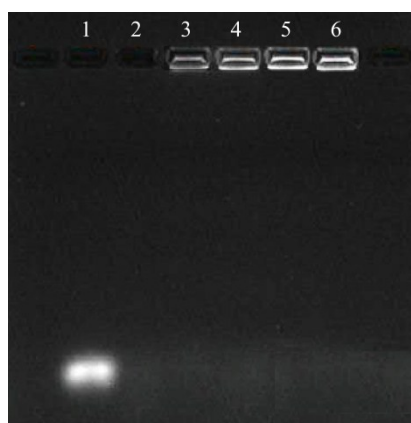


Fig. S3. Fabrication of FA-BSA-MnO₂/DOX/siRNA NPs with various molar ratios of siRNA to carrier: (1) free siRNA, (2) carrier, (3) 1:12, (4) 1:24, (5) 1:36, (6) 1:48, respectively.

S3. Degradation of H₂O₂ by nanohybrid

For the degrading experiments, FA-BSA-MnO₂/DOX/siRNA NPs (90 μM, contains 150 μM MnO₂) were placed in cell medium containing 10% FBS at 37°C, and H₂O₂ (1mM) was added to initiate the reaction. The residual concentration of H₂O₂ was determined over time using a PeroXOquant assay kit (Solarbio, USA) at 37°C. Cell medium with 10% FBS was considered as the control group. The hypoxic air bag was used to create a hypoxic environment during the experiment.

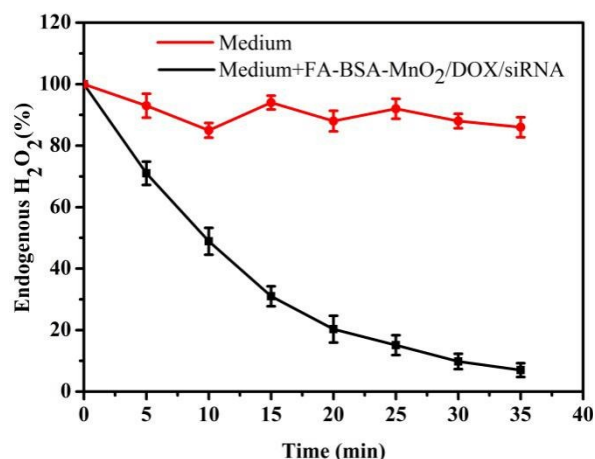


Fig. S4. Degradation of H₂O₂ (1 mM) by the FA-BSA-MnO₂/DOX/siRNA NPs.

S4. *In vitro* DOX release

To investigate pH/H₂O₂-responsive release, FA-BSA-MnO₂/DOX/siRNA NPs containing 1 mg DOX were loaded into dialysis bag (MWCO of 14, 000) and dialysate was 30 mL PBS (pH 7.4, 5.0, pH 5.0 containing 100 μM H₂O₂, respectively), shaking at 150 rpm (37°C). At different time intervals, 2 mL of the release medium was removed and same volume fresh medium was added. The release of DOX was quantified by UV-vis test at 485 nm.

S5. *In vitro* siRNA release

To study pH/H₂O₂-responsive release, FA-BSA-MnO₂/DOX/siRNA NPs containing 0.5 OD siRNA were loaded into dialysis bag (MWCO of 14, 000) and dialysate was 30 mL PBS (pH 7.4, 5.0, pH 5.0 containing 100 μM H₂O₂, respectively), shaking at 150 rpm (37°C). At different time intervals, 2 mL of the release medium was removed and same volume fresh medium was added. The release medium was placed in an ultrafiltration tube with a molecular weight of 3 KD to remove DOX (3500 rpm, 20 min), and the siRNA released in the inner tube was measured by fluorescence intensity quantitation with the excitation wavelength set at 485 nm and emission wavelength set at 518 nm.

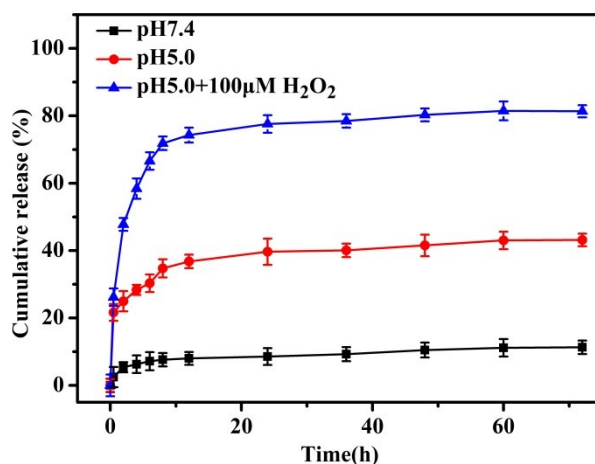


Fig. S5. *In vitro* release profiles of siRNA

S6. Cell culture

DOX-resistant breast cancer MCF-7/ADR cells and 4T1 cells were obtained from the iCell Bioscience Inc (Shanghai, China). The 4T1 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U mL⁻¹ penicillin and 100 mg mL⁻¹ streptomycin. The MCF-7/ADR cells were cultured in RPMI-1640 medium supplemented with 15% FBS, 1 μM DOX and 1% penicillin/streptomycin. Cell culture was maintained in a humidified atmosphere containing 5% CO₂ at 37°C.

S7. Study on intracellular generation of O₂ from the FA-BSA-MnO₂/DOX/siRNA NPs

The intracellular generation of O₂ was investigated with Ru(dpp)Cl₂ (RDPP) (Aladdin, USA) by a fluorescence microscope. MCF-7/ADR cells were incubated with 1 × 10⁻⁵ μM RDPP for 4 h and further incubated with FA-BSA-MnO₂/DOX/siRNA NPs in hypoxic air bags for 0, 4, 8 and 12 h. Fluorescence quenching of RDPP was observed by fluorescence microscopy.

S8. Cell uptake of siRNA

MCF-7/ADR cells (Human anti-DOX breast cancer cell lines) were inoculated in 6-well culture plates for 24 h and the cell density was 2 × 10⁵ cells/well, followed by treatment with free siRNA, BSA-MnO₂/DOX/siRNA and FA-BSA-MnO₂/DOX/siRNA NPs (the concentration of siRNA: 10

nM) in normal culture medium. We labeled BSA with the packaged DOX. After 12 h of incubation, cells were washed three times with PBS. Followed by adding 4% paraformaldehyde to soak for 10 min. Then DAPI staining solution ($5 \mu\text{g mL}^{-1}$) was mixed to each well in the plate to dye nuclei for 10 min. Cell imaging was conducted by a fluorescence microscope.

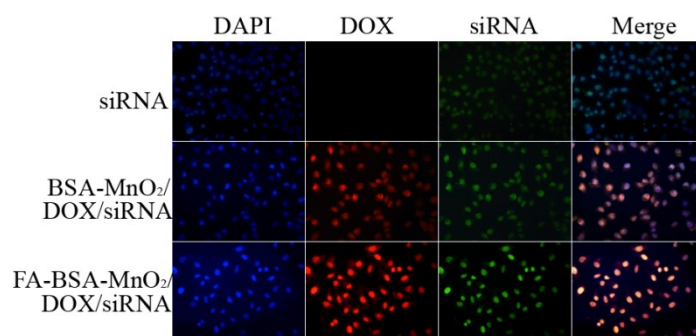


Fig.S6. Fluorescent micrographs of MCF-7/ADR cells treated with siRNA, BSA-MnO₂/DOX/siRNA and FA-BSA-MnO₂/DOX/siRNA for 12 h.

S9. Animal model

All animal experiments were performed under a protocol approved by the Henan Laboratory Animal Center. The MCF-7/ADR tumor models were generated by subcutaneous injection of 1×10^6 MCF-7/ADR cells in 100 μL PBS into the right mammary gland of female BALB/c nude mice (18 ± 2 g, obtained from Hunan SJA Laboratory Animal Co., Ltd). Female BALB/c mice (18 ± 2 g, obtained from Hunan SJA Laboratory Animal Co., Ltd) were used to investigate the inhibition efficacy of tumor growth and lung metastasis *in vivo*. Briefly, 100 μL PBS containing 1×10^6 4T1 cells were subcutaneously implanted in the right mammary gland of female BALB/c mice, which also were used for the biodistribution and *in vivo* MRI. Establishment of lung metastasis model: 4T1 cell suspension was injected into the tail vein of the mice at 5.0×10^5 cells per mouse in 50 μL PBS.

S10. The efficacy of cooperative therapy *in vivo*

The MCF-7/ADR tumor model was established to evaluate the efficacy of resistant breast cancer of different NPs. Briefly, the mice were randomly divided into 7 groups, and each experimental group was consisted of six mice. Mice in different groups were intravenously injected with saline, FA-BSA-MnO₂, free DOX, FA-BSA-MnO₂/siRNA, FA-BSA-MnO₂/DOX, BSA-MnO₂/DOX/ siRNA, FA-BSA-MnO₂/DOX/siRNA NPs (DOX dose: 5 mg kg⁻¹, siRNA dose: 47 μg kg⁻¹) via tail vein, respectively. The experiment was carried out once every two days for 2 weeks. The changes of tumor size and body weight were detected every two day. As experiment ended, the tissues were removed and rinsed with PBS. Finally, the tissue was fixed in 4% paraformaldehyde solution for H&E staining.

S11. HE slices of organs

To evaluate the efficacy of resistant breast cancer of different NPs, the tissues were removed and rinsed with PBS when the experiment ended. Finally, the tissues were fixed in 4% paraformaldehyde solution for H&E staining.

The potential toxicity of the animal organs was further studied by HE staining (**Fig. S7**). Cardiomyocytes in the DOX group showed nuclear detachment and cytoplasm shrinkage, indicating that DOX has toxic side effects on the heart. The organs of other groups (heart, liver, spleen, lung, kidney) grew well, the cell structure was intact, the edge was clear, and there was no obvious cell damage. This result further proves that FA-BSA-MnO₂/DOX/siRNA NPs have good biosafety.

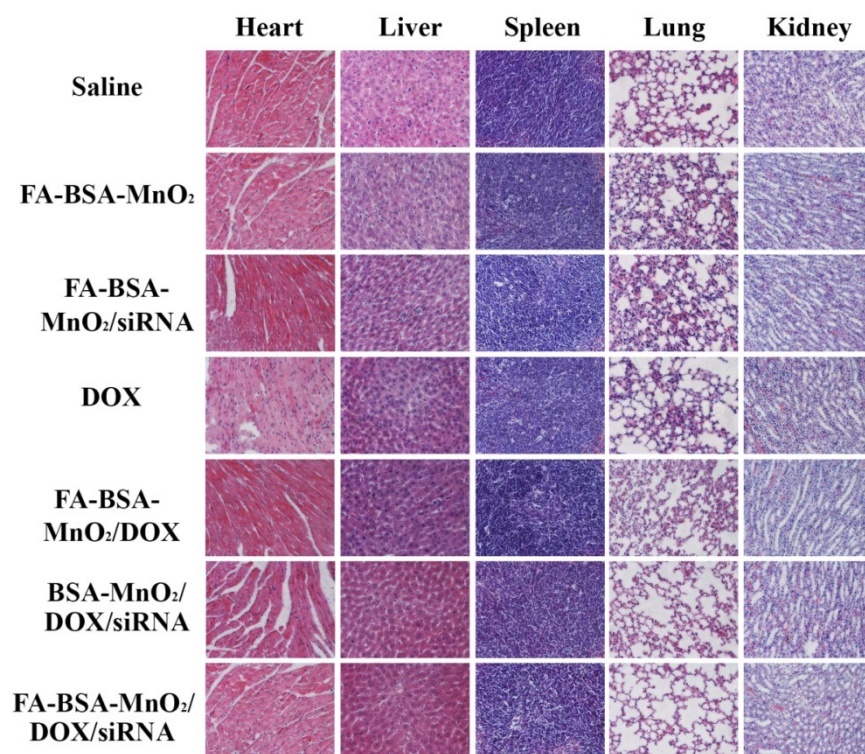


Fig. S7. H&E stained heart, liver, spleen, lung and kidney harvested from the mice with different treatments.

S12. Ex vivo Immunofluorescence Staining

The MCF-7/ADR tumor bearing mice were treated with intravenous injection: saline, FA-BSA-MnO₂, FA-BSA-MnO₂/siRNA, FA-BSA-MnO₂/DOX, FA-BSA-MnO₂/DOX/siRNA, and FA-BSA-MnO₂/DOX/siRNA (DOX dose: 5 mg kg⁻¹, siRNA dose: 47 μg kg⁻¹). After 24 h of administration, the tumor was removed from mice and immunofluorescence was performed⁶. The sections were incubated with mouse anti-HIF-1α antibody and 488-conjugated goat anti-mouse secondary antibody to detection of HIF-1α. Staining of blood vessels was performed with rabbit anti-CD31 antibody and Cy3-conjugated goat anti-rabbit secondary antibody. Cell nuclei were stained with DAPI. The sections were observed and photographed under a fluorescence microscopy. To analyze the tumor hypoxia image with Image J software. The hypoxic positive area (%) = visible

hypoxic marker in tumor tissue section/total area ($n = 3$).

S13. Transwell assay

4T1 cells were cultured on the upper chamber of transwells by density of 8×10^4 cells per chamber. 100 μL of medium containing 1% FBS with free DOX, FA-BSA-MnO₂, FA-BSA-MnO₂/siRNA, FA-BSA-MnO₂/DOX/siRNA NPs (DOX dose: $1 \mu\text{g mL}^{-1}$) were added into the upper chamber, while 800 μL of media containing 10% FBS were added to the lower chambers. After 24 h incubation, the upper media were removed, and a cotton swab was applied to take away the remaining cells. Then, the transwell chambers were washed three times with PBS, and the migrating cells were fixed with methanol for 15 min and stained with crystal violet for 20 min. Finally, the upper chambers were washed twice with PBS, and every chamber was photographed using a fluorescence microscope. Image J software was used to quantify the proportion of cells migrated from the upper chamber to the lower chamber.

The invasion ability of 4T1 cells was evaluated using matrigel-coated transwell chambers following the similar procedure applied for migration staining assay.

S14. Inhibition of lung metastasis in vivo

In brief, 4T1 cell suspension was injected into the tail vein of the mice at 5.0×10^5 cells per mouse in 50 μL PBS. After injection of tumor cells, all mice were randomly assigned to 7 groups ($n = 6$). After 10 days, the mode of administration and grouping were consistent with the above pharmacodynamic experiments. After treatment for 14 d, the lungs were excised, rinsed with PBS and soaked in a 4% paraformaldehyde solution, embedded with paraffin for H&E staining. To investigate the lung metastasis, the lungs were photographed for counting the numbers of metastatic nodules on the lung, and the percentage of metastatic nodules was derived from the following

equation: metastatic nodules (%) = (metastatic nodules numbers after cure)/ (metastatic nodules numbers of blank control) \times 100%.

S15. The biodistribution and *in vivo* MRI

To study the tumor accumulation capacity of FA-BSA-MnO₂/DOX/siRNA NPs in 4T1 tumor mice, the biodistribution and tumor accumulation were observed using a noninvasive near-infrared optical imaging system. In this experiment, near-infrared dye IR783 was loaded in NPs⁷⁻⁹. Free IR783 or IR783-labeled NPs were injected intravenously via the tail vein into mice (IR783 dose: 0.5 mg kg⁻¹). Then, the imaging data were collected at different time points. The mice were sacrificed 12 h after intravenous injection. Tumors and major organs were collected for *ex vivo* imaging. The acquisition parameters were as follows: λ_{em} = 790 nm, λ_{ex} = 720 nm, binning = 1, exposure time = 0.1 s. The contents of MnO₂ in the tissues were detected by ICP-MS (**Fig. S8**).

Under the authorization of the regional ethics committee, *in vivo* experiments were carried out on the 4T1 tumor mice. The mice were injected intravenously with the FA-BSA-MnO₂/DOX/siRNA NPs (doses: DOX 5 mg kg⁻¹, MnO₂ 1.3 mg kg⁻¹) and imaged at 0, 1, 2, 4, 8, 12 and 24 h using a 3-T clinical MR scanner.

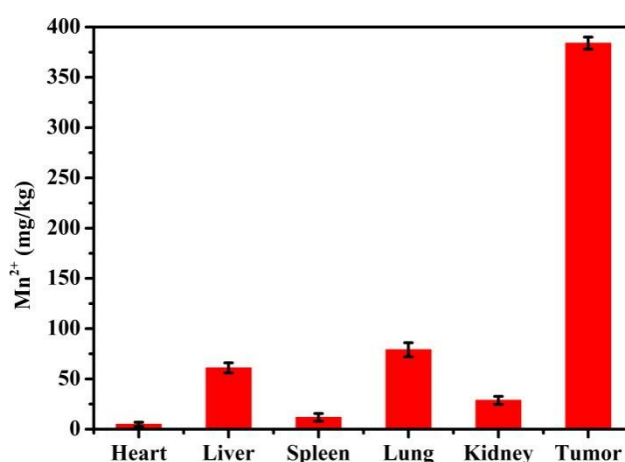


Fig. S8. Quantitation the amount of MnO₂ presence in the tissues by ICP-MS.

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