

Electronic Supplementary Information

of

**A MSN-Based Tumor-Targeted Nanoplatfom to Interfere with
Lactate Metabolism to Induce Tumor Cell Acidosis for Tumor
Suppression and Anti-Metastasis**

Zhao-Xia Chen,[§] Miao-Deng Liu,[§] Deng-Ke Guo, Mei-Zhen Zou, Shi-Bo Wang, Han
Cheng,* Zhenlin Zhong, Xian-Zheng Zhang*

Key Laboratory of Biomedical Polymers of Ministry of Education & Department of
Chemistry, Wuhan University, Wuhan 430072, PR China

* Corresponding authors.

E-mails: chm128256@163.com (H. Cheng), xz-zhang@whu.edu.cn (X. Z. Zhang).

[§]Z. X. Chen and M. D. Liu contributed equally to this work.

Materials: Tetraethyl orthosilicate (TEOS), 3-mercaptopropyl trimethoxysilane (MPTMS), polyethyleneimine (PEI, Mw = 1800 Da) and glutathione (GSH) were purchased from Sigma-Aldrich (USA). Hydrochloric acid (HCl), methanol, cetyltrimethylammonium chloride (CTAC), potassium permanganate (KMnO₄), and triethanolamine (TEA) were purchased from Shanghai Chemical Reagent Co., Ltd. (China). NHS-PEG-FA (Mw = 2000 Da) was obtained from Shanghai Ponsure Biotech, Inc. (China). 1,1-Dimethylbiguanide hydrochloride (Me) was obtained from Tianjin Heowns Biochemical Technology Co., Ltd. (China). Fluvastatin sodium (Flu) was purchased from Shanghai Bide Pharmatech Ltd. (China). 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) and BCECF-AM fluorescent probes were purchased from Beijing innoChem Science&Technology Co., Ltd. (China). HEPES buffer solution was purchased from Shanghai Macklin Biochemical Technology Co., Ltd. (China). The apoptosis/necrosis detection kit and Hoechst33342 were obtained from Beyotime Biotechnology (China). Agarose, trypsin, fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), RPMI 1640, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium-bromide (MTT), phosphate buffered saline (PBS), and penicillin-streptomycin were obtained from Lonza Group Ltd. (Switzerland). All other reagents and solvents were of analytical grade and used directly.

Apparatus: The morphology of the MSN and MSN@MnO₂ nanoparticles was obtained by transmission electron microscopy (TEM, JEM-2100) and scanning electron microscopy (SEM, Sigma). TGA was measured by a Shimadzu

thermogravimetric analyzer (DTG-60, Japan). Zeta potentials and hydration particle size were tested on a zeta sizer (Nano ZS, Malvern Instruments). The UV-vis absorbance of Me and Flu were measured by UV-vis spectroscopy (Lambda Bio40). The coating amount of manganese dioxide on MSN was measured by ICP-AES (IRIS Intrepid II XSP, USA Thermo Elemental). MR imaging experiments were performed in a Bruker BioSpec 4.7 T/20 cm system (Bruker, Ettlingen, Germany). Blood biochemistry analysis was examined by biochemical auto analyzer (MNCHIP, China).

Synthesis of MSNs: Mesoporous silicon nanoparticles (MSNs) were synthesized based on previous literature.^{S1} Briefly, 10 g of CTAC and 0.4 g of TEA were dissolved in 100 mL of deionized water and vigorously at 95 °C for 1 h. Then, 7.5 mL of TEOS was slowly added dropwise and the mixture was stirred for 1 h. The white solid (CTAC@MSN) was obtained by centrifugation at 11 000 rpm for 30 min and washed with deionized water and methanol for several times. After vigorously stirring the mixture of CTAC@MSN solution and concentrated hydrochloric acid overnight, the CTAC was removed by refluxing at 60 °C for 48 h to obtain the MSNs.

Synthesis of MSN-SH: 3 mL of MPTMS was thoroughly mixed with 100 mg of CTAC@MSN in 50 mL of methanol. After stirring for 72 h at room temperature, CTAC@MSN-SH NPs were collected by centrifugation and vacuum drying. Finally, MSN-SH NPs were obtained after removing the CTCA template and stored at 4 °C.

Synthesis of Me&Flu@MSN: First, excess Me (4 mL, 30 mg/mL) and Flu (4 mL, 30 mg/mL) were separately dissolved in a mixed solvent (H₂O: DMF = 1: 1) to obtain a stock solution. Then, 20 mg of MSN-SH NPs were added to the as-obtained stock solution (20 mL, 1 mg/mL) and stirred overnight. The superfluous Me and Flu were removed by centrifugation and washed with deionized water.

Synthesis of Me&Flu@MSN@MnO₂: 30 mL of KMnO₄ solution (1 mg/mL) was added dropwise to the same volume of aqueous solution consisting of Me&Flu@MSN NPs (1 mg/mL). After stirring for 10 min, Me&Flu@MSN@MnO₂ NPs were washed for several times with deionized water and re-dispersed in water for further use.

Synthesis of Me&Flu@MSN@MnO₂-FA: 10 mL of Me&Flu@MSN@MnO₂ (4 mg/mL) NPs were poured to the vial containing 40 mL of PEI (5 mg/mL) solution under ultrasound and vigorously stirred at room temperature for 24 h. Subsequently, the free PEI was removed by centrifugation and the PEI-modified Me&Flu@MSN@MnO₂ NPs were added to 20 mL of NSH-PEG-FA solution (2 mg/mL). After stirring for 24 h, the solid materials were obtained by centrifugation and washed with deionized water for three times. The PEG-FA-modified Me&Flu@MSN@MnO₂ NPs were re-dispersed in deionized water for further characterization and analysis.

In Vitro Drug Release Study: Me@MSN@MnO₂-FA and Flu@MSN@MnO₂-FA (15 mg) were dispersed into 3 mL of deionized water and divided into three parts. The dialysis bag (Mw = 3000 Da) containing the mixture (1 mL) was immersed in 10 mL solution with different concentrations of GSH (0 mM, 5 mM, 10 mM) and placed on a shaker at 37 °C. The dialysate was taken out at a predetermined time point and the released Me and Flu were measured by UV-vis spectroscopy (Lambda Bio40).

Cell Culture: Human breast tumor cells (MCF-7) were cultured with DMEM medium. Murine mammary carcinoma cells (4T1) and colon adenocarcinoma cells (CT26) were incubated with RPMI 1640 medium. All cells were cultured at 37 °C in a humidified atmosphere containing 21% O₂ and 5% CO₂.

Preparation of Multicellular Tumor Spheroids: Avascular MCF-7 tumor

spheroids were prepared as our previous report.^{S2} Briefly, MCF-7 cells were incubated and seeded in the 96-well cell culture plates, which were pre-coated with agarose. After one week, the MCF-7 multicellular tumor spheroids (MTSs) were obtained.

Intracellular pH Detection: Firstly, the standard curve was prepared based on the fluorescence intensity of BCECF in PBS of different pH (pH = 4.6, 5.6, 6.5, 7.4, 8.0). Secondly, BCECF-AM fluorescence probe was used to monitor the intracellular pH changes. MCF-7 cells were seeded in glass culture dishes for 24 h. After co-incubating with Me@MSN@MnO₂-FA, Flu@MSN@MnO₂-FA, and Me&Flu@MSN@MnO₂-FA (100 µg/mL) for 0 h, 4 h, 6 h or 8 h, all cells were treated with BCECF-AM in HEPES buffer solution for 30 min at 37 °C. Subsequently, the cell samples were observed by super-resolution microscope (Leica TCS SP8 STED, Germany) and the data were analyzed with ImageJ.

Lactate Efflux Detection: To investigate the inhibitory effect of the two drugs on the intracellular lactate efflux, the changes of extracellular lactate concentration in MCF-7 MTSs were monitored for 96 hours. When the MCF-7 MTSs were incubated at 37 °C for 7 days, the cell culture medium were carefully removed. The drug-loaded materials containing Me@MSN@MnO₂-FA, Flu@MSN@MnO₂-FA, and Me&Flu@MSN@MnO₂-FA (100 µg/mL) were incubated with the tumor spheroids for 72 h. As scheduled, 50 µL of the medium was taken out from the well one by one. The lactate concentration of all experimental groups was detected by lactate assay kit, and the experimental procedures have been illustrated in the previous report.^{S3}

In Vitro Cytotoxicity: The cytotoxicity of different nanomaterials was measured by MTT assay. MCF-7 cells were incubated in DMEM medium and seeded in 96-well plates. To evaluate the cytotoxicity of the nanocarrier materials in vitro, MSN-SH,

MSN@MnO₂ or MSN@MnO₂-FA (200 µg/mL) were added into the well plates, respectively. Moreover, the cells were co-incubated with drug-loaded materials for 24 h to demonstrate the different killing effects of Me@MSN@MnO₂-FA, Flu@MSN@MnO₂-FA, and Me&Flu@MSN@MnO₂-FA (the concentration ranging from 0 to 300 µg/mL) on tumor cells. The absorbance of each well at 570 nm was measured on the microplate reader (Bio-Rad, 550, USA). The cells without materials treatment were used as the control.

Apoptosis/Necrosis Analysis: MCF-7 cells were seeded in 6-well plates at a density of 5×10^5 cells per well. After 24 h, the cells were incubated with Me@MSN@MnO₂-FA, Flu@MSN@MnO₂-FA or Me&Flu@MSN@MnO₂-FA (200 µg/mL) for 24 h. The cells without any treatment were used as the control. Then all of the cells were washed with PBS for three times, digested by trypsin, and collected by centrifugation. The cells were evenly dispersed in 1 mL of annexin binding buffer after being washed with PBS for three times. All cells were stained with PI and Annexin-V-FITC in the dark for 20 minutes at room temperature. Finally, flow cytometry (BD FACSAria TM III) was used to detect apoptosis/necrosis of the cells.

Wound-Healing Assay: MCF-7 cells were seeded in 6-well plates at a density of 1×10^6 cells per well and incubated for 24 h. After drawing five parallel vertical lines at the bottom of the cell-filled plate by a 10 µL pipette tip, the detached cells were washed with PBS for three times. The MCF-7 cells were treated with Me@MSN@MnO₂-FA, Flu@MSN@MnO₂-FA or Me&Flu@MSN@MnO₂-FA (100 µg/mL), at different time points, the migration of different groups of cells were observed under the inverted fluorescence microscope. The groups without materials treatment were used as the control.

Transwell Invasion Assay: MCF-7 cells were pretreated with DMEM medium

only (control groups), Me@MSN@MnO₂-FA, Flu@MSN@MnO₂-FA or Me&Flu@MSN@MnO₂-FA (dispersed in medium, 100 µg/mL). Then, 100 µL of cells (dispersed in medium) were seeded into transwell chamber (8 µm pore), and the chamber was soaked in 24-well plate, which were filled with 500 µL of DMEM medium in each well. Finally, the nucleus of migrated cells was labeled by Hoechst33342 for conveniently counting under the microscope. Migration inhibition rate (%) = 100 × (1-Quantity (samples)/Quantity (control)), where Quantity (samples) and Quantity (control) represent the average number of migrated cells in the experimental groups and control groups, respectively.

Animals and Tumor Model: Female BALB/c-nu mice (4-5 weeks old) from Wuhan University Animal Biosafety Level III Lab were chosen as animal model for the experiments in vivo. All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of the Animal Experiment Center of Wuhan University (Wuhan, China). All mouse experimental procedures were performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of People's Republic of China. MCF-7 tumor model was established by injecting MCF-7 cells (1×10^7) subcutaneously into the right hind limb of the mouse. About one week, the tumor volume reached approximately 50 mm³ for further experimental study.

In Vitro and In Vivo MR Imaging: Bruker BioSpec 4.7T/20 cm system was used to test the in vitro MR imaging property of Me&Flu@MSN@MnO₂-FA. For in vitro MR imaging, Me&Flu@MSN@MnO₂-FA (the initial concentration of Mn was 0.284 mM) was diluted step by step and dispersed in 10 mM of GSH at pH 7.4 or 6.0. The samples without GSH were used as the blank control. T₁-Weighted MRI scans were performed by a non-linear fit to the changes in the mean signal intensity of each

sample as a function of repetition time (TR). Finally, the r_1 relaxation efficiency was determined through the curve fitting of the $1/T_1$ relaxation time (s^{-1}) versus the sample concentration (mg/mL).

For in vivo MR imaging, 100 μ L of MSN@MnO₂ or MSN@MnO₂-FA (dispersed in medium) was injected into MCF-7 tumor-bearing nude mice, and the MRI images were captured at the scheduled time (0 h, 3 h, 6 h). The nude mice treated with PBS were only used the control.

In Vivo Antitumor Study: To evaluate the antitumor effect in vivo, MCF-7 tumor-bearing nude mice were randomly divided into 4 groups (six mice in each group). The mice were treated with 1) PBS, 2) Me@MSN@MnO₂-FA, 3) Flu@MSN@MnO₂-FA, 4) Me&Flu@MSN@MnO₂-FA (5.5 mg/mL). From the first day of drug injection, the body weight and tumor volume of the mice were recorded every other day. After 14 days of treatment, three mice of each group were sacrificed, and the tumor tissues and main organs were removed for histological analysis.

Blood Biochemistry Analysis: MCF-7 tumor-bearing nude mice were tail vein injected with 100 μ L of MSN-SH, MSN@MnO₂, Me@MSN@MnO₂-FA, Flu@MSN@MnO₂-FA or Me&Flu@MSN@MnO₂-FA (dispersed in PBS, 5.5 mg/mL), respectively. After 24 h, the blood was collected and analyzed by Blood Biochemistry Analyzer (MNCHIP POINTCARE).

In Vivo Anti-Metastasis Research: To evaluate the metastasis of MCF-7 tumors, the MCF-7 tumor-bearing nude mice were divided into 4 groups and the mice were tail vein injected with PBS (Group 1), Me@MSN@MnO₂-FA (Group 2), Flu@MSN@MnO₂-FA (Group 3) and Me&Flu@MSN@MnO₂-FA (Group 4) (5.5 mg/mL). After 14 days, three mice of each group were sacrificed, and the liver were removed for H&E staining analysis. To further investigate the effects of different

materials on tumor metastasis in vivo, the remaining nude mice of each group were continued to be reared for 30 days. After 30 days, the lungs of all the mice were dyed by India ink, and the images of the lung metastasis were obtained under an inverted fluorescence microscope.

Statistical Analysis: Statistical significance was analyzed by one-way ANOVA. P values less than 0.05 were considered to be statistically significant.

References

- S1. J. Zhang, Z. F. Yuan, Y. Wang, W. H. Chen, G. F. Luo, S. X. Cheng, R. X. Zhuo, and X. Z. Zhang, *J. Am. Chem. Soc.*, 2013, **135**, 5068-5073.
- S2. Q. Lei, S. B. Wang, J. J. Hu, Y. X. Lin, C. H. Zhu, L. Rong and X. Z. Zhang, *ACS Nano*, 2017, **11**, 7201-7214.
- S3. Z. X. Chen, M. D. Liu, M. K. Zhang, S. B. Wang, L. Xu, C. X. Li, F. Gao, B. R. Xie, Z. L. Zhong and X. Z. Zhang, *Adv. Funct. Mater.*, 2018, **28**, 1803498.

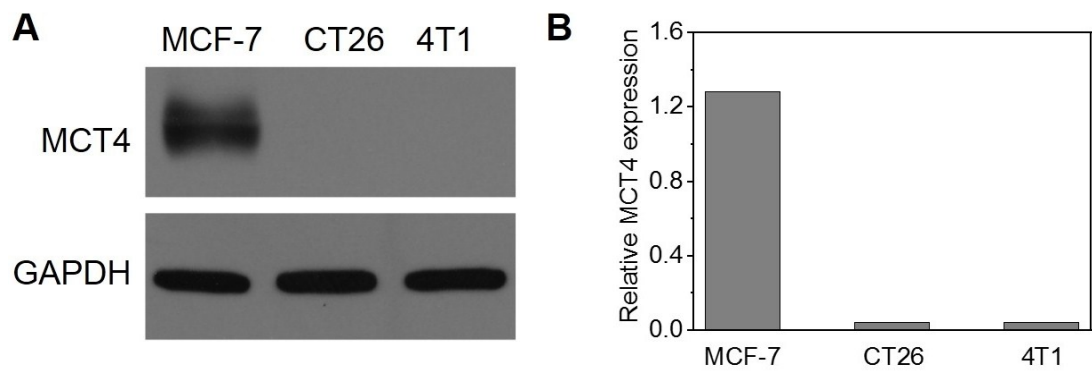


Fig. S1 (A) Western blotting analysis of MCT4 expression in MCF-7, CT26, and 4T1 cells (B) corresponding gray values.

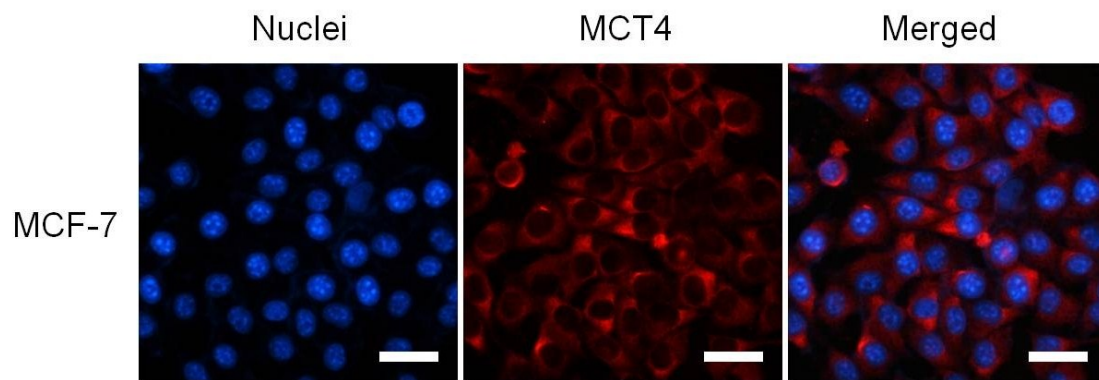


Fig. S2 Immunofluorescence images of MCT4 expression in MCF-7 cells. Scale bar = 20 μm .

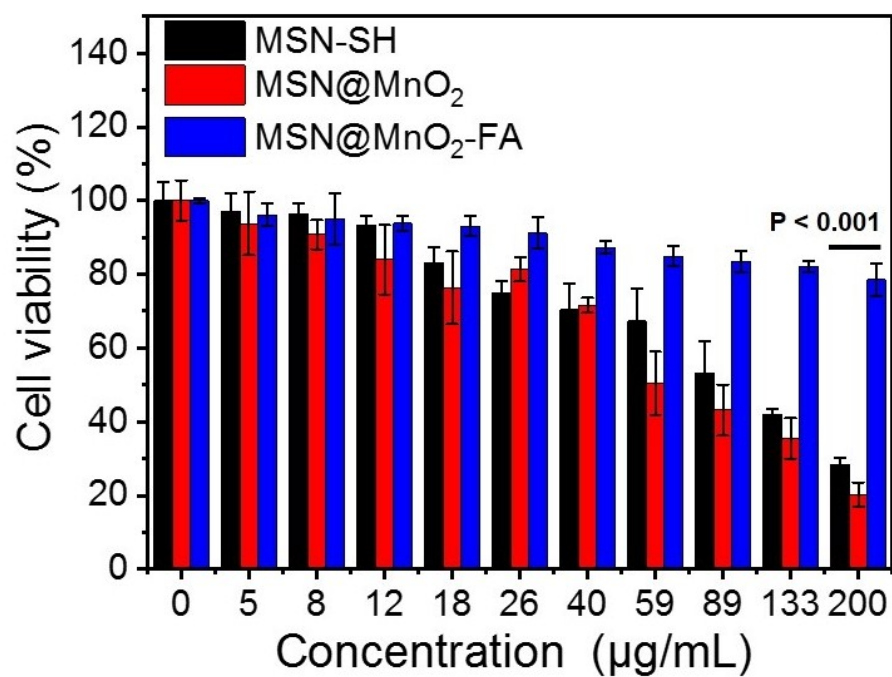


Fig. S3 Viability of MCF-7 cells treated with MSN-SH, MSN@MnO₂ or MSN@MnO₂-FA (P < 0.001).

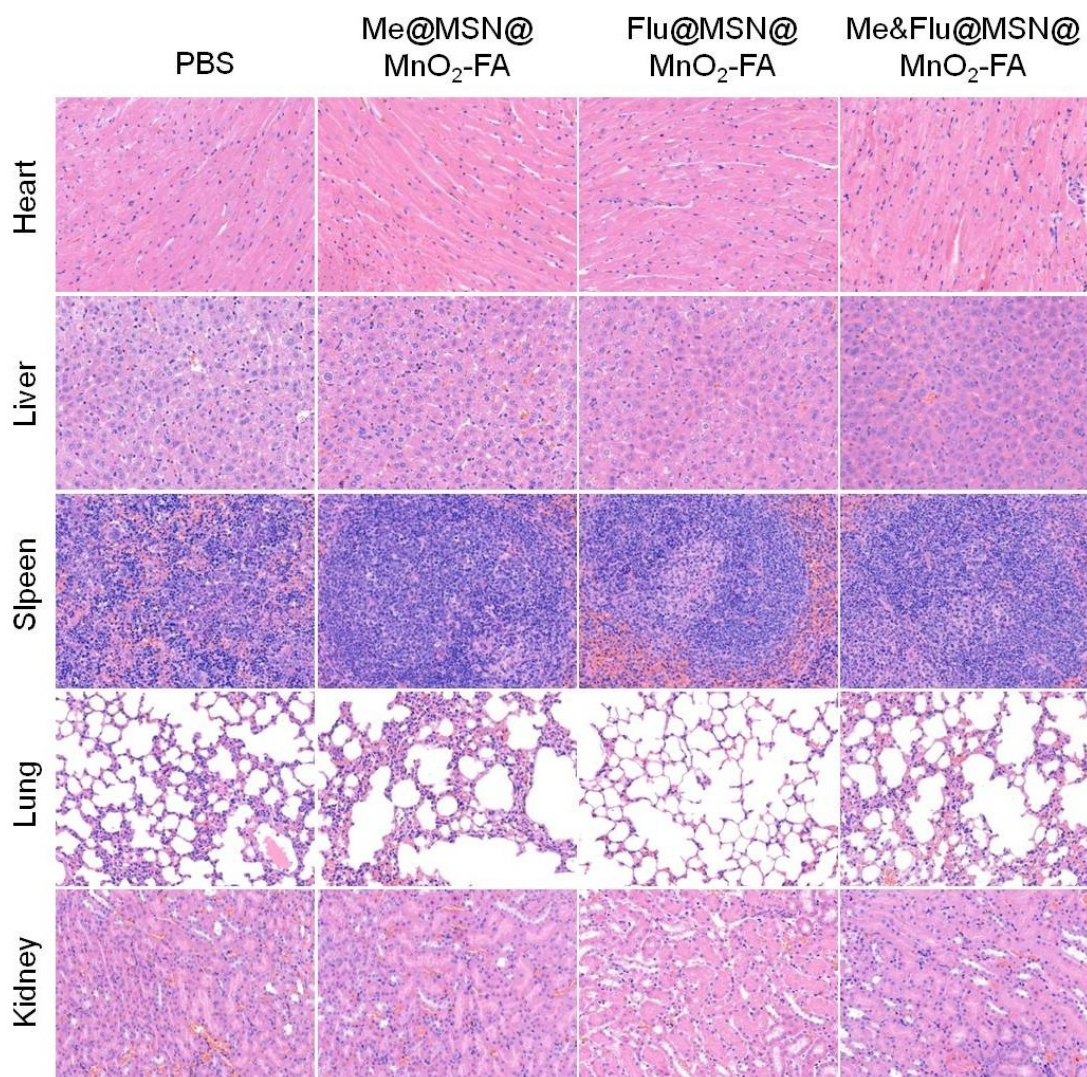


Fig. S4 H&E staining images of heart, liver, spleen, lung and kidney tissues after various treatments by tail vein injection on MCF-7 tumor-bearing mice. Different groups: PBS, Me@MSN@MnO₂-FA, Flu@MSN@MnO₂-FA, and Me&Flu@MSN@MnO₂-FA.

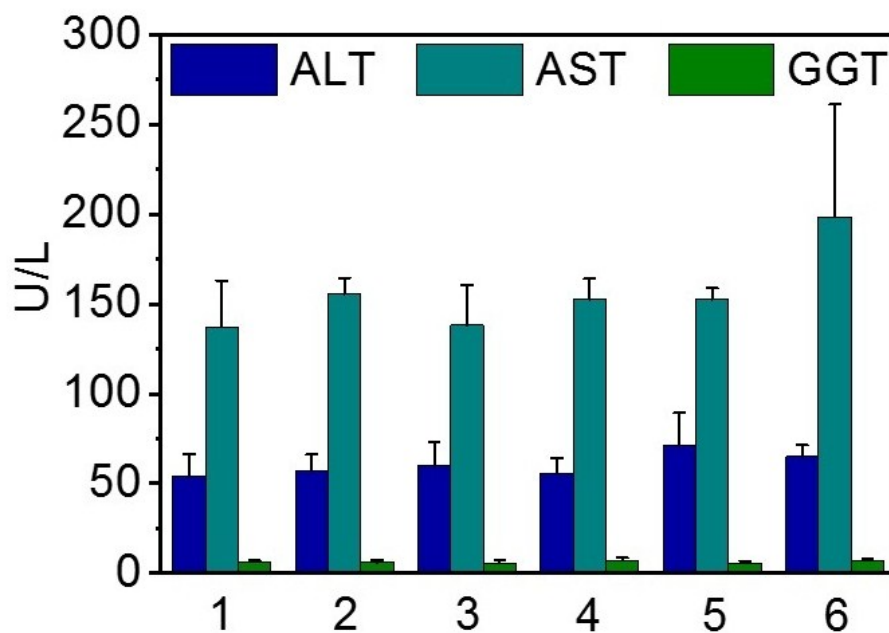


Fig. S5 Blood biochemical analysis of six groups after different treatments: Alanine aminotransferase (ALT), aspartate aminotransferase (AST), Glutamyltranspeptidase (GGT) levels. Different groups: 1) PBS, 2) MSN-SH, 3) MSN@MnO₂, 4) Me@MSN@MnO₂-FA, 5) Flu@MSN@MnO₂-FA, 6) Me&Flu@MSN@MnO₂-FA.

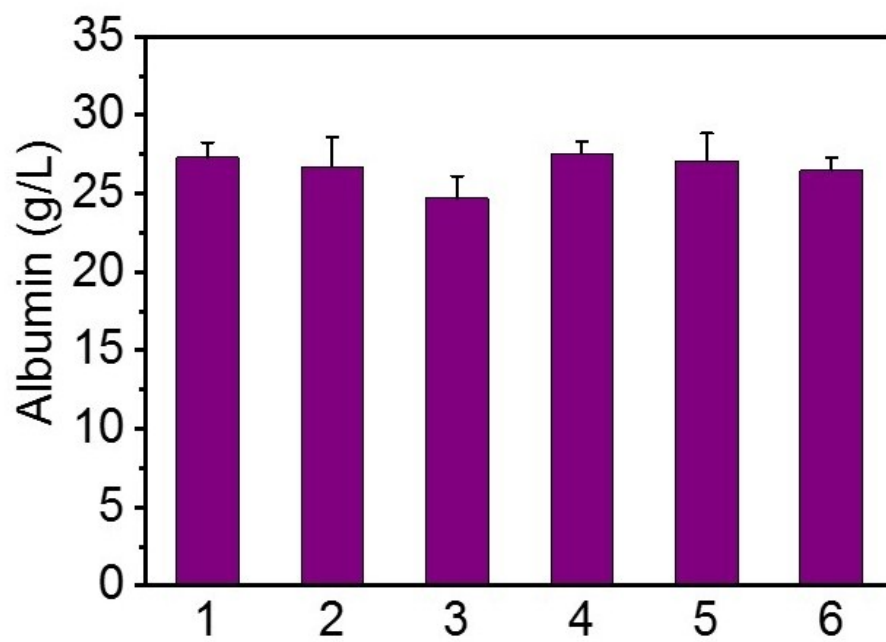


Fig. S6 Albumin concentration of six groups after different treatments. The six groups:
Different groups: 1) PBS, 2) MSN-SH, 3) MSN@MnO₂, 4) Me@MSN@MnO₂-FA, 5)
Flu@MSN@MnO₂-FA, 6) Me&Flu@MSN@MnO₂-FA.

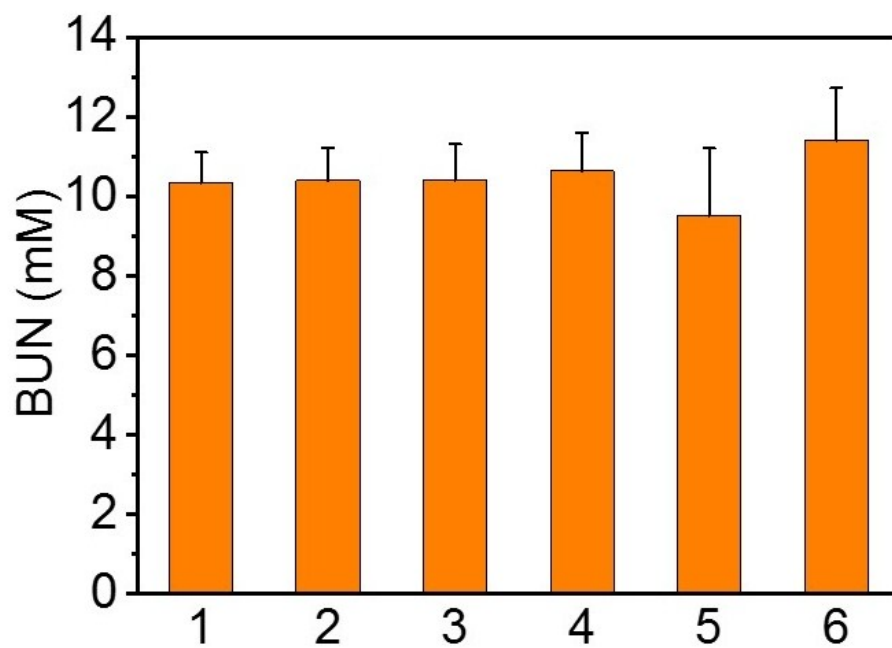


Fig. S7 Blood urea nitrogen (BUN) concentration of six groups after different treatments. Different groups: 1) PBS, 2) MSN-SH, 3) MSN@MnO₂, 4) Me@MSN@MnO₂-FA, 5) Flu@MSN@MnO₂-FA, 6) Me&Flu@MSN@MnO₂-FA.