Supporting Information

A pH-responsive iron/manganese bimetallic organic framework nanosystem for synergistic therapy of colon cancer

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1. Materials and methods

1.1 Materials

Iron (III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), 2-aminoterephthalic acid, potassium permanganate ($KMnO_4$), citric acid, mitoxantrone (MTO), sodium alginate (SA), Cy5.5 (sulfo-cyanine 5.5 carboxylic acid), glutathione, phosphate buffered saline, 5,5-dithiobis (2-nitrbenzoic acid) (DTNB) and methylene blue (MB) were received from Aladdin (Shanghai, China). Calcein-AM/PI double stain kit, 1,1',3,3'-tatraethyl-5,5',6,6'-tetrachloroimidacarbocyanine iodide (JC-1), lipid peroxidation MDA assay kit, 7-dichlorofluorescin diacetate (DCFH-DA) and 3-(4,5-dimethyi-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) were provided by Beyotime Biotechnology (Shanghai, China). Rhodamine B was purchased from Macklin (Shanghai, China). Reduced glutathione (GSH), reactive oxygen species assay kit and DAPI were purchased from Solarbio (Beijing, China).

1.2 Preparation of FMMS NPs

$FeCl_3 \cdot 6H_2O$ (0.675 g) and H_2BDC (1.36 g) were dissolved in DMF (15 mL). The mixture was stirred for 10 min and then treated at 70 ^oC for 24 h. The final resultant was centrifuged and washed with DMF three times, ethanol three times, water three times in sequence, MIL-101 was obtained. Fe nanoparticles (5 mg) was dispersed in 40 mL of water and ultrasonically added with $KMnO_4$ (15 mg). Then, citric acid (7 mg) was added and stirred for 1.5 h under ultra-sonication to get the FM NPs. 3 mg of MTO was added to 8 mg of FM NPs and stirred for 12 h. RO water was centrifuged three times to get FMM NPs. After half an hour of mixing, 2 mg of SA was added to 1 mg of FMM NPs to get FMMS NPs.

1.3 Response of *in vitro* pH to drug release

FMMS NPs (1 mg) was placed in 1.5 mL PB EP tubes with different pH values (pH = 7.4 or 5.5) at 37 ^oC with slow stirring. The test solution (1 mL) was then collected at different time intervals (0, 2, 4, 6, 24, 48, 72, and 96 h) and the amount of MTO released by the FMMS NPs was determined by UV-VIS spectrophotometer. Meanwhile, 1.0 mL of the same pH PB was added to the beaker.

1.4 ROS generation and GSH-depletion ability

FMMS NPs with concentrations of 20, 40, 60, 80, and 100 μg/mL were added to 10 μL of MB solution. After half an hour of reaction, the absorption of MB was measured on a UV-VIS.

FMMS NPs with concentrations of 20, 40, 60, 80, and 100 μg/mL were added to the GSH solution, incubate in the dark for 2 h, and then DTNB was added. The absorption at 412 nm was measured by a UV-VIS spectrophotometer.

1.5 Cellular uptake *in vitro*

CT26 cells (2×10^5 cells) in 24-well plates were treated with FM NPs (1 mL, 2 mg/mL) labeled with RB (0.05 mL, 10 μM) for different time periods (2, 4, and 6 h) and observed by an inverted microscope.

1.6 Cytotoxicity assay

We tested the cytotoxicity of FMMS NPs by MTT assay. CT26 cells, NIH3T3 cells, and L929 cells (obtained from ATCC) were employed for the detection of the cytotoxicity of FMMS NPs. Firstly, the cells were seeded in 96-well plates and incubated with 37 °C and 5 % CO_2 for 24 h. Subsequently, the cells were treated with different concentrations of free MTO, FM NPs, FMM NPs, and FMMS NPs (0, 20, 40, 60, 80, and 100 μg/mL) for 24 h. Finally, the cells were washed PBS three times, then the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5 mg/mL) solution was used to determine the cytotoxicity of cells. After 4 h incubation, the MTT solution was discarded, 150 μL DMSO was added to each well, then it was shaken in the dark for 20 min. The absorbance of each well at 492 nm was measured by the enzyme-labeled instrument (DeTie, China).

1.7 Colony formation assay

Cell growth and colony forming ability were monitored using the colony formation assay. Inoculate CT26 cells into a 6-well plate (600 cells/well). Incubate cells with FM NPs, free MTO, FMM NPs, and FMMS NPs at 37 °C in 5 % CO_2. The medium was changed every three days until colonies appeared. Colonies were fixed in 4 % paraformaldehyde for 10 min and then stained with 1 % crystal violet for 10 min at room temperature. Observe colony growth by counting the number of colonies.

1.8 Wound healing assay

Cell migration was studied by *in vitro* scratch healing. CT26 cells and L929 cells were inoculated in 6-well plates, adhered, and then linearly scratched on the cell monolayer with the tip of a 200 μL pipette. Cells were then incubated with FM NPs, FMM NPs, and FMMS NPs, and the scratches were observed at different times with a light microscope.

1.9 Mitochondrial membrane potential detection

CT26 cells were inoculated in 6-well plates and co-incubated with FMMS NPs. After 24 h of treatment, JC-1 was added and incubated for 30 min, and finally the fluorescence was observed by fluorescence microscopy.

1.10 Intracellular ROS detection *in vitro*

CT26 cells (2×10^5 cells) in 24-well plates were incubated with different formulations (PBS, FM NPs, MTO, FMM NPs, and FMMS NPs), stained with 2',7'-dichlorofluorescin diacetate (DCFH-DA), the intracellular ROS level was detected using an inverted fluorescence microscope.

1.11 Immunofluorescence staining *in vitro*

CT26 cells were plated in 24-well plates, and incubated with PBS, FM NPs, MTO, FMM NPs, FMMS NPS overnight, washed three times with PBS. Then, the cells were fixed with paraformaldehyde for 30 min, washed 3 times with PBS and then ruptured with 0.5 % Triton 100 for 10 min, and washed 3 times with PBS, blocked with 3 % Bovine Serum Albumin (BSA) and 10 % goat serum for 1 h at room temperature, and stained with antibodies against GPX4, CRT or HMGB1 at 4 ^{o}C overnight. Following this, the cells were washed 3 times with PBS, and incubated with secondary antibody for 1 h. After 3 times washing, the cells were stained with DAPI for 10 min, then observed by a fluorescence microscope.

1.12 Western blot analysis

CT26 cells were treated with PBS, FM NPs, MTO, FMM NPs, and FMMS NPs respectively for 24 h. Cells were collected and treated with lysate on ice for 30 min. The protein concentration of the samples was detected using the BCA protein assay kit, and the same concentration of protein was scraped into SDS-PAGE gel electrophoresis

and transferred to PVDF membrane. The PVDF membrane was incubated with primary antibodies (GPX4, CRT, and HMGB1) again overnight at 4 °C, and then incubated with secondary antibodies for 2 h at room temperature, respectively. Finally, the bands were detected using ECL Western blot.

1.13 MDA assay

The Lipid Peroxidation MDA Assay Kit was used to assess the relative levels of malondialdehyde (MDA) in cell lysates. CT26 cells were formed into a homogenate with the MDA lysate on ice and centrifuged at 12,000 rpm for 10 min to remove insoluble components. A mixture of 100 μL of standard buffer and 200 μL of thiobarbituric acid solution (TBA) was incubated at 100 °C for 15 min to produce MDA-TBA adducts with proportional coloration (OD = 532 nm). Each 200 μL sample was cooled to room temperature and transferred to a 96-well plate and analyzed for absorbance at 532 nm by an enzyme labeling analyzer. The MDA concentration was calculated from the standard curve.

1.14 Mice subcutaneous CT26 tumor model and therapeutic effects evaluation

CT26 cells (1×10^6 cells) were implanted subcutaneously into the right hind leg of Balb/c mice. When tumor volume reached 100 mm^3, CT26 tumor-bearing mice were randomly divided into five groups: PBS, FM NPs, MTO, FMM NPs, and FMMS NPs. CT26 tumor-bearing mice received tail vein injections of the respective drug every 3 days, with precise measurements of mouse body weight and tumor volume taken at each injection. Following the treatment period, mice were euthanized. Tumors and organs (heart, liver, spleen, lung, and kidney) were harvested, preserved in 4 % paraformaldehyde, and subsequently embedded in paraffin for haematoxylin and eosin (H&E) staining and immunofluorescence analysis.

All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Jilin University (Protocol Approval Number: 2022427). All animal experiments were conducted in strict compliance with China's national laws and guidelines, such as the Regulations on the Management of Laboratory Animals.

1.15 Hemocompatibility test

The hemocompatibility of FMMS NPs was determined by hemolysis assay. Fresh mouse blood was centrifuged at 1,500 rpm for 5 min at 4 °C to separate erythrocytes from plasma and washed three times with sterile PBS. The erythrocyte precipitate was mixed with saline to obtain a 2 % erythrocyte suspension. Dissolve various concentrations of FMMS NPs in 200 μL of saline and mix with 200 μL of erythrocyte suspension. After incubation at 37 °C for 4 h, the mixture was centrifuged at 3,500 rpm for 5 min and the supernatant was labeled with an enzyme to monitor absorption at 540 nm.

1.16 Statistical analysis

Statistical calculations were performed by GraphPad Prism 9. All data were expressed as mean ± SD. Significant differences between groups were indicated by $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$, ns, not significant.

2. Supplementary Results

	1	2	3	4	5	6
FeMn/MTO	4/0.75	4/1	4/1.5	2/1	4/3	1/1
Particle	291.6±	271.9±	282.8±	1161.0±	1381.0±	1961.0±
Size (nm)	38.8	1.8	2.7	61.6	21.1	61.9
Zeta (mV)	-9.6±1.0	-12.6±0.7	-18.3±0.1	-3.3±0.5	-3.0±0.4	11.9±0.2
DLC (%)	15.6±1.1	19.6±0.5	26.6±0.6	/	/	/
DLE (%)	98.3±0.5	97.3±1.0	96.4±0.7	/	/	/

Table S1 Different ratios of FM NPs and MTO were compared and screened to obtain the optimum ratio.

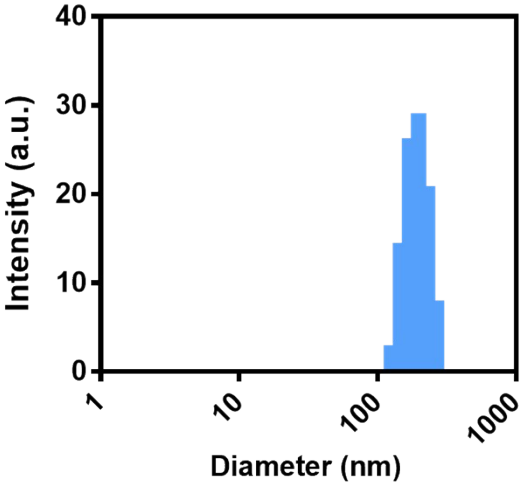

Fig. S1 Hydrodynamic diameters of FMMS NPs.

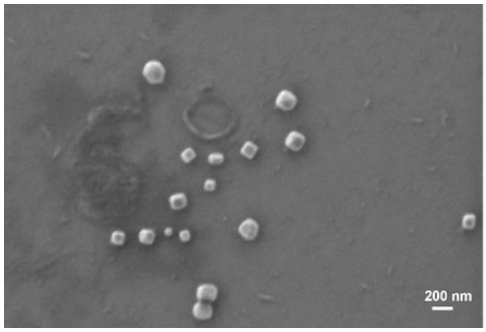

Fig. S2 SEM image of FM NPs.

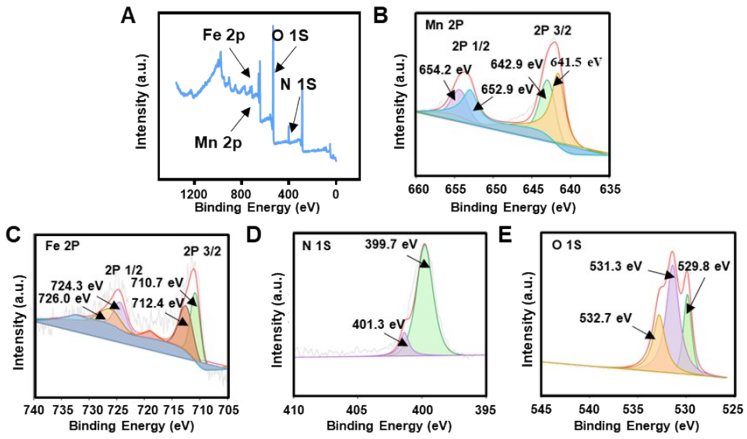

Fig. S3 (A) XPS spectra of FMMS NPs. (B) Mn 2p spectrum of FMMS NPs. (C) Fe 2p spectrum of FMMS NPs. (D) N 1s spectrum of FMMS NPs. (E) O 1s spectrum of FMMS NPs.

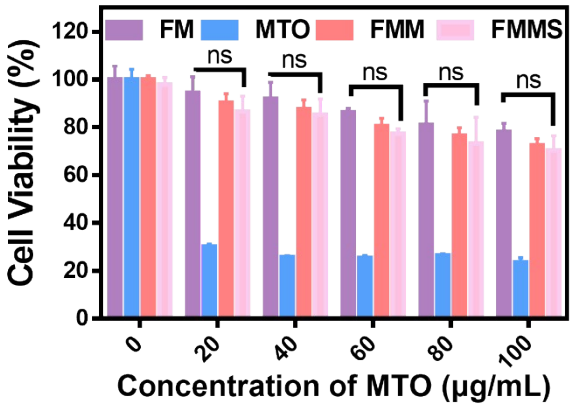

Fig. S4 Cytotoxicity of different formulations against NIH3T3 cells after 24 h of incubation (n=3).

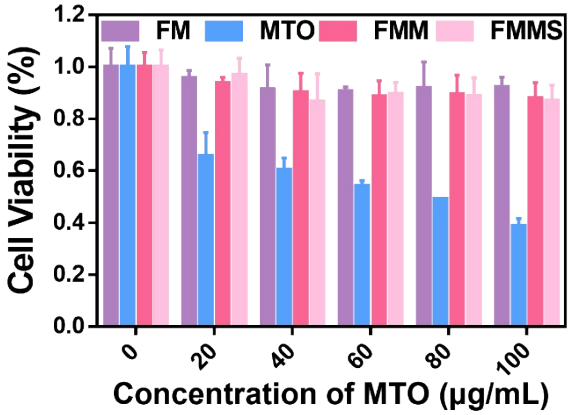

Fig. S5 Cytotoxicity of different formulations against L929 cells after 24 h of incubation (n=3).

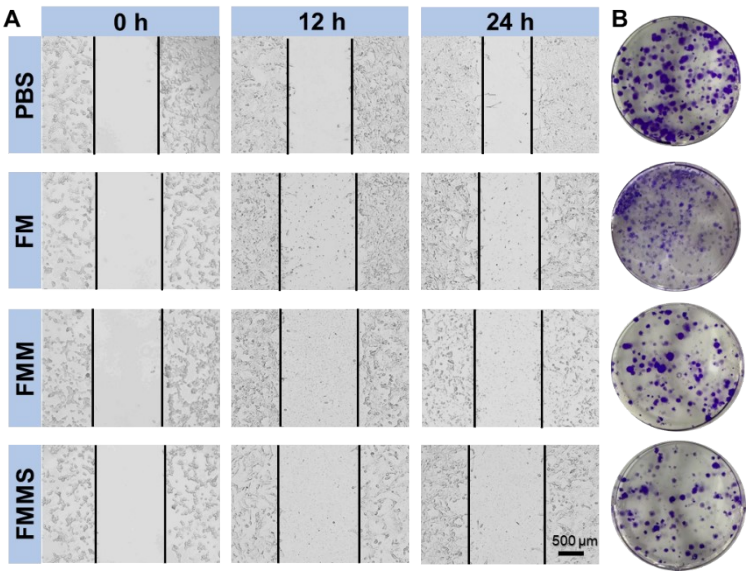

Fig. S6 (A) Wound healing assay of CT26 cells under various interventions. (B) Clonogenic assays of CT26 cells under various interventions.

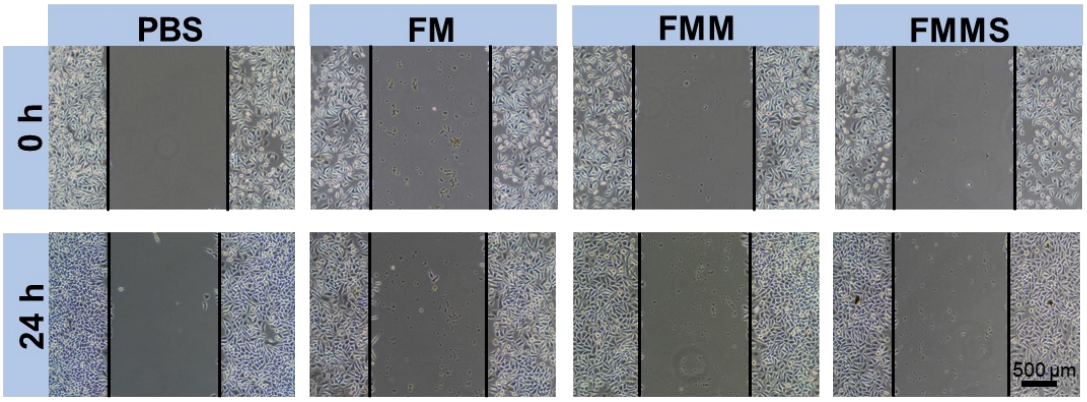

Fig. S7 Wound healing assay of L929 cells under various interventions.

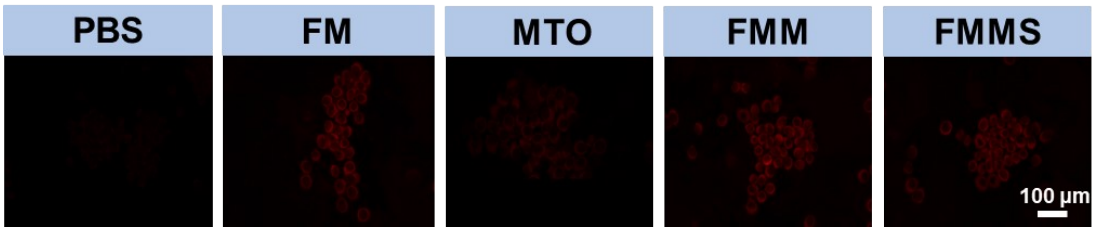

Fig. S8 Detection of intracellular Fe concentration.

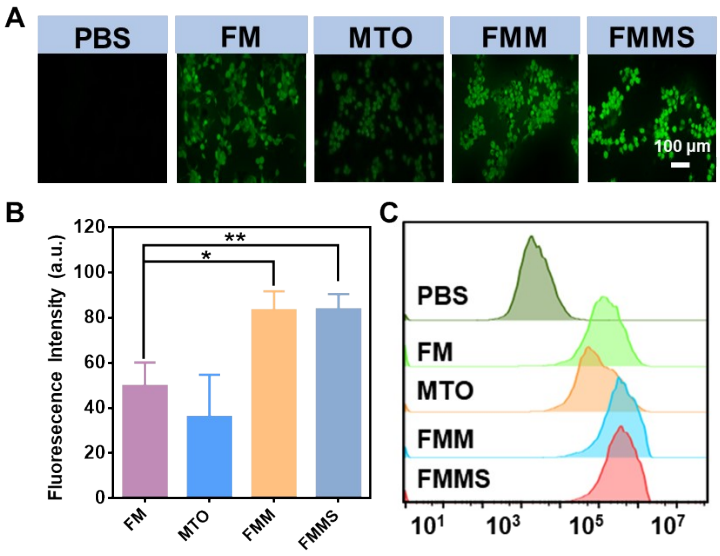

Fig. S9 (A) Fluorescence pictures evaluation of ROS formation in CT26 cells. (B) Detecting ROS with plotting histograms in CT26 cells (n=3). (C) Detecting ROS with cell flowmetry in CT26 cells. Statistical significance was set as follows: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

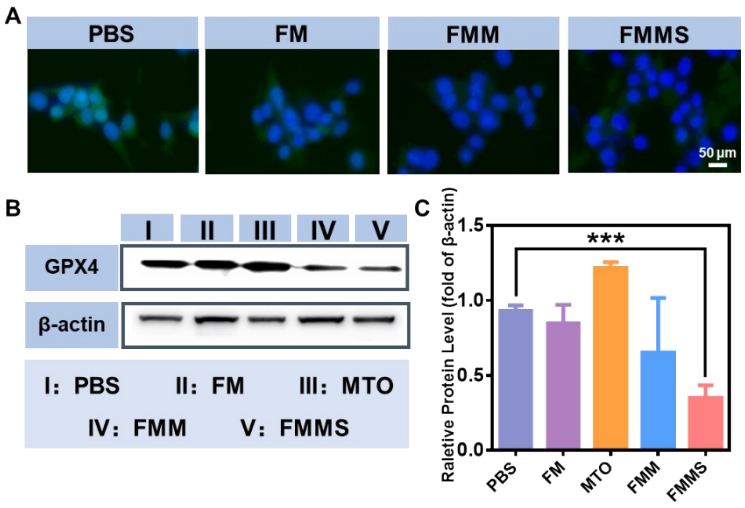

Fig. S10 (A) Immunofluorescence images of CT26 tumor cells stained with GPX4, (B-C) Western blot analysis of proteins represented GPX4 effect in CT26 cells (n=3). Statistical significance was set as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

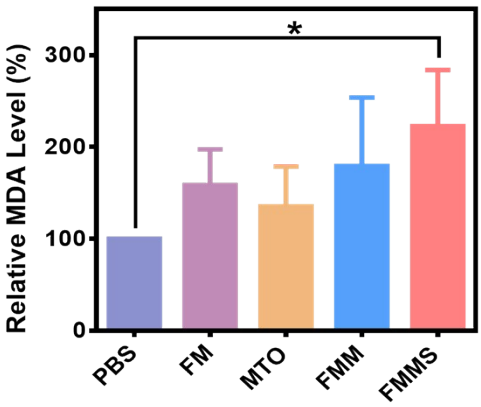

Fig. S11 Changes in MDA under different conditions (n=3). Statistical significance was set as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

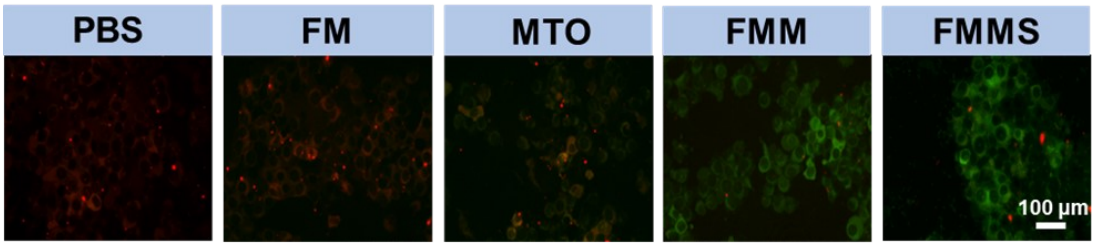

Fig. S12 Mitochondrial membrane potential changes were detected by JC-1 probe.

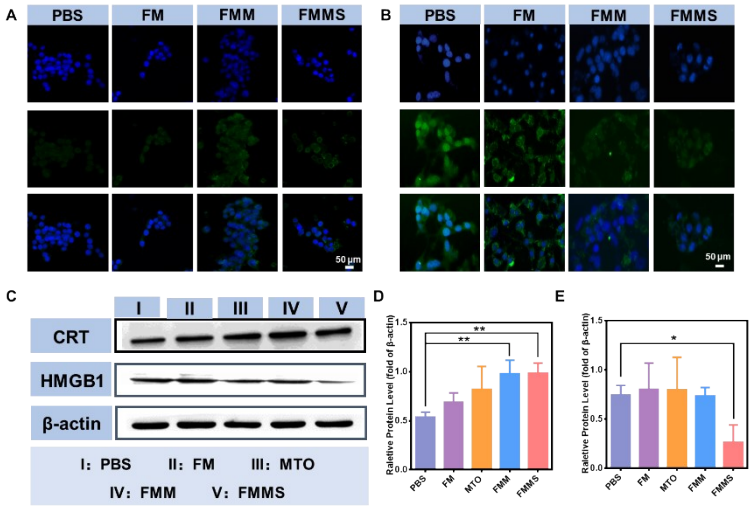

Fig. S13 Immunofluorescence images of CT26 tumor cells stained (A) CRT and (B) HMGB1 after different treatments. (C) Western blot analysis of proteins represented for CRT and HMGB1 effect in CT26 cells. (D) Quantitative analysis of CRT protein in CT26 cells (n=3). (E) Quantitative analysis of HMGB1 protein in CT26 cells (n=3). Statistical significance was set as follows: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

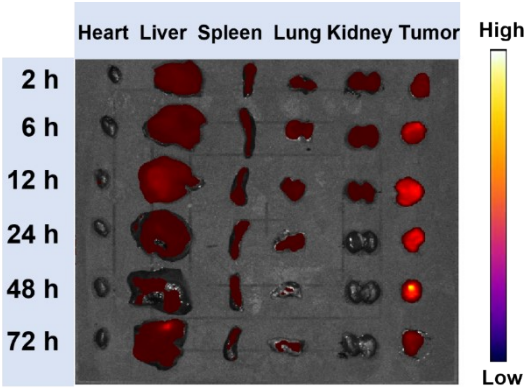

Fig. S14 The major organs of CT26 tumor-bearing mice after intravenously administration with FMMS NPs labeled by fluorescent probe Cy5.

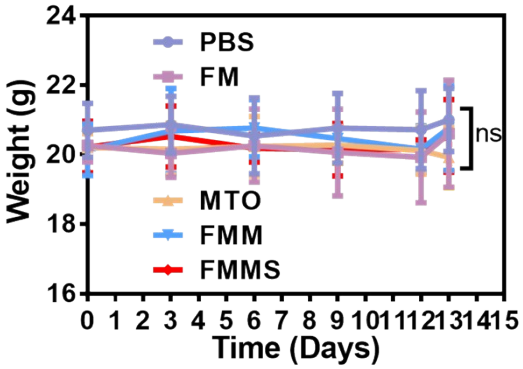

Fig. S15 The body weight profiles of tumor-bearing mice after different treatments (n=5). Data were presented as mean ± SD (ns, not significant).

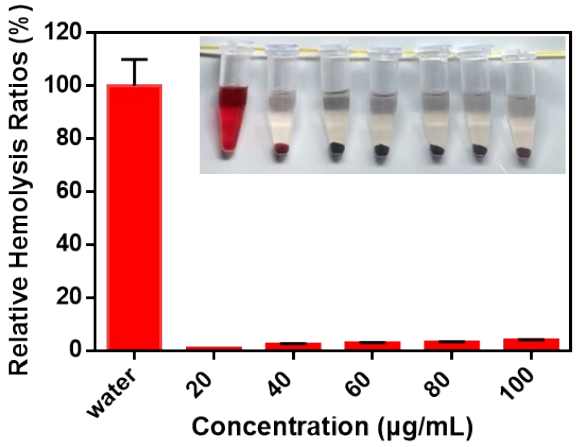

Fig. S16 Relative hemolysis ratios of different concentrations of FMMS NPs (n=3, incubation time was 4 h).

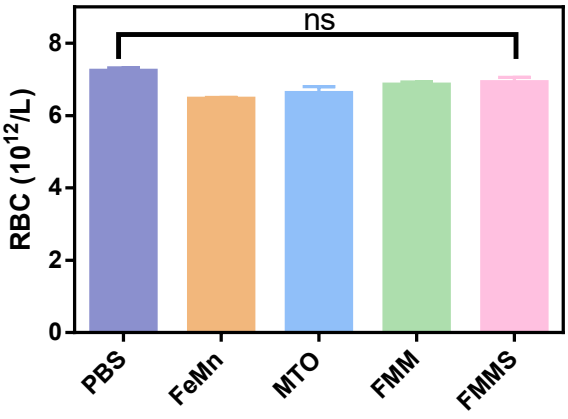

Fig. S17 The assessment of red blood cell (RBC) count in various groups (n=5). Data were presented as mean ± SD (ns, not significant).

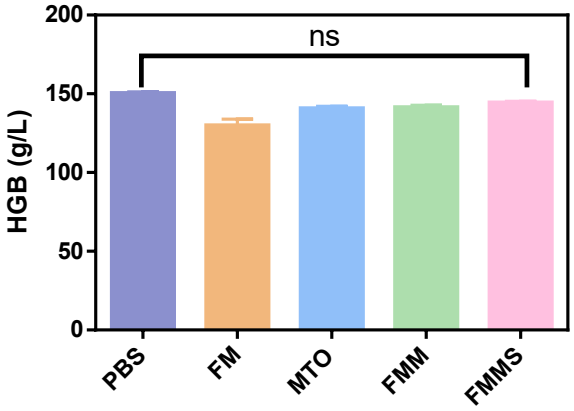

Fig. S18 The assessment of hemoglobin (HGB) concentration count in various groups (n=5). Data were presented as mean ± SD (ns, not significant).

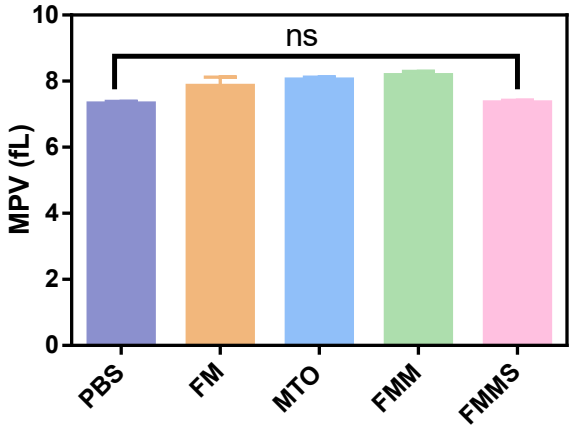

Fig. S19 The assessment of mean platelet volume (MPV), count in various groups (n=5). Data were presented as mean ± SD (ns, not significant).

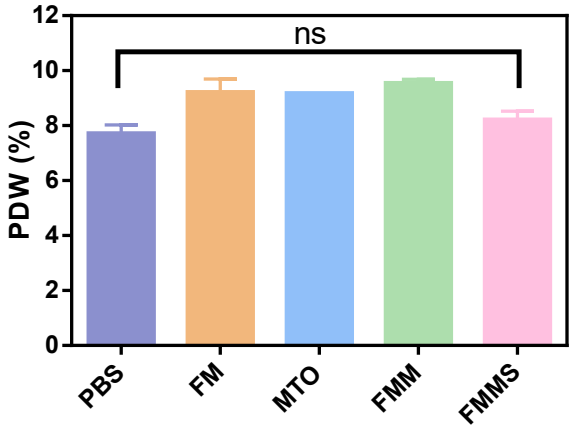

Fig. S20 The assessment of platelet distribution width (PDW) count in various groups (n=5). Data were presented as mean ± SD (ns, not significant).

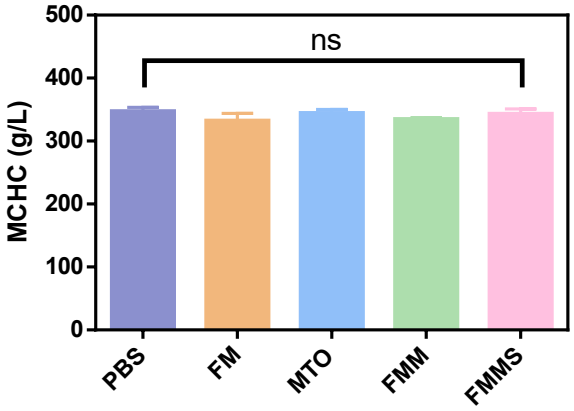

Fig. S21 The assessment of mean corpuscular hemoglobin concentration (MCHC) count in various groups (n=5). Data were presented as mean ± SD (ns, not significant).

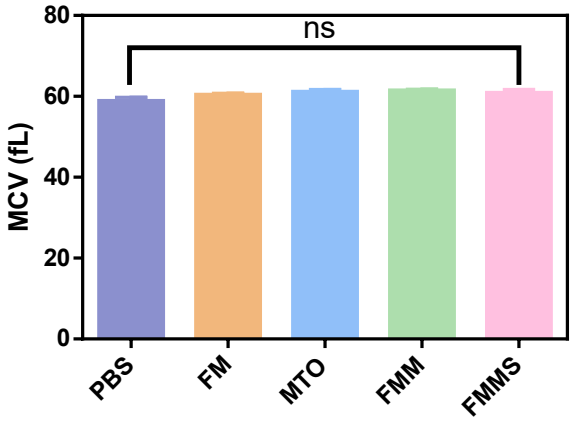

Fig. S22 The assessment of mean corpuscular volume (MCV) count in various groups (n=5). Data were presented as mean ± SD (ns, not significant).

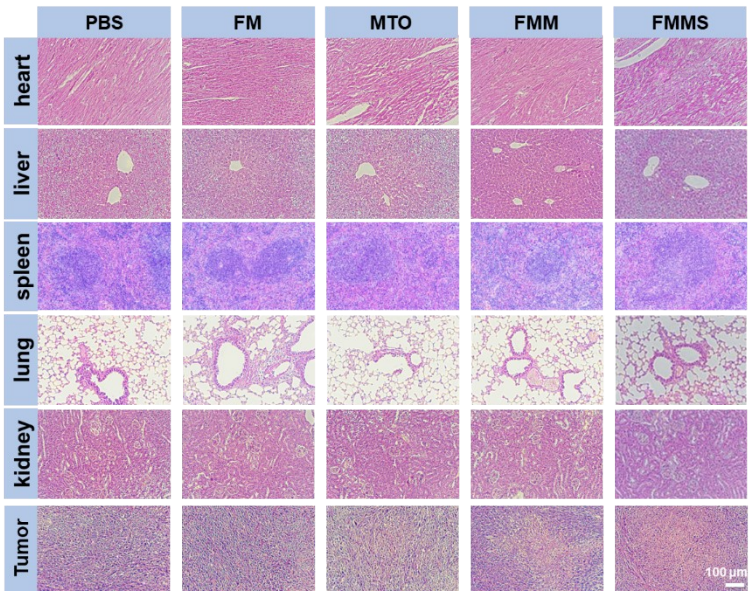

Fig. S23 Representative H&E stained images of major organs from different groups.