Iron Oxide-EDTA Nanoparticles for Chelation-Enhanced Chemodynamic Therapy and Ion Interference Therapy

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Experimental Section

Chemicals. All chemical reagents were used directly without further purification. 1-hexanol (98%), n-dodecane (98%) and concentrated H_2O_2 (30%) were purchased from Aladdin. Pentacarbonyl iron ($Fe(CO)_5$, >97%) was purchased from Aldrich. Diethylene glycol and hexadecyltrimethylammonium bromide (CTAB, >99%) were purchased from Sinopharm chemical reagent co. LTD. Ethylenediaminetetraacetic acid disodium salt (EDTA-2Na, >99%) was purchased from Tokyo Chemical Industry. DSPE-PEG_{2000}-NH_2(>99%) was obtained from Ponsure Biotechnology (Shanghai).

Cell lines and animals. 4T1 cells were first cultured in Roswell Park Memorial Institute (RPMI) 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS, GIBCO), 100 units per mL of penicillin and 100 units per mL of streptomycin (Sigma) in an atmosphere of 5% CO_2 at 37 °C. Female Balb/c mice (19-22 g) were purchased from the Center for Experimental Animals, Jilin University (Changchun, China). All mice were handled using the protocol approved by the Institutional Animal Care and Use Committee of Jilin University.

Material Characterization. The transmission electron microscopy (TEM) images of samples were obtained using a FEI Tecnai G2 S-Twin with a field emission gun operating at 200 kV. Their crystal structure was determined by X-ray powder diffraction (Bruker) equipped with Cu-Kα radiation (λ=0.154 nm). The X-ray photoelectron spectra (XPS) were taken on a Thermo SCIENTIFIC ESCALAB 250Xi electron spectrometer using AI Kα (1487.20 eV) as the excitation source. Concentrations of Fe, Na and Ca were tested by inductively coupled plasma-mass

spectrometer (ICP-MS) or inductively coupled plasmaoptical emission spectrometer (ICP-OES). The dynamic light scattering was performed on a Malvern instrument Zatasizer Nano. The flow-cytometry assay was performed on a Flow Cytometer (guava easyCyteTM).

Synthesis of AIO. The synthesis of AIO NPs was the same as that of the above AIO-EDTA NPs, except that 0.5 mL of deionized water was added instead of 0.5 mL of an aqueous solution of EDTA-2Na.

In vitro antitumor effect. First, 4T1 cells were seeded into 96-well culture plates at a density of 8000 cells per well and then incubated with EDTA-2Na, AIO NPs or AIO-EDTA NPs with different concentrations (EDTA-2Na: 0, 1.3, 2.5, 5, 10 and 20 μg mL^{-1}; AIO NPs: 0, 15.7, 31.3, 62.5, 125 and 250 μg mL^{-1};) for 24 h. The cell viability was tested by a typical methyl thiazolyl tetrazolium (MTT) assay.

In vitro •OH detection. First, 4T1 cells were seeded into 6-well culture plates at a density of 1.5×10^6 cells per well and treated with (a) PBS, (b)EDTA-2Na (5 μg mL^{-1}), and (c) AIO NPs (62.5 μg mL^{-1} of AIO), (d) AIO-EDTA NPs (62.5 μg mL^{-1} of AIO) respectively. After 4 h of incubation in the darkness, the cells were washed three times with PBS. Then, 2′,7′-dichlorofluorescin diacetate (DCFH-DA) dispersed in fresh culture media was added and the mixture was incubated for 30 min at 37 °C. Lastly, the fluorescence from 2′,7′-dichlorofluorescein (DCF) was detected using a fluorescence inversion microscope system.

Lysosomal membrane integrity. The acridine orange (AO) staining method was used to evaluate lysosomal membrane integrity. Briefly, 4T1 cells were seeded into 12-well

culture plates at a density of 0.8×10^6 cells per well and treated with (a) PBS, (b)EDTA-2Na (10 μg mL^{-1}), and (c) AIO NPs (125 μg mL^{-1} of AIO), and (d) AIO-EDTA NPs (125 μg mL^{-1} of AIO) for 12 h, respectively. Then, the cells were washed three times with PBS. An AO probe dispersed in PBS (0.1 mg mL^{-1}) was added to each well and the mixture was incubated at 37 °C for 5 min. Finally, fluorescence was detected by fluorescence inversion microscope system.

Measurement of mitochondrial membrane potential. To evaluate whether AIO-EDTA can decrease the mitochondrial membrane potential of 4T1 cells, 4T1 cells were seeded in 12-well plate at a density of 1.0×10^6 cells per well in 1.0 mL of medium, and cultured for 24 h. Then, the medium was replaced with 1.0 mL of fresh medium with (a) PBS, (b)EDTA-2Na (5 μg mL^{-1}), and (c) AIO NPs (62.5 μg mL^{-1} of AIO), and (d) AIO-EDTA NPs (62.5 μg mL^{-1} of AIO) for 6 h. After washing with PBS, 4T1 cells were stained with JC-1 and detected by fluorescence microscope.

Flow-cytometry apoptosis assay. For this assay, 4T1 cells were seeded into 6-well culture plates at a density of 3×10^6 cells per well and treated with (a) PBS, (b)EDTA-2Na (5 μg mL^{-1}), (c) AIO NPs (62.5 μg mL^{-1} of AIO), and (d) AIO-EDTA NPs (62.5 μg mL^{-1} of AIO) at 37 °C for 24 h. After that, the cells were washed with PBS and then handled by trypsinization to obtain a single cell suspension. Lastly, the obtained single cell suspension was stained with annexin-FITC and PI for flow-cytometry apoptosis assay.

In vivo anti-tumor therapy. The 4T1 tumor models were successfully established by subcutaneous injection of 4×10^6 cells suspended in 100 μL of PBS into the left axilla

of each mouse. The mice were treated when the tumor volumes reached 80-100 mm^3. The tumor-bearing mice were randomly divided into four groups (n = 5, each group), and treated with (i) pure PBS (control group), (ii) EDTA, (iii) AIO NPs, and (iv) AIO-EDTA NPs, respectively. The body weight and tumor volume of each mouse were monitored every two days, and after 14 days treatment, the tumors were dissected and weighed to evaluate the therapeutic efficacy. In a typical calculation, the tumor volume was calculated based on the following equation

$$Vx = \frac{Lx \times Wx^2}{2}$$

where Vx, Lx, and Wx represent the volume, length, and width of 4T1 tumors after indicated treatments for x days. The mice were killed on day 14 and the tumors were collected for staining. Briefly, tumor sections were stained with haematoxylin and eosin (H&E) and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL), and then examined using a fluorescence microscope, respectively.

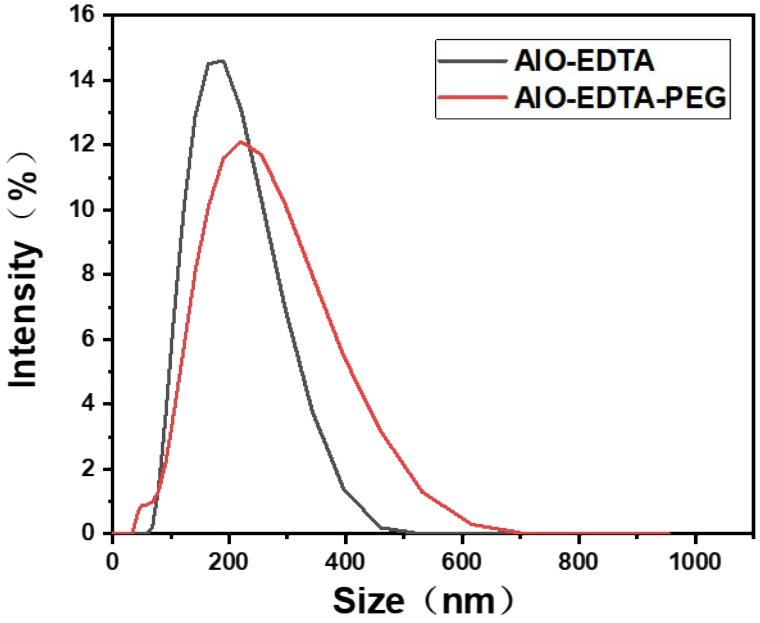

Figure S1. The hydrodynamic sizes of AIO-EDTA NPs and AIO-EDTA-PEG NPs in H_20 detected by dynamic light scattering.

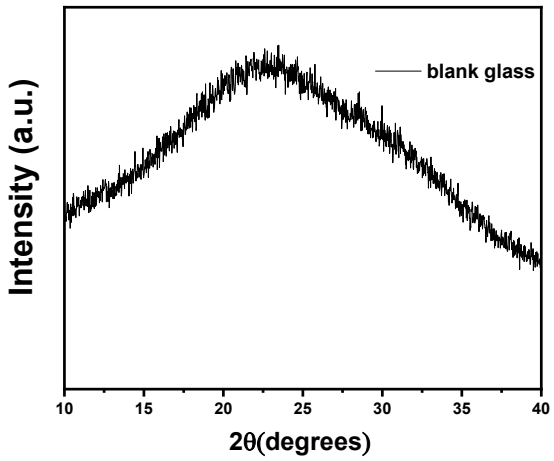

Figure S2. PXRD pattern of blank glass sheet.

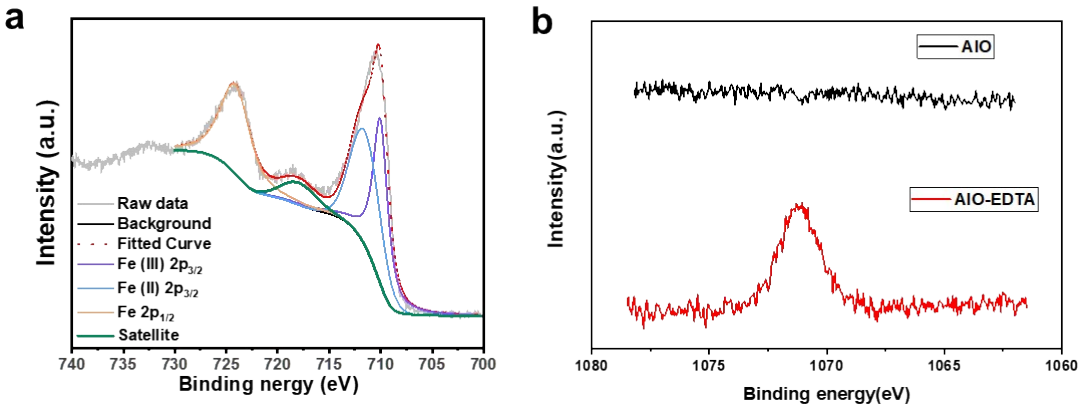

Figure S3. (a) X-ray photoelectron spectroscopy (XPS) high-resolution scans of Fe 2p peaks in AIO NPs. (b) XPS high-resolution scans of Na 1s peaks in AIO NPs and AIO-EDTA NPs. Compared with AIO NPs, the appearance of Na 1s peaks in AIO-EDTA NPs suggests EDTA is successfully loaded into AIO NPs.

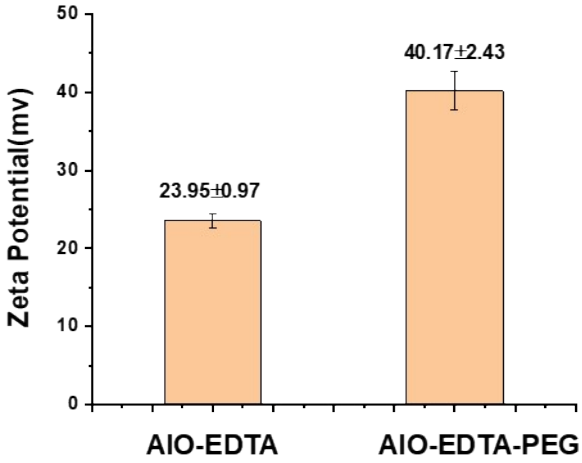

Figure S4. Zeta potentials of AIO-EDTA NPs with or without PEG modifications detected by dynamic light scattering.

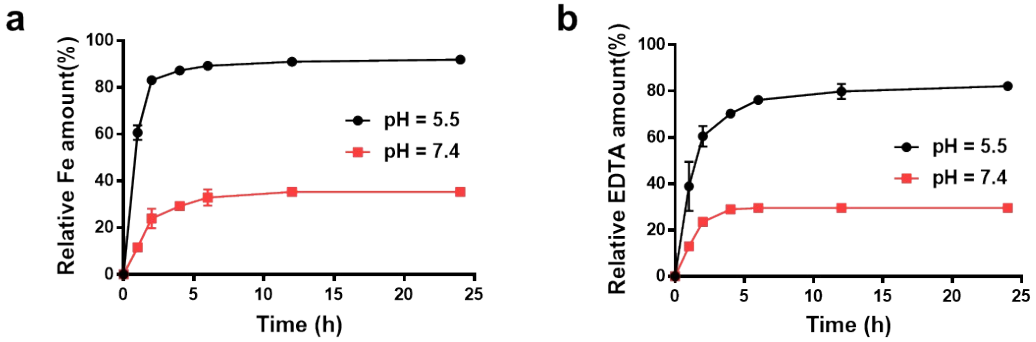

Figure S5. The (a) Fe and (b) EDTA release profiles from AIO-EDTA in PBS solution under different pH (5.5 and 7.4).

Figure S6. The photograph of TMB solution color change. (1) TMB; (2) TMB+H_2O_2; (3) TMB+H_2O_2+AIO; (4) TMB+H_2O_2+AIO+EDTA; (5) TMB+H_2O_2+AIO-EDTA.

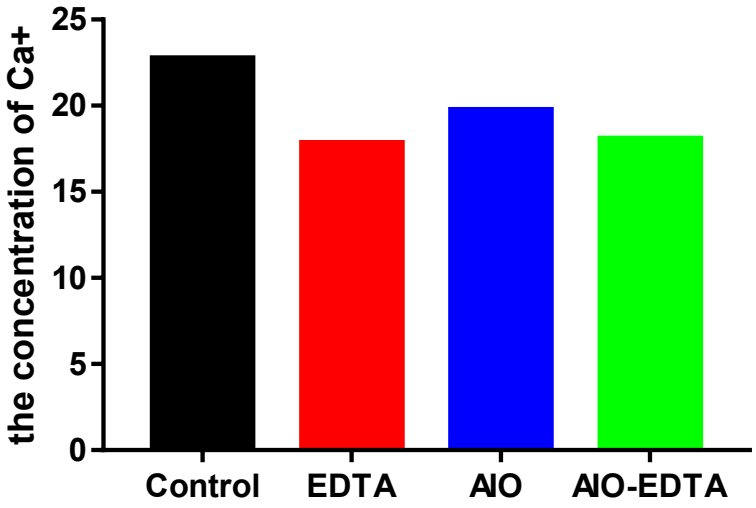

Figure S7. The extracellular Ca^{2+}concentrations treated with RPMI 1640, EDTA, AIO NPs or AIO-EDTA NPs.

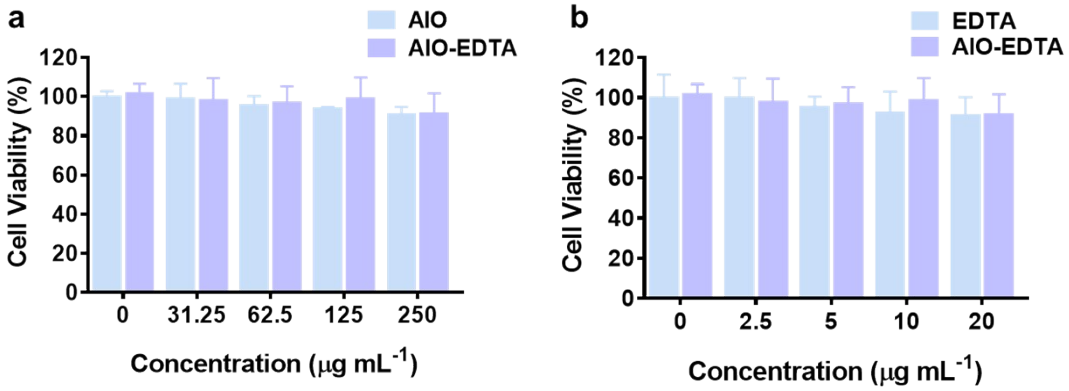

Figure S8. (a) Cell viability of L929 cells treated with AIO or AIO-EDTA NPs at different concentrations for 24 h. (b) Cell viability of L929 cells treated with EDTA or AIO-EDTA NPs at different concentrations for 24 h.

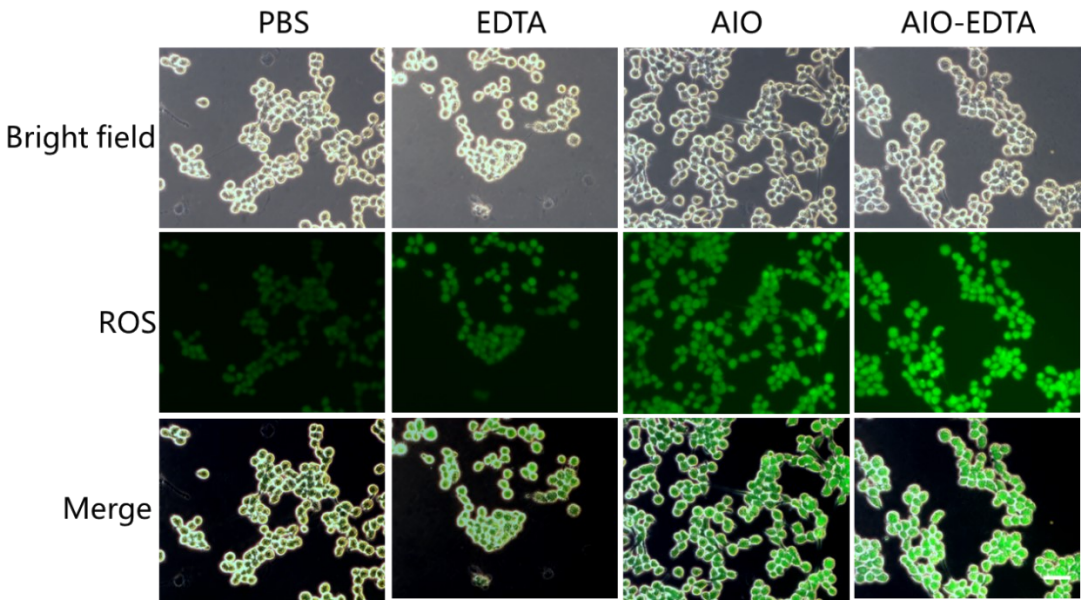

Figure S9. Intracellular ROS detections of 4T1 cells treated with PBS, EDTA, AIO NPs or AIO-EDTA NPs stained with 2′,7′-dichlorodihydrofluorescein (DCFH-DA) which can be rapidly oxidized to 2′,7′-dichlorofluorescein (DCF) with green fluorescence by ROS. Scale bar: 20 μm.

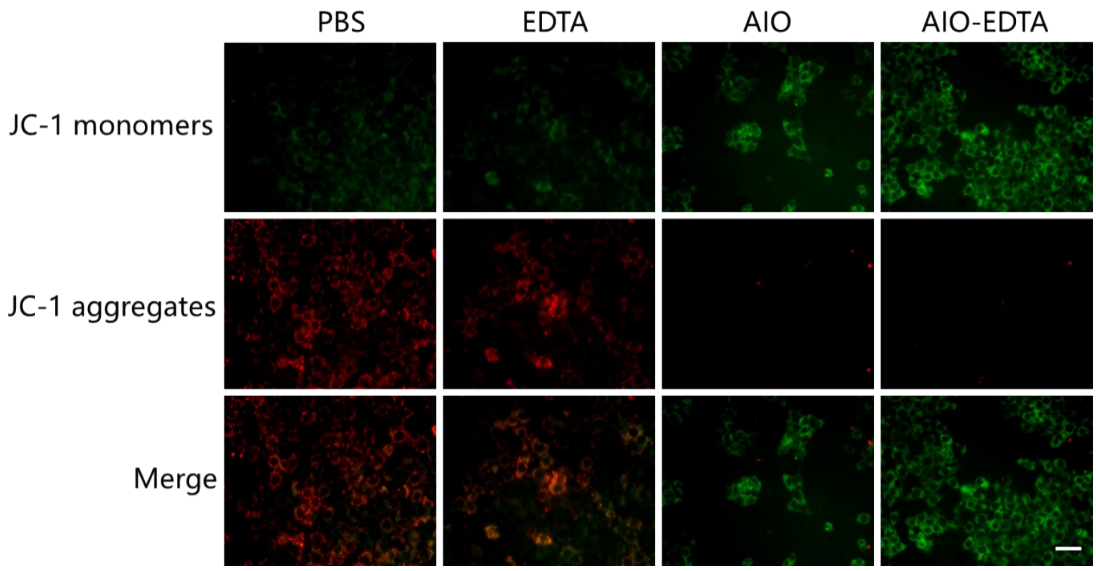

Figure S10. The detection of Mitochondrial membrane potential of 4T1 cells treated with PBS, EDTA, AIO NPs or AIO-EDTA NPs stained with 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide (JC-1). Scale bar: 20 μm.

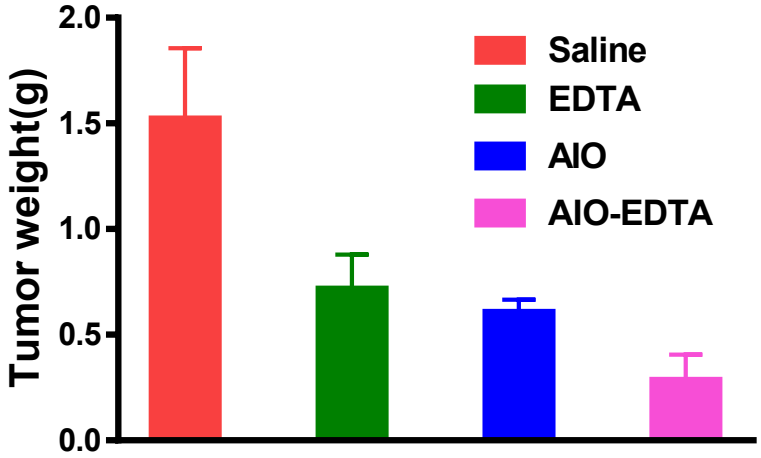

Figure S11. The excised tumors weight after 14 days of different treatments.

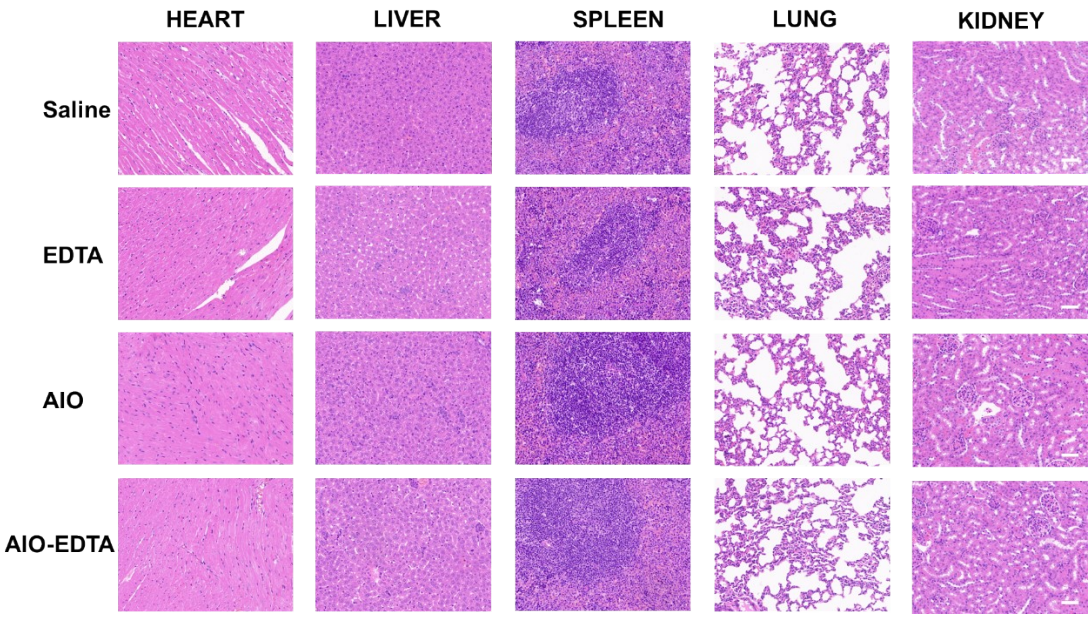

Figure S12. H&E-stained images of major organs of BALB/c mice after different treatments (Saline, EDTA, AIO or AIO-EDTA NPs) in antimetastatic studies, including heart, liver, spleen and kidney. Scale bar: 20 μm.