Supporting Information

Dehydrogenase-like stacked MoS_2 nanozymes for cancer treatment through disrupting tricarboxylic acid cycle

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Experimental methods

Chemicals

Ammonium molybdate tetrahydrate ($(NH_4)_6Mo_7O_{24}\cdot 4H_2O$, 99%), ethanol (99.5%), oxalic acid ($H_2C_2O_4$), α-Ketoglutaric acid (α-KG), fumaric acid (Fum), 3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di-phenytetrazoliumromide (MTT) and nitro blue tetrazolium chloride (NBT) were obtained from Aladdin. Thiourea (NH_2CSNH_2) and Succinic Acid (Suc) were purchased from Macklin. Polyethyleneglycol-2000 (PEG-2000) and polyethyleneglycol-400 were obtained from Mreda. Mitochondrial membrane potential assay kit with JC-1 was purchased from Beijing Solarbio Science & Technology Co., Ltd. All chemicals were of analytical grade and used as received without further purification. Deionized water (18.5 MΩ) was purified using a Milli-Q system.

Synthesis of MSs and MS-f

For the synthesis of MoS_2 nanosheets (MSs), 0.1766 g of $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$, 0.305 g of thiourea, 0.3 g of oxalic acid and 0.5 g of PEG-2000 were dissolved in 40 mL ultrapure water and stirred for 30 minutes until a homogeneous solution was obtained. This solution was then transferred to a hydrothermal reactor and heated at 180 ℃ for 24 hours. The resulting product was centrifuged at 12000 r min^{-1}, washed three times with water and ethanol, respectively, and finally dried at 60 ℃ for 4 hours to obtain MS-4. To obtain MS-6, MS-8 and MS-10, 0.457 g, 0609 g, and 0.761 g of thiourea was used, respectively.

To prepare MoS_2 nanoflowers (MS-f), 0.6385 g of $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ and 1.3736 g of thiourea were dissolved in 25 mL ultrapure water. The mixture was stirred for 30 minutes while acetic acid was added to adjust the pH to 2 until a homogeneous solution was obtained. The solution was then transferred to a hydrothermal reactor and heated at 220 ℃ for 24 hours. The product was centrifuged at 12000 r min^{-1}, washed three times with water and ethanol, respectively, and then dried at 60 ℃ for 4 hours to yield the MS-f.

Characterizations

Scanning electron microscopy (SEM) images were obtained on a Nova field emission microscope (NanoSEM 450). Nanoparticle morphology and size was evaluated using transmission electron microscopy (TEM) and high resolution transmission electron microscope (HRTEM). The crystal

structure was investigated by X-ray powder diffraction (Bruker D8 advanced) with a copper Kα source (wavelength, 1.54 Å). Data were recorded from 10 to 80 degrees and the obtained peaks were indexed using JCPDS card No.73-1508. Zeta potential (Smoluchowski model) and average hydrodynamic diameters were measured by dynamic light scattering (DLS) on a BECKMAN Nanosizer (DelsaMax PRO). Electron spin resonance (ESR) measurements were conducted using a Brucker E 500 spectrometer. The chemical composition and chemical valence states were analyzed by X-ray photoelectron spectroscopy (XPS, Omicron Inc.). The raw data were processed and deconvoluted by Avantage surface chemical analysis software (version 6.6.0) and indexed using the standard library.

Dehydrogenase-like activity and kinetic assay

Dehydrogenase-like activity was determined using MTT and NBT as probes. Dehydrogenase can reduce tetrazole to a formazan structure with an absorption maximum at 510 nm [1], which is similar to the reaction between MTT and mitochondrial dehydrogenase. Specifically, MSs and MS-f (25 μg mL^{-1}) was reacted with MTT (5 mg mL^{-1}) in a pH 7.4 PBS buffer, and the absorbance of the solution was measured using a UV-3600 Shimadzu spectrometer. To detect the dehydrogenase-like activity of MSs, NBT was used as the probe in place of MTT.

To measure the kinetics of dehydrogenase-like catalysis, α-KG was used as substrate and the absorbance at 320 nm was recorded every two seconds. The kinetic parameters were calculated according to the Michaelis-Menten equation (Equation S1):

$$v_0 = \frac{v_{max} \cdot [S]}{K_M + [S]} \qquad \text{(Equation S1)}$$

The maximum initial velocity (v_{max}) and Michaelis-Menten constant (K_M) were obtained using the Lineweaver-Burk plot (Equation S2):

$$\frac{1}{V_0} = \frac{K_M}{v_{max}} \cdot \frac{1}{[S]} + \frac{1}{v_{max}} \qquad \text{(Equation S2)}$$

Where v_0 represents the initial rate, v_{max} represents the maximum rate conversion, *[S]* denotes the substrate concentration, and K_M is the apparent Michaelis-Menten constant. All experiments were carried out in the dark.

NMR analysis

5.0 g of α-KG was dissolved in 25 mL ultrapure water, and 100 mg MS-4 was added. The mixture was allowed to react for 24 h and subsequently centrifuged at 12,000 g for 10 min at 4 ℃. Following centrifugation, the supernatant was immediately frozen in liquid nitrogen, and then freeze-dried at -80 ℃ until further analysis. 20 mg of the freeze-dried powder was dissolved in 500 μL of DMSO-d_6 and 50 μL of PBS-D_2O (pH 7.4, 99 % D_2O) was added. After thorough mixing, the solution was centrifuged at 12,000 rpm min^{-1} for 10 min at 4 ℃. 500 μL of the supernatant was transferred into a 5 mm NMR tube for analysis (BRUKER AVANCE 400). Chemical shifts were referenced to the residual solvent signals (^1H δ 2.50 ppm).

LC-MS/MS analysis

LC-MS/MS analysis was performed on a Thermo Ultimate 3000 UHPLC coupled to a Q Exactive hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, Germany) with a heated ESI source. 2.041g of α-KG was dissolved in 10 mL water, with 100 mg MS-4 added. The mixture was allowed to react for 24 h and subsequently centrifuged at 12,000 g for 10 min at 4 ℃. Chromatographic separation was achieved on an Agilent TC - C18, 100 × 2.1 mm reversed-phase column maintained at 50 ℃. The mobile phase consisted of (A) 0.1 % formic acid in deionized water and (B) 0.1 % formic acid in methanol delivered at 0.4 mL min^{-1}. The gradient was: 0 - 1 min, 100 % A; 1 - 16 min, 100 % - 0% A, 0 % - 100 % B; 16 - 20 min, 100 % B. The injection volume was 10 μL. Full-scan MS was acquired at 70,000 resolution (m/z 20-2,000) in a positive/negative switching mode (ESI^+/ESI^-). Data were processed with Xcalibur 4.1. All experiment processes were conducted in dark.

Cell culture

4T1 murine breast cancer cells and L929 fibroblasts obtained from the American Type Culture Collection (ATCC) were cultured in high-glucose Dulbecco′s Modified Eagle′s Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 1% streptomycin, and 1% penicillin. The cells were incubated at 37 ℃ in a CO_2 incubator (Thermo Fisher) with 5% CO_2 and passaged every 2-3 days at a ratio of 1:3.

Crystal violet assay

Biocompatibility of MS-4 was assessed using L929 fibroblasts as a normal cell model. Cells were seeded into 96-well plates at a density of 5000 cells per well in 200 μL of DMEM medium. Various concentrations of MS-4 (6.25 μg mL^{-1}, 12.5 μg mL^{-1}, 25 μg mL^{-1}, and 50 μg mL^{-1}) was added to each well and incubated for 24 h. After incubation, the medium was removed, and each well was washed three times with 200 μL PBS buffer. Subsequently, 100 μL of methanol was added to each well for 15 minutes to fix the cells. The methanol was then removed, and the wells were allowed to air dry. To stain the cells, 100 μL 1% crystal violet solution was added to each well and incubated at room temperature for 5 minutes. After staining and removing the solution, 100 μL of 33% glacial acetic acid solution was added to each well, and the plate was incubated at 37 ℃ for 30 minutes to dissolve the crystal violet. The absorbance at 590 nm was measured using a microplate reader (iMark, BIO-RAD).

For cancer therapy, 4T1 cells were seeded into 96-well plates at a density of 5000 cells per well in 200 μL of DMEM medium. After 24 h of culture, the medium was removed. For the hypoxia group, cells were pre-incubated under hypoxic conditions for 4 hours prior to treatment, while the normoxia group proceeded directly to treatment. Then 25 μg mL^{-1} of MS-4, along with different concentrations of α-KG (125 μg mL^{-1}, 250 μg mL^{-1}, 500 μg mL^{-1}, 1000 μg mL^{-1} and 2000 μg mL^{-1}) was added to the wells. Following an additional 24 h incubation, crystal violet assay was conducted to evaluate the cell viability.

Fluorescence analysis of mitochondrial membrane potential

Mitochondrial membrane potential was measured by adding JC-1 to the cells. Hypoxic cells were pre-incubated under hypoxia for 4 hours prior to treatment, while normoxic cells received immediate treatment. All cells were then incubated with various formulations for 6 hours under their respective oxygen conditions. After a 20 min incubation, red fluorescent JC-1 aggregates and green fluorescent JC-1 monomers were observed using a Confocal laser scanning microscope (Leica SP8). Relative fluorescence intensity was quantified using Image J software.

SDH activity assay

SDH activity was measured using a Succinate Dehydrogenase Activity Assay Kit (Suzhou Comin

Biotechnology Co.,Ltd.) according to the manufacturer's instructions with slight modifications. For the hypoxia group, cells were pre-incubated under hypoxic conditions for 4 hours prior to treatment, while the normoxia group was maintained under normoxic conditions. Both groups were then treated with various formulations for 2 h. Then cells were harvested, washed twice with PBS, and homogenized in SDH Assay Buffer. After centrifugation at 10,000 g for 5 min, the supernatant was collected and added to a 96-well plate. The reaction was initiated by adding SDH Substrate Mix and Probe, and the absorbance at 600 nm was monitored in kinetic mode for 10–30 min at 25°C. SDH activity was quantified as per the manufacturer's guidelines.

Assay of intracellular ATP levels

Intracellular ATP content was determined using an ATP assay kit (Beyotime). Briefly, cells were collected in a centrifuge tube, and 0.4 mL cell lysate was added. The cells were lysed on ice for 30 min and then centrifuged at 10,000 g for 10 min at 4 ℃. The supernatant was collected and absorbance at 340 nm was measured to calculate ATP content according to the kit instructions.

Transcriptome analysis

4T1 cells were divided into 4 groups: (a) Control; (b) α-KG; (c) MS-4; (d) MS-4+α-KG. RNA was extracted from these cells, and transcriptomic analysis was conducted by Bioprofile Biotechnology Co. Ltd (Shanghai, China). Differentially expressed genes (DEGs) were identified based on $|\log2FoldChange| > 1$, $p < 0.05$.

Hemolysis assay

Different concentrations of MS-4 (25, 50, 100, 200, 400 and 800 μg mL^{-1}) were added to mouse red blood cells. Water and PBS were set as the positive and negative controls, respectively. The mixtures were incubated at room temperature for 6 hours, and the supernatants were collected. Absorbance at 540 nm was measured using a Shimadzu UV-3600 spectrometer. The hemolysis rate was calculated using the formula (Equation S3):

$$Hemolysis = \frac{A_{sample} - A_{negative}}{A_{positive} - A_{negative}} \quad \text{(Equation S3)}$$

Animals

BALB/c and ICR female mice, approximately 8 weeks, were provided by Beijing Vital River Laboratory Animal Technology Co., Ltd.. The animal handling procedures were in strict compliance with the Beijing Administration Rule of Laboratory Animals and the national standards Laboratory Animal Requirements of Environment and Housing Facilities (GB 14925-2001). The animal experiments were approved by the Committee on Ethics of Beijing Institute of Nanoenergy and Nanosystems (Approval Number: 2024A043). The animal studies were performed in strict accordance with the ARRIVE guidelines and the institutional ethical guidelines for the care and use of laboratory animals.

***In vivo* systematic toxicity assessment**

Twelve female ICR mice, 8 weeks old with body weights of approximately 20 g, were randomly divided into four groups (n = 3). The mice were intravenously injected with 0, 50, 100 or 200 mg kg^{-1} of MS-4 dissolved in PBS. Over a 14-day evaluation period, the normal activity, health status, food intake, water intake and body weight of the mice were recorded. After 14 days, blood routine and blood biochemical indexes were analyzed. The mice were euthanized by painless cervical dislocation, and histopathological analysis of major organs (heart, liver, spleen, lungs, and kidneys) was performed.

***In vivo* cancer therapy**

4T1 cells (1×10^6) dispersed in PBS (100 μL) were subcutaneously injected the right posterior abdomen of female BALB/c mice. When the tumor volume reached approximately 100 mm^3, the mice were randomly divided into six groups (n = 5): (1) PBS; (2) α-KG; (3) MS-f; (4) MS-f+α-KG; (5) MS-4; (6) MS-4+α-KG. A 16-day treatment cycle involved twice administration of nanomaterials on day 0 and day 4, followed 30 minutes later each time by an α-KG injection. The body weight of the mice was recorded every 2 days, and the subcutaneous tumor volume was measured and calculated using the following equation (Equation S4):

$$Volume = \frac{Length \times Width^2}{2} \quad \text{(Equation S4)}$$

At the terminal of the 16-day treatment, the mice were euthanized, and the tumor tissues were

collected, photographed and weighed. Further pathological analysis of the tumors was performed using H&E staining and Ki-67 immunohistochemistry. Major organs including the heart, liver, spleen, lungs and kidneys were subjected to H&E staining to observe pathological changes. The tumor growth inhibition (TGI) rate of each group was calculated using the tumor weights at the terminal of the treatment (Equation S5):

$$TGI = \frac{W_{control} - W_{treatment}}{W_{control}} \times 100\% \qquad \text{(Equation S5)}$$

Statistical analysis

All experiments were expressed as mean values ± S.D. Statistical differences were calculated using the two-tailed Student′s t test.

Supporting Figures

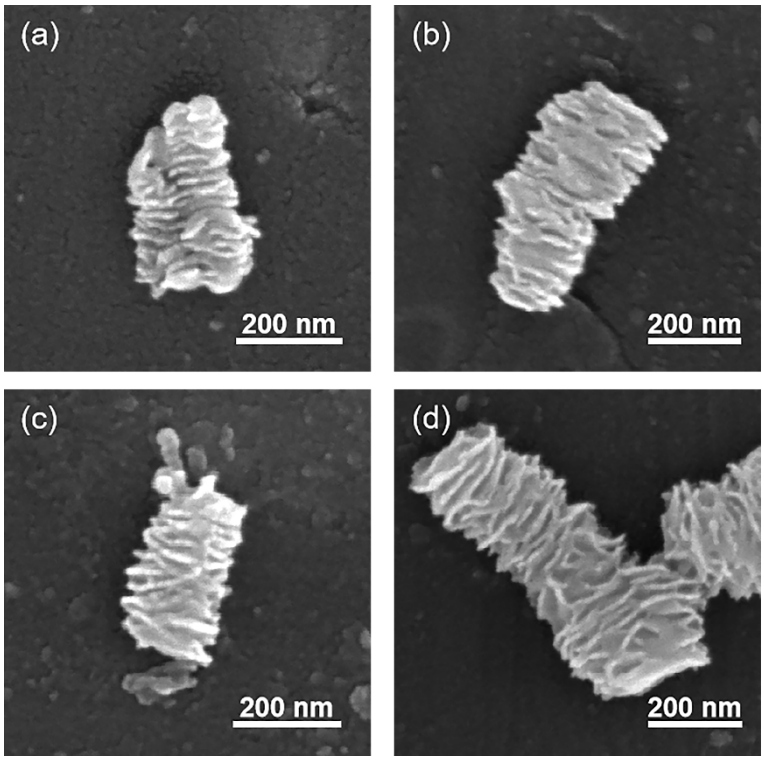

Fig. S1 SEM images of (a) MS-4, (b) MS-6, (c) MS-8 and (d) MS-10.

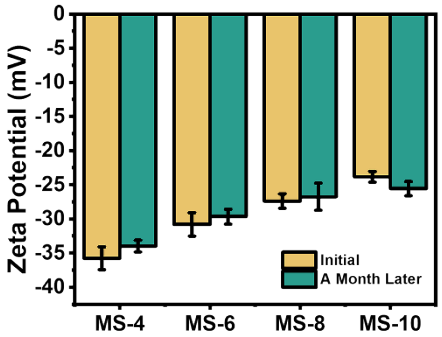

Fig. S2 Change in Zeta potentials of the samples distributed in PBS at pH 7.4 over a one-month period.

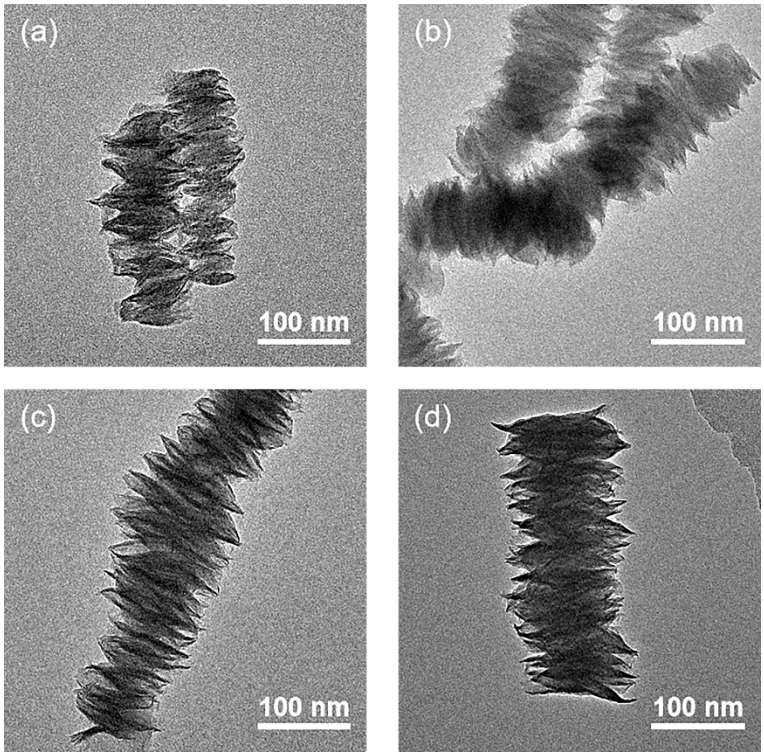

Fig. S3 TEM images of (a) MS-4, (b) MS-6, (c) MS-8 and (d) MS-10.

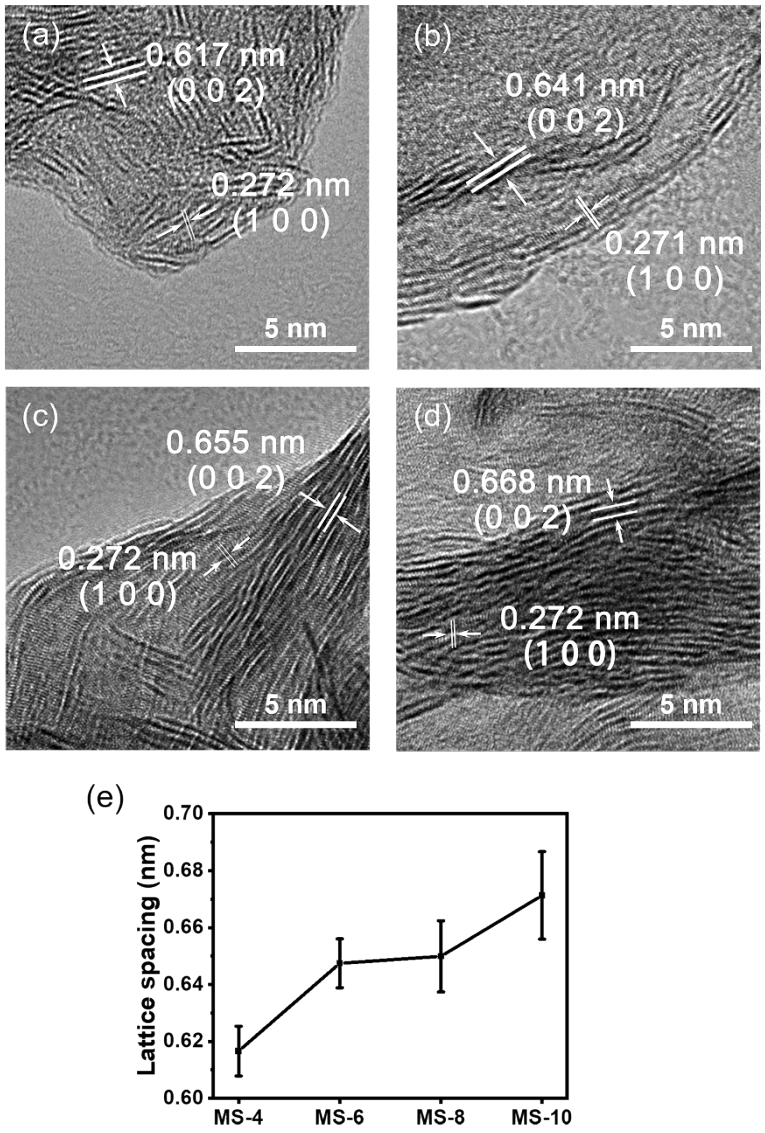

Fig. S4 HRTEM images of (a) MS-4, (b) MS-6, (c) MS-8, and (d) MS-10. (e) Comparison of lattice spacing across different samples. The values are determined by randomly selecting (002) regions in different images (n = 5).

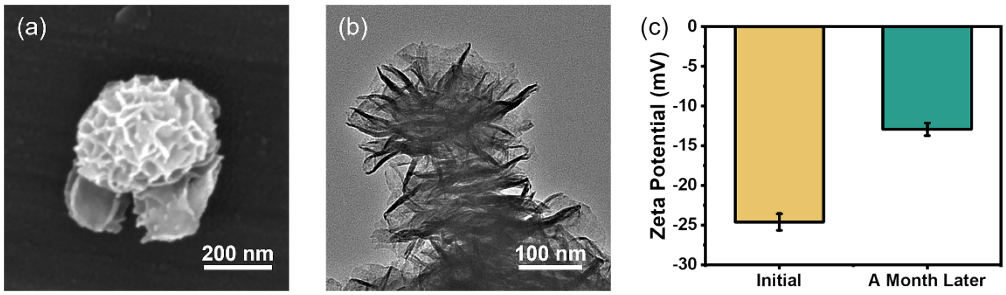

Fig. S5 (a) SEM images and (b) TEM images of MS-f, (c) Change in Zeta potential of MS-f distributed in PBS at pH 7.4 over a one-month period.

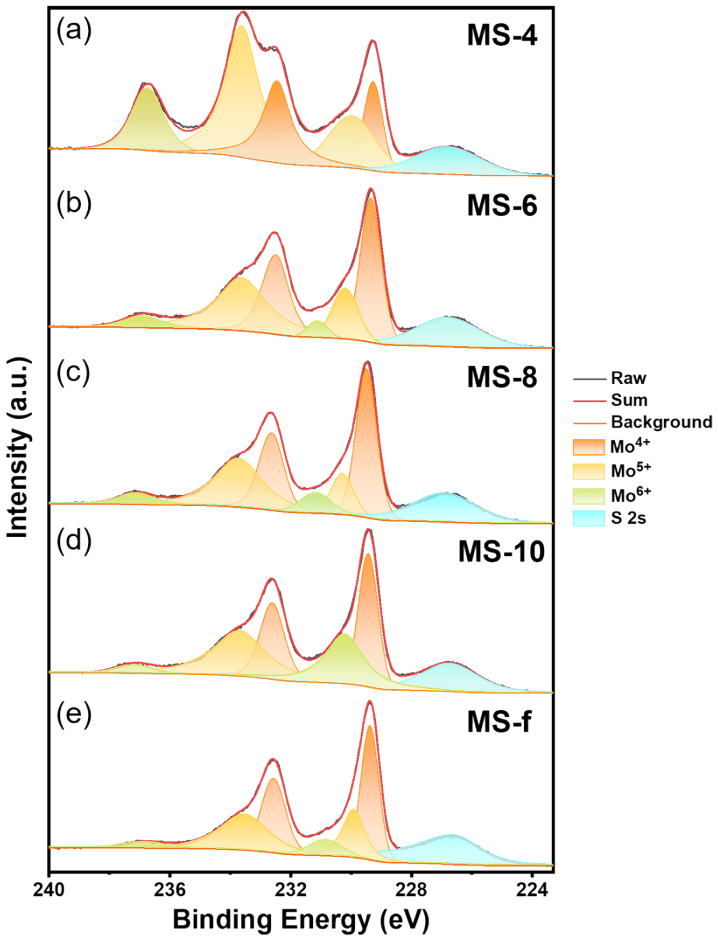

Fig. S6 High-resolution Mo 3d XPS spectrum of (a) MS-4, (b) MS-6, (c) MS-8, (d) MS-10 and (e) MS-f.

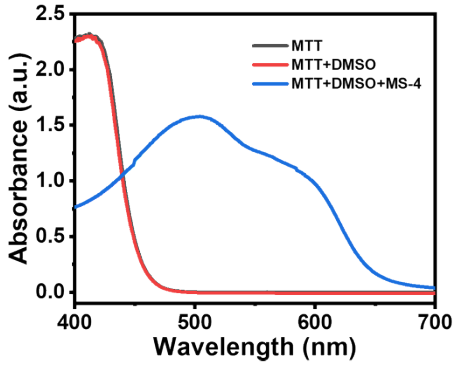

Fig. S7 Catalytic production of formazan from MTT by MS-4.

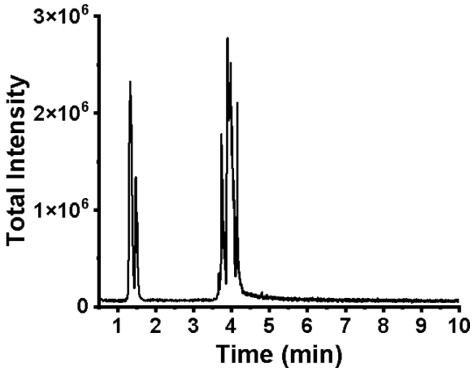

Fig. S8 Total ion chromatogram of LC-MS test.

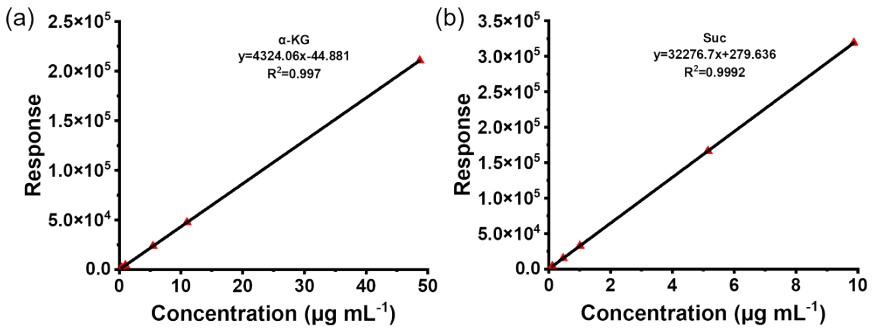

Fig. S9 Concentration standard curve of (a) α-KG and (b) Suc.

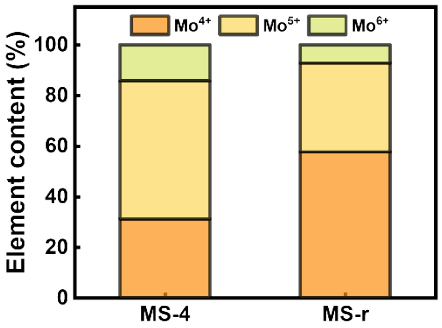

Fig. S10 Molar ratios of Mo^{4+}, Mo^{5+} and Mo^{6+} in MS-4 and MS-r.

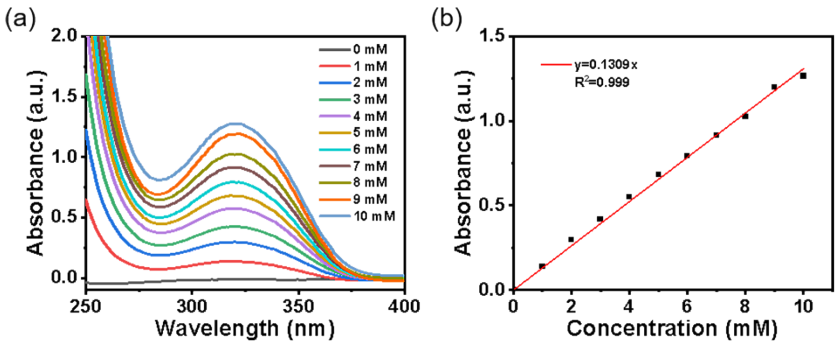

Fig. S11 (a) UV absorption spectra of different concentrations (0 - 10 mM) of α-KG. (b) Standard curve showing the relationship between absorbance at 320 nm and α-KG concentration.

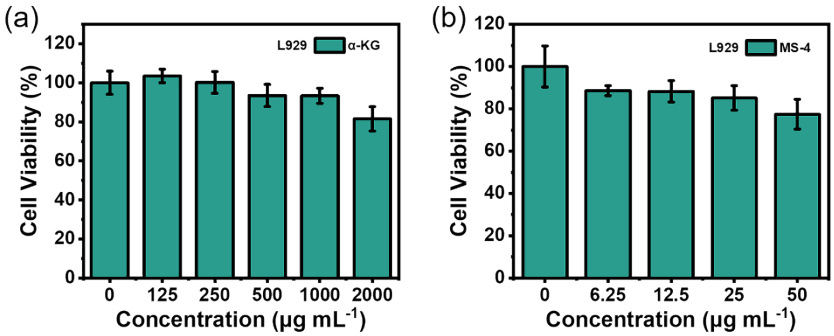

Fig. S12 Viability of L929 cells incubated with different concentrations of (a) α-KG, (b) MS-4.

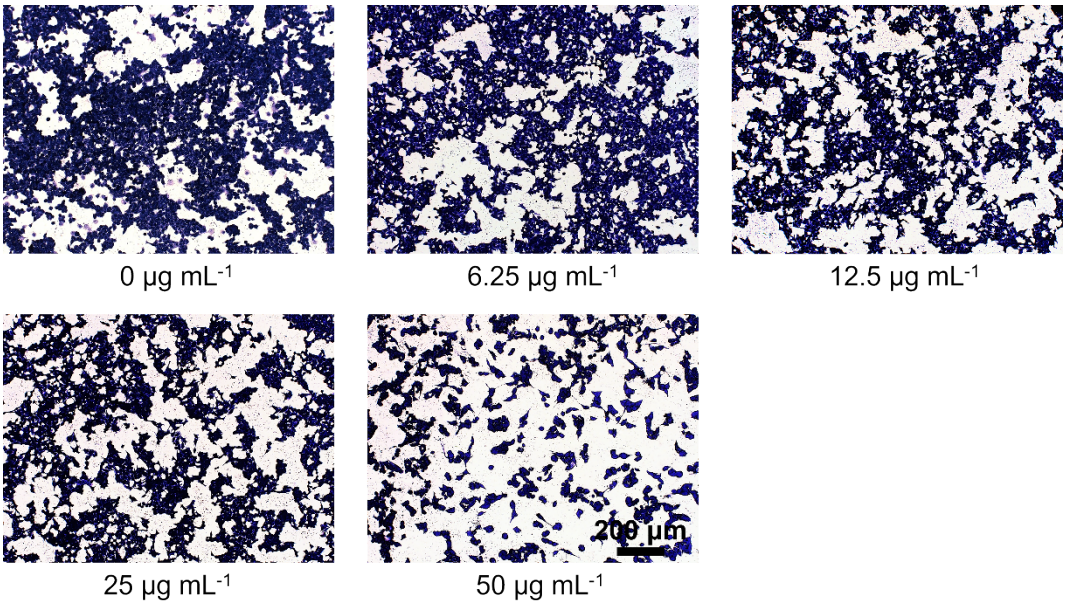

Fig. S13 Crystal violet staining of 4T1 cells after 24 h co-culture with different concentration of MS-4.

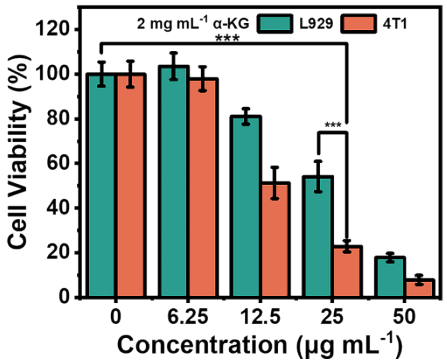

Fig. S14 Viability of L929 and 4T1 cells after co-treatment with 2 mg mL^{-1} α-KG and different concentrations of MS-4.

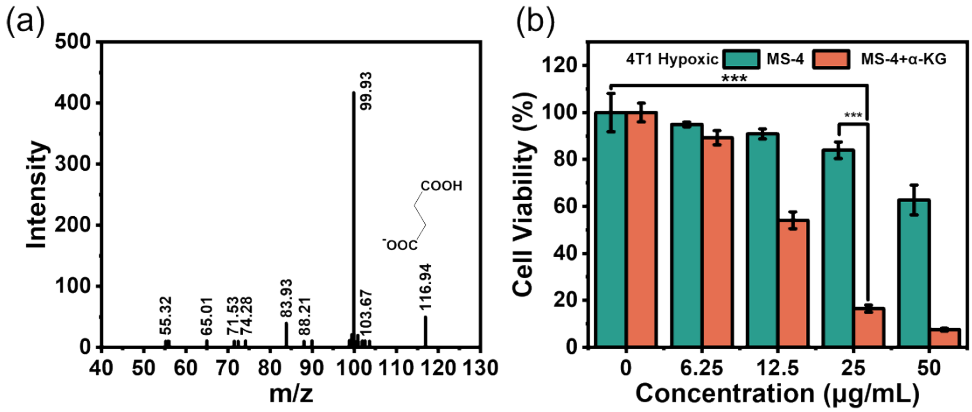

Fig. S15 (a) MS analysis of reaction products from MS-4 + α-KG under hypoxic conditions (1% O_2). (b) Cell viability of 4T1 cells following treatment with MS-4, MS-4+α-KG under a hypoxic condition (1% O_2, 24 h).

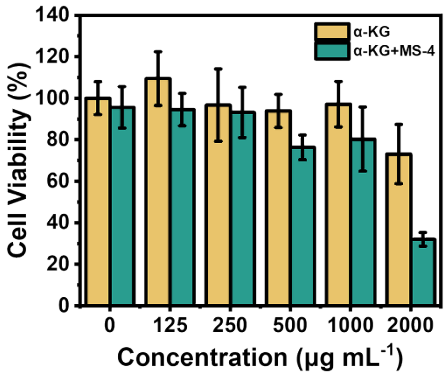

Fig. S16 Viability of B16F10 cells after different treatment.

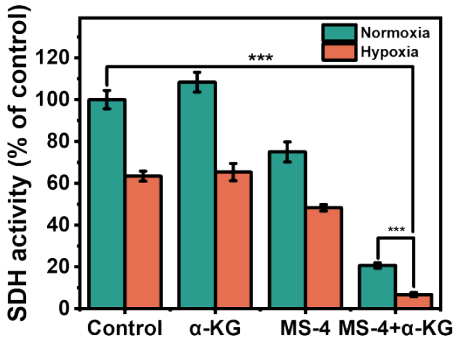

Fig. S17 SDH activity under normoxic (21% O_2) and hypoxic (1% O_2) conditions.

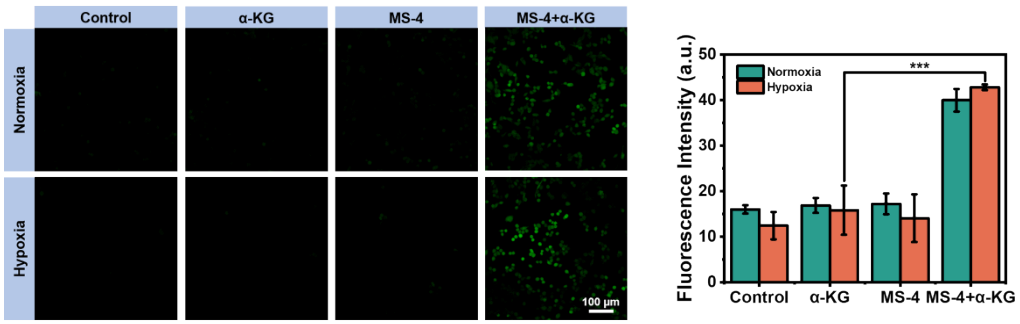

Fig. S18 Intracellular ROS detection by DCFH-DA fluorescence and corresponding semi-quantitative analysis.

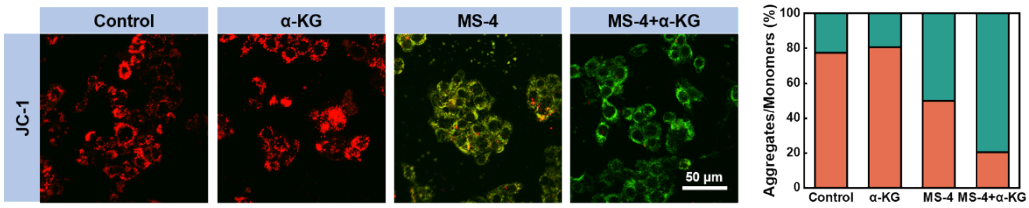

Fig. S19 Mitochondrial membrane potential by JC-1 staining under hypoxic conditions.

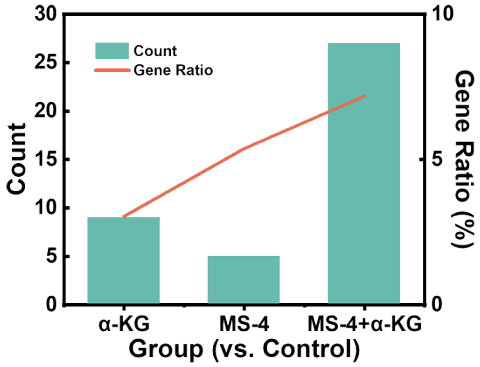

Fig. S20 Count and ratio of DEGs located in mitochondria in each group compared with the control group.

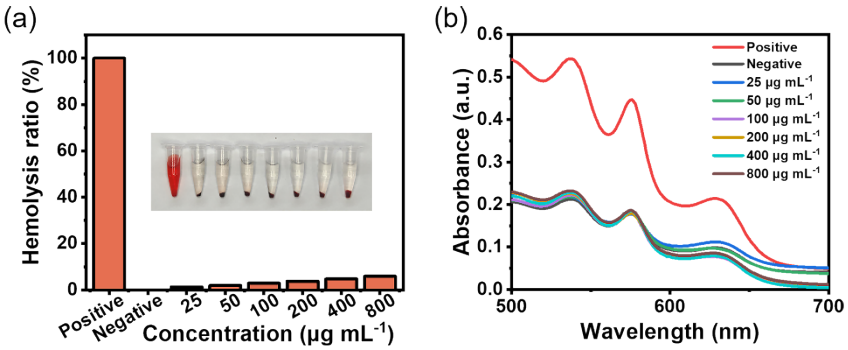

Fig. S21 (a) Hemolytic rate of MS-4. Inset shows the corresponding digital photograph. (b) UV-Vis absorption spectra of the supernatants.

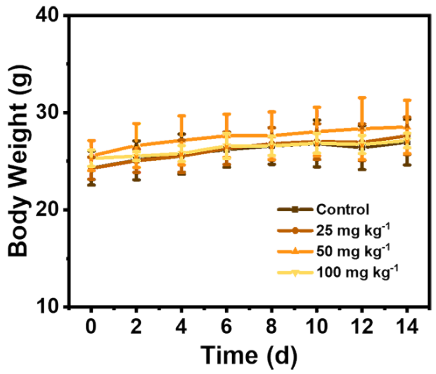

Fig. S22 Body weight of mice after intravenous injection of 25 mg kg^{-1}, 50 mg kg^{-1} and 100 mg kg^{-1} of MS-4 (n = 3).

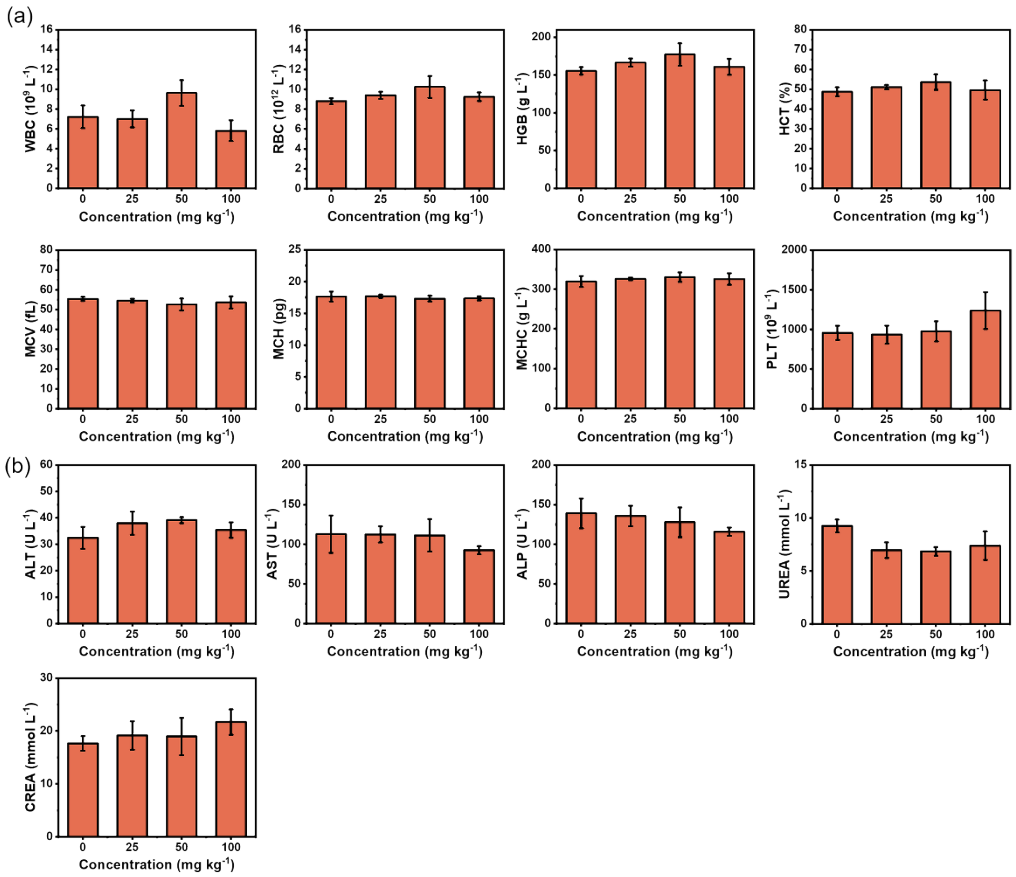

Fig. S23 *In vivo* acute toxicity of MS-4 on female ICR mice. (a) Blood routine indexes and (b) blood biochemistry on the day 14 after i.v. injection of MS-4 with different doses (0, 25, 50, and 100 mg kg^{-1}, n = 3).

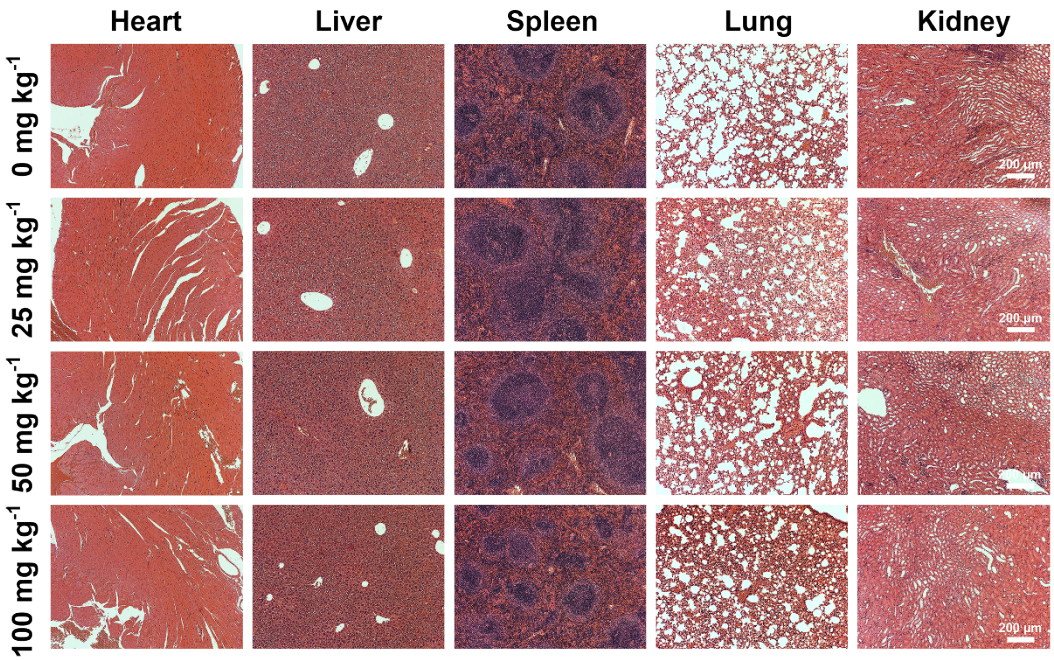

Fig. S24 H&E-stained organs of the mice on day 14.

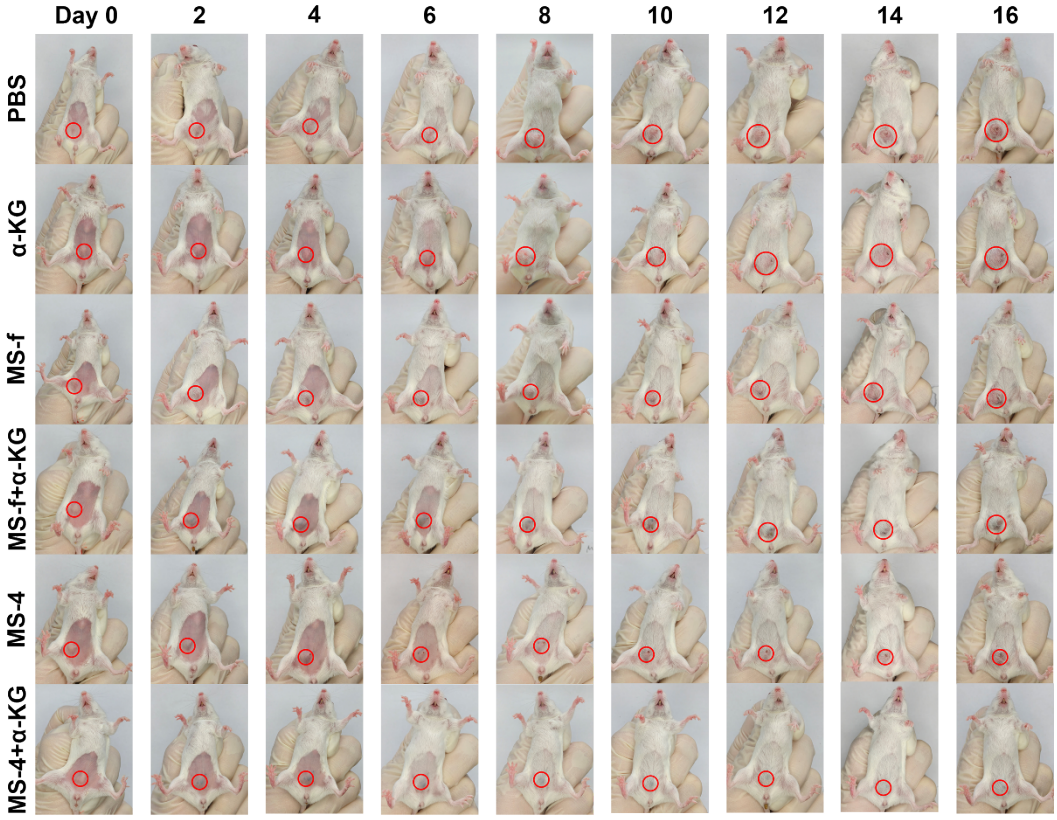

Fig. S25 Photographs of mice during the treatment process.

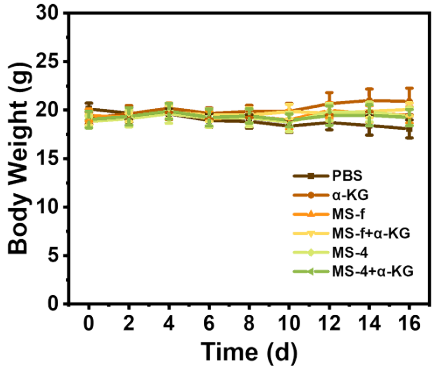

Fig. S26 Body weight change curves of the mice during the 16 days of treatment (n = 5).

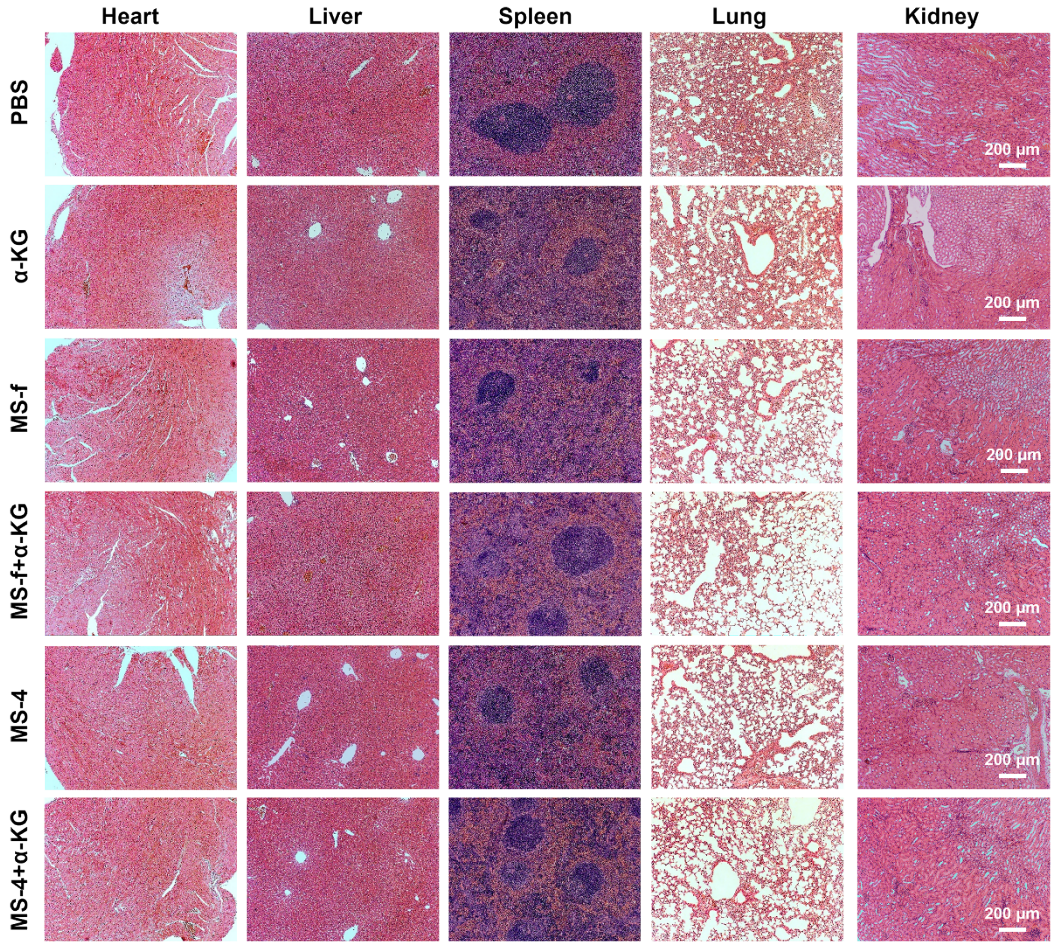

Fig. S27 H&E slices of major organs (heart, liver, spleen, lungs and kidneys) with different treatments.

Table S1 Kinetic parameters of dehydrogenase-like activity of MSs and MS-f.

Sample	K_M (mM)	v_{max} (M s^{-1})
MS-4	33.13	2.43×10^{-7}
MS-6	39.42	1.43×10^{-7}
MS-8	47.69	1.10×10^{-7}
MS-10	72.11	1.30×10^{-8}
MS-f	73.04	1.34×10^{-8}

Table S2 Comparison of kinetic parameters of dehydrogenase-like nanozymes in references

Sample	Substrate	K_M (mM)	v_{max} (M s^{-1})	Reference
MS-4	α-KG	33.13	2.43×10^{-7}	This work
AP-mSi	H_2O_2	16.86	2.175×10^{-8}	40
Dis-SAzyme	Glutathione	34.80	3.60×10^{-8}	41
Rh SANs	Glucose	56.96	7.67×10^{-7}	42
LDO	Lactate	0.4228	2.72×10^{-7}	43
CDs	3,3’,5,5’-Tetramethylbenzidine	0.22	2.74×10^{-8}	44

Supporting Reference

[1] J. A. Plumb, R. Milroy and S. B. Kaye, *Cancer Res*, 1989, **49**, 4435-4440.