Supporting information

The LDHs biological hybrid promotes electron transfer via dual starvation enhancing tumor Ferroptosis/Apoptosis.

Materials&Instruments

$MgCl_2 \cdot 6H_2O$, $FeCl_3 \cdot 6H_2O$, glucose, lactic acid, glutaraldehyde and methylene blue were purchased from Sinopharm Chemical Reagent Co., Ltd and used as received. Doxorubicin hydrochloride (DOX), glucose oxidase (GOx), 5,5'-Dithiobis-(2-nitrobenzoic acid) (DTNB) were obtained from Beijing Yinuokai Technology Co., Ltd. Tryptone, soy peptone, sodium chloride and agar powder were obtained from Wuhan Huashun Biotechnology Co., Ltd. Dulbecco's modified eagle medium (DMEM), trypsin, fetal bovine serum (FBS), phosphate buffer solution (PBS), penicillin–streptomycin, Cell Counting Kit-8 (CCK-8), 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), calcein-AM/propidium iodide (PI), Annexin V-FITC/PI apoptosis detection kit, Cy5.5, DAPI, tris(4,7-diphenyl-1,10-phenanthroline)ruthenium(II) dichloride, lipid peroxidation assay kit, lyso-tracker red and mitochondrial membrane potential assay kit with JC-1 were purchased from Beyotime Biotechnology Co., Ltd. Anti-Glutathione Peroxidase 4 Mouse mAb, Recombinant Anti-beta Actin antibody (Mouse mAb）, Ferritin light chain Polyclonal antibody, Caspase 3/P17/P19 Polyclonal antibody, GAPDH Monoclonal antibody were obtained from Wuhan Servicebio Technology Co., Ltd.

Morphology and element mapping of the NPs were measured with a transmission electron microscope (TEM, FEI Talos F200 microscope, 200 kV, the Netherlands). The size and zeta potential of the NPs were measured through dynamic light scattering (DLS) (Malvern Nano-ZEN3690, Malvern, U.K.). X-ray photoelectron spectroscopy (XPS) was performed on an x-ray photoelectron spectrometer (PHI5000 Versaprobel, Japan). The absorbance of the obtained solution was measured on a UV–Vis spectrophotometer (UV-1800, Shimadzu). Fluorescence was detected by a fluorescence spectrophotometer (F-380, Gangdong Technology). ROS species were measured by an EMXmicro-6/1 electron paramagnetic resonance spectrometer. Optical microscopy images were taken using a multifunctional fluorescence microscope (BZ-X800E, Japan). Characterization analysis of confocal laser scanning microscopy (CLSM) was implemented with a Leica TCS SP8 microscope.

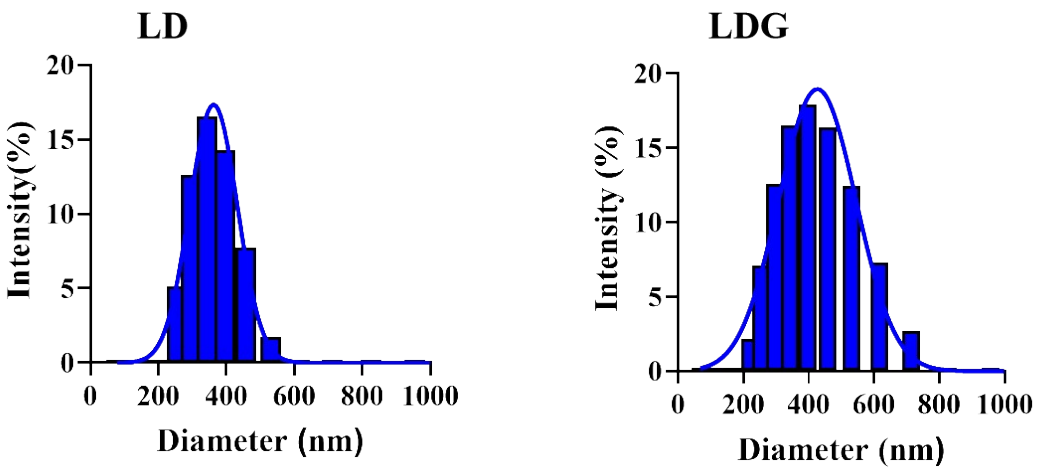

Figure S1. The hydrodynamic diameter of individual LD and LDG

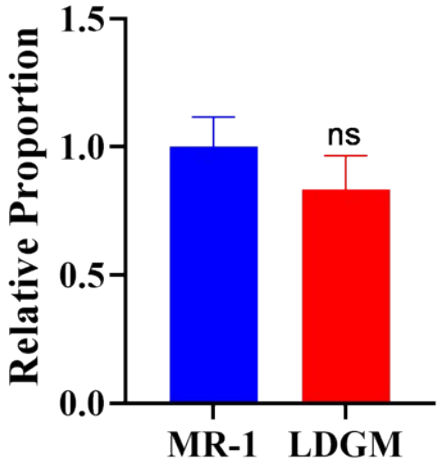

Figure S2. Relative proportion of colony counts

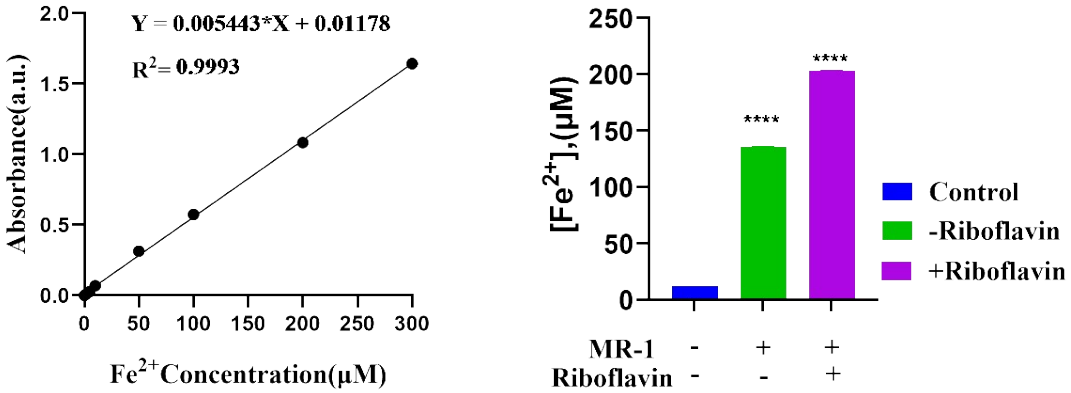

Figure S3. Standard curve of Fe^{2+} and verification of MR-1 electron transfer chain

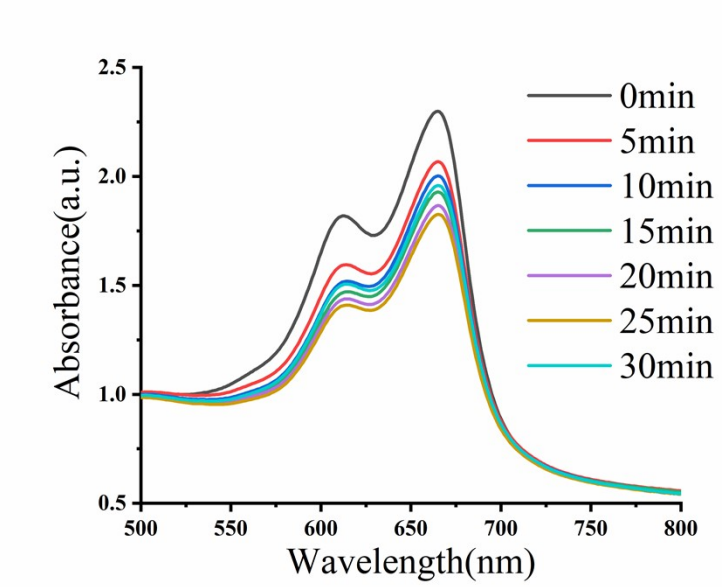

Figure S4. The UV-vis absorption spectra of the MB solutions with different time

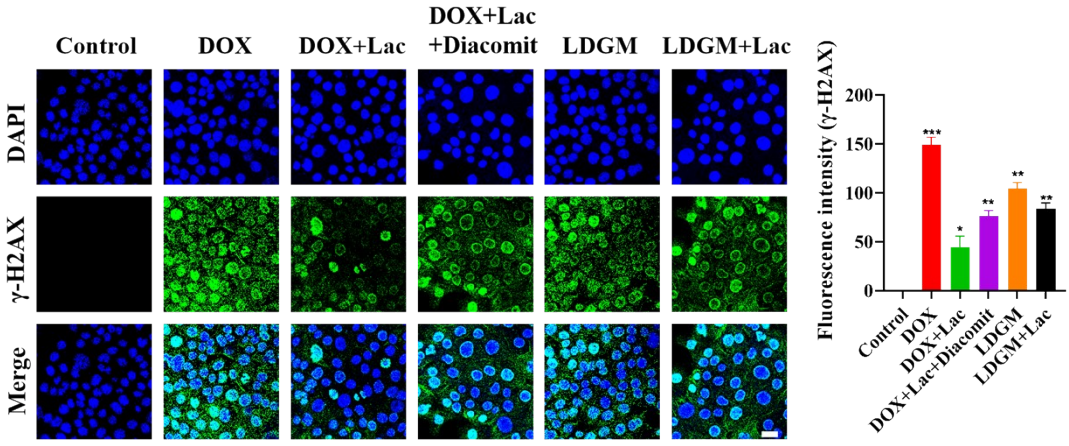

Figure S5. The multidrug resistance of tumor cells to chemotherapy drugs (scale bar: 20 μm)

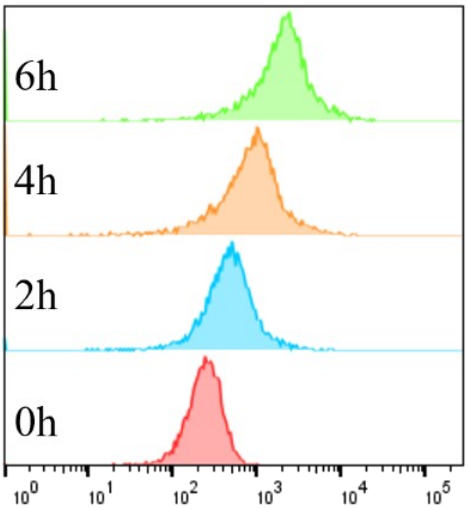

Figure S6. Flow cytometry data show the LDH endocytosis amount after different times

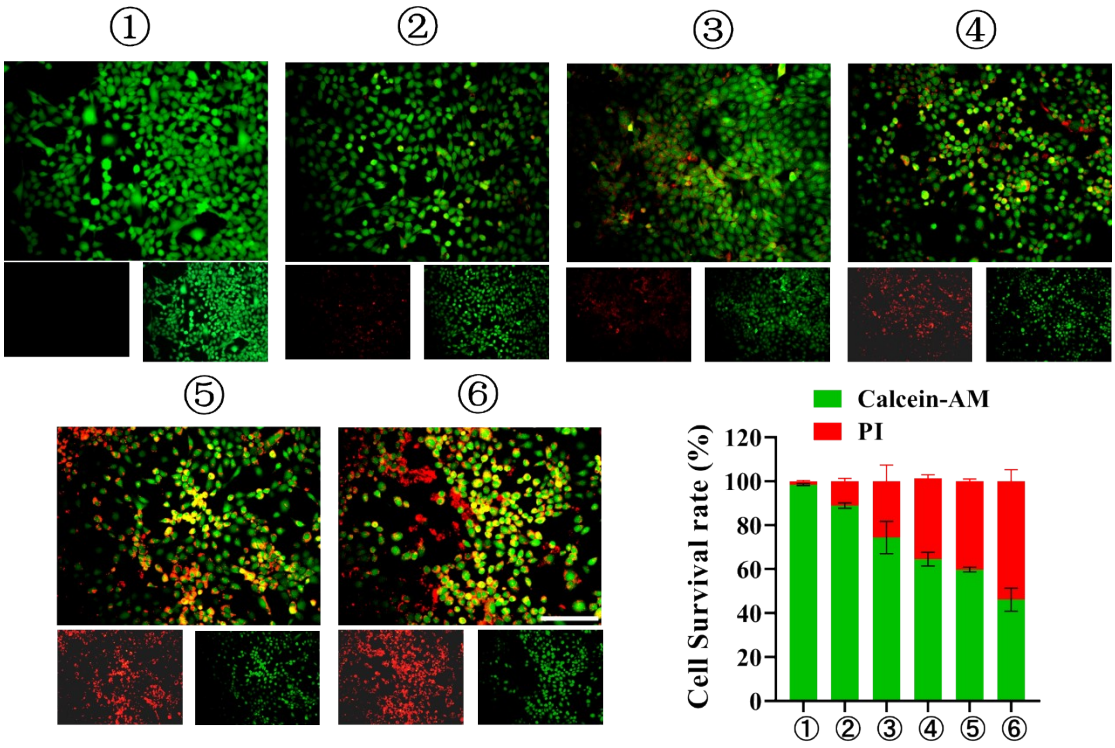

Figure S7. The images of 4T1 cells stained with calcein AM (green, live cells) and PI (red, dead cells) after different treatments (①L, ②LD, ③LDG, ④LDGM+Glu, ⑤LDGM+Lac, ⑥LDGM+Glu+Lac). (scale bar: 150 μm)

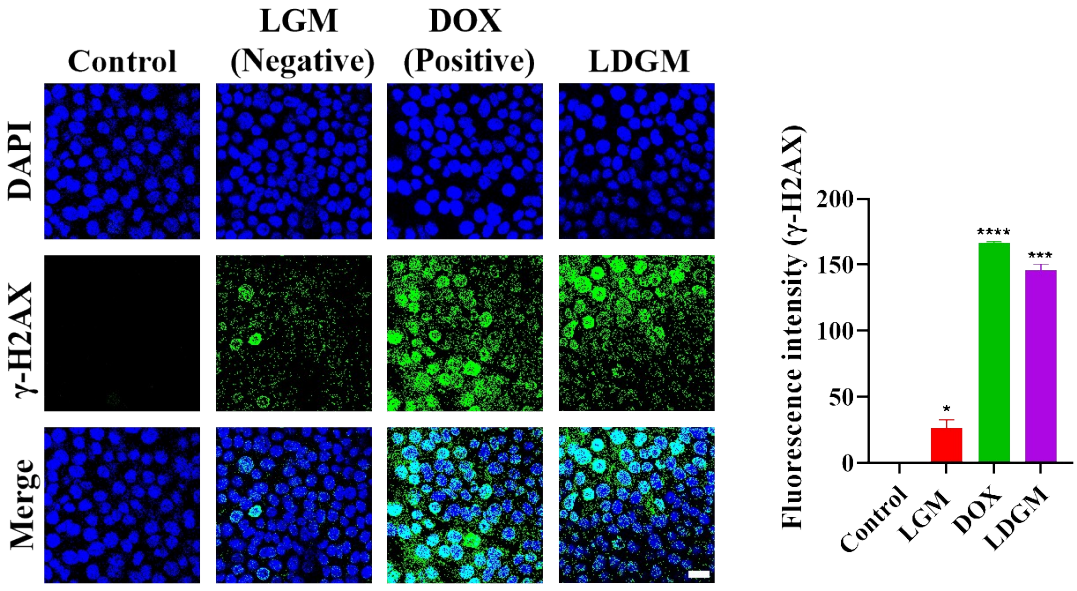

Figure S8. The DNA damage verification caused by DOX (LGM: LDGM not include DOX, scale bar: 20 μm)

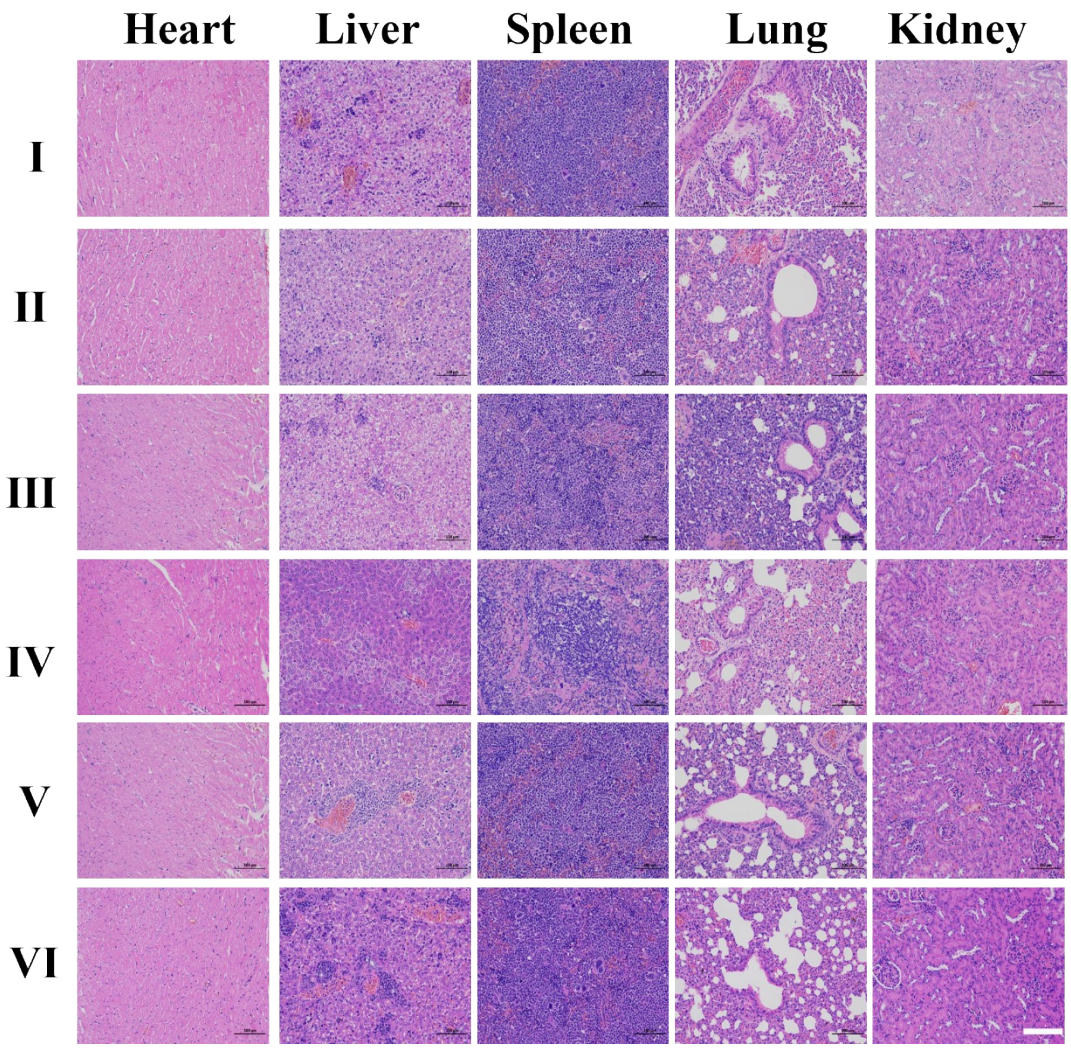

Figure S9. Representative H&E stained sections of liver, spleen, kidney, lung and heart from mice treated with I. Control, II. L, III. LD, IV. DOX, V. LDG, VI. LDGM. The scale bar in the last image applies to the others (scale bar: 150 μm)

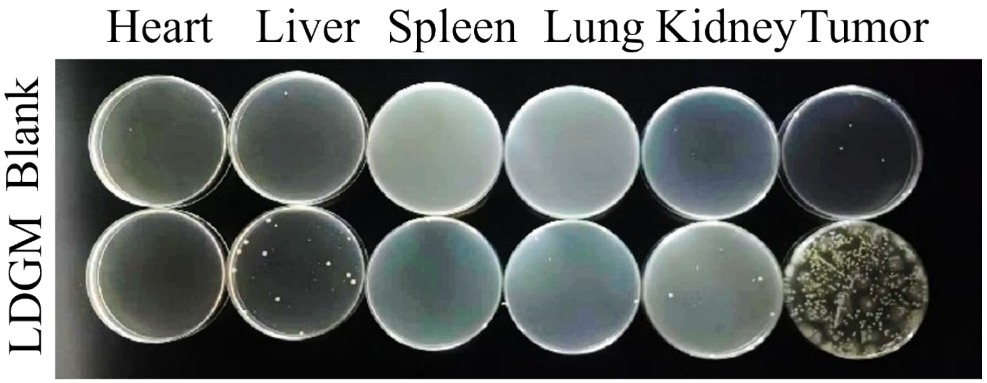

Figure S10. Study of LDGM accumulation in different tissues by using a dilution coating on the LB agar plates after the intravenous injection for 72 h

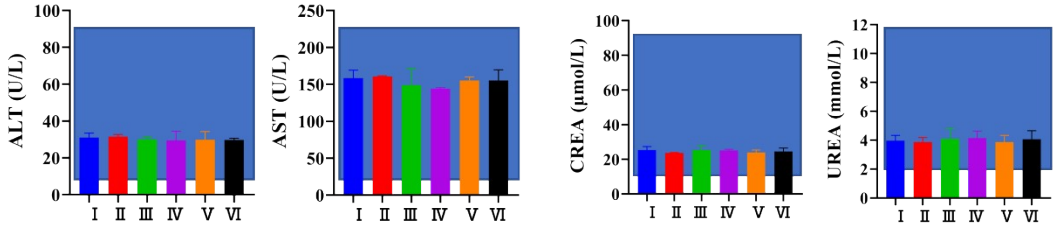

Figure S11. Biochemical analysis of ALT, ATS, BUN, and CR after intravenously injection of I. Control, II. L, III. LD, IV. DOX, V. LDG, VI. LDGM into the healthy BALB/c mice

Experiments

4.1 The preparation of MgFe-LDH (DOX)@GOx/MR-1 (LDGM)

MgFe-LDH (L) was synthesized by the hydrothermal method. Magnesium chloride ($MgCl_2$•$6H_2O$) and ferric chloride ($FeCl_3$•$6H_2O$) were weighed in a molar ratio of 3:1 and dissolved in 10 mL of mixed metal salt solution. An alkaline solution was added to adjust the pH to 10 ± 0.01 under N_2 atmosphere at 37°C. The mixture was then stirred for half an hour, followed by centrifugation and discarding of the supernatant to obtain the initial product. The initial precipitate of L was resuspended in 40 mL of deionized water and reacted in a hydrothermal reactor at 100°C for 16 hours. After cooling, centrifuge and wash twice, finally lyophilize to obtain MgFe-LDH, referred to as L. Take 10 mg of MgFe-LDH nanoparticles and disperse them in 10 mL of ultrapure water (nanoparticle mass concentration: 1 mg/mL), followed by the addition of 5 mL of DOX solution (1 mg/mL). After stirring for 24 hours, the DOX-loaded MgFe-LDH nanoparticles were collected via centrifugation (10,000 rpm) and washed repeatedly with ultrapure water until the supernatant became colorless. The resulting product, designated as MgFe-LDH (DOX) (abbreviated as LD), was lyophilized for storage.To determine the drug loading efficiency of DOX, the supernatant after centrifugation was collected, and the absorbance of free DOX was measured at 480 nm using a UV spectrophotometer. The content of unencapsulated DOX was calculated based on the standard curve. The drug loading (DL%) was calculated as (mass of loaded DOX / total mass of carrier) × 100%; the encapsulation efficiency (EE%) was calculated as (mass

of loaded DOX / initial mass of DOX) × 100%. After calculation, the drug loading of DOX in the LD system was 25%, and the encapsulation efficiency was 88.2%. Subsequently, 1 mg of GOx was dissolved in 500 μL of ultrapure water to prepare a 2 mg/mL solution. Then, 200 μL of the GOx solution was mixed with 20 mL of LD suspension (2 mg/mL) and stirred for 24 hours. The GOx-loaded LD nanoparticles were collected by centrifugation (10,000 rpm) and washed with ultrapure water until the supernatant was colorless. After lyophilization, MgFe-LDH(DOX)@GOx (abbreviated as LDG) was obtained. Finally, 1 mL of MR-1 suspension (10^8 CFU/mL) was combined with 2 mg of LDG (1 mg/mL) in ultrapure water and gently agitated for 60 seconds to prepare MgFe - LDH /MR-1. The mixture was centrifuged (6,000 × g, 3 min) and washed with PBS to yield the LDH (DOX)/GOx@MR-1 biohybrid material, abbreviated as LDGM.

4.2 The release of DOX

The release of doxorubicin hydrochloride in vitro was evaluated by dialysis method. In brief, different samples (LDGM, LDGM + Glu, LDGM + Lac, LDGM + Glu + Lac) with the same dose of DOX were uniformly dispersed in PBS solution with pH 5.0 and loaded into dialysis bags (molecular weight (MW): 3500). Then, the dialysis bags were placed in PBS solution with the same pH and stirred at 200 r/min. At different time points of 0.5, 1, 2, 4, 8, 12, 22, 32, 46, 60, and 74 h, 2 mL of the suspension was drawn out and replaced with the same volume of PBS solution. The absorbance of each sample was measured at 480 nm using a UV spectrophotometer to analyze the drug release situation.

4.3 The detection of •OH *in vitro*

The LDGM suspension (1 mg/mL), glucose (20 mM), and lactic acid (20 mM) were mixed in PBS solution for 15 minutes, then adding 3 μL of the free radical capture agent DMPO and H_2O_2 (0.1 mM) to detect the •OH signal using ESR; To study the time-dependent generation of hydroxyl free radicals, the LDGM suspension (1 mL, 1 mg/mL), glucose solution (200 μL, 20 mM), and lactic acid solution (200 μL, 20 mM) were added to the MB solution (200 μL, 25 μg/mL) for 60 minutes at room temperature, recording the change in absorbance at 665 nm using UV-Vis spectroscopy; To study the production of reactive oxygen free radicals (•OH) in different groups, the MB solution (200 μL, 25 μg/mL) was added to the LDGM, LDGM+Glu, LDGM+Lac, and LDGM+Glu+Lac suspensions (1 mL, 1 mg/mL) respectively to obtain the mixed suspensions for 30 minutes at room temperature.

4.4 The consumption of GSH *in vitro*

The depletion of glutathione by different nanoparticles was detected using the DTNB probe. In brief, 100 μL (11 mg/mL) of LDG+Glu, LDGM+Glu, LDGM+Lac, and LDGM+Glu+Lac were mixed with 100 μL of glutathione (10 mM) in 3.8 mL distilled water, and then the mixture was incubated at 37°C for 30 minutes. Subsequently, the mixture was centrifuged at 9000 rpm/min for 10 minutes, and then 600 μL of the supernatant was mixed with 3 mL DTNB solution (0.02 mg/mL) in Tris-HCL buffer (pH = 6.5). The absorbance at 412 nm was measured by ultraviolet spectrophotometry; the depletion of glutathione by nanoparticles at different times was detected using the

DTNB probe, following the same method. The absorbance at 412 nm was measured by ultraviolet spectrophotometry at 0, 15, 30, 45 and 60 min.

4.5 The content of O_2 *in vitro*

To study the oxygen consumption capacity of different groups, the dissolved oxygen content of L+Glu, LDG+Glu, and LDGM+Glu solutions (L, LDG, LDGM are 100 μL, 200 μg/mL, and Glu is 50 μL, 20 mM) was measured every minute using an oxygen meter, until the oxygen content remained constant.

4.6 The metabolism of lactic acid *in vitro*

These were dissolved in 1 mL of PBS buffer solution (where the lactate and glucose concentrations were 20 mM, and the concentrations of LDG and LDGM were 200 μg/ml). After 24 hours, the lactate concentration was determined using the lactate assay kit.

4.7 Bacterial experiment

The Shewanella marcescens MR-1 strain was cultivated aerobically on the soybean casein agar medium TSA (1.5g peptone, 0.5g soybean peptone and 1.5g sodium chloride) at 30°C.

4.8 Cell experiment

4.8.1Cell viability assay

In the cell viability experiment, 4T1 and L929 cells were seeded into 96-well plates (1×10^4 cells per well), and incubated at 37°C overnight. In L929 cells, 100 μL of 31.25, 62.5, 125, 250, 500 μg/mL of L and L/MR-1 were added respectively. In 4T1 cells, 100 μL of 31.25, 62.5, 125, 250, 500 μg/mL of L, LD, LDG, LDGM+Glu, LDGM+Lac, LDGM+Glu+Lac were added respectively. After 24 hours of incubation, fresh medium with 10 μL of CCK-8 were added and incubated for 4 hours. The absorbance of each well was measured using microplate reader.

4.8.2 Detection of intracellular reactive oxygen species (ROS)

The total level of ROS was detected using the standard probe DCFH-DA. In brief, 4T1 cells were cultured in 24-well plates with 10% serum medium (7×10^4 cells per well), and cultured for 24 hours. Then, the cells were washed with PBS and incubated with 200 μg/mL PBS, LDGM+Glu, LDGM+Lac, LDGM+Glu+Lac for 24 hours, and then washed with PBS and incubated with 1 μL of DCFH-DA (10 mM) for 20 minutes. Fluorescence imaging was recorded using a multifunctional fluorescence microscope.

4.8.3 Detection of cell apoptosis/necrosis

4T1 cells (2.5×10^5 cells per well in six-well plates) were seeded overnight and treated with 200 μg/mL of L, LD, LDG, LDGM+Glu, LDGM+Lac, LDGM+Glu+Lac for 24 hours. Then, the cells were washed three times with PBS, treated with the V-FITC/PI cell apoptosis detection kit, and analyzed using a flow cytometer. Cells without drug treatment were set as the control group.

4.8.4 Cell live/dead staining

For live/dead cell staining, 4T1 cells (7×10^4 cells per well in 24-well plates) were seeded overnight and treated with 200 μg/mL of L, LD, LDG, LDGM+Glu, LDGM+Lac, LDGM+Glu+Lac for 24 hours. Then, calcium yellow-green fluorescent albumin and PI staining was performed for 30 minutes, and fluorescence was observed

using a multifunctional fluorescence microscope; Red cells represent dead cells, and green cells represent live cells.

4.8.5 Glutathione consumption in vivo

4T1 cells (7×10^4 cells per well in 24-well plates) were seeded overnight and treated with 200 μg/mL of LDG+Glu, LDGM+Glu, LDGM+Lac, LDGM+Glu+Lac for 24 hours. Then, the cells were washed three times with PBS, and lysed using a cell homogenizer, centrifuged (10000rpm, 30min). Then, 600 μL of the supernatant was mixed with 3 mL of DTNB solution (0.02 mg/mL) in Tris-HCL buffer (pH = 6.5) and the absorbance at 412 nm was measured by UV spectrophotometry.

4.8.6 Detection of mitochondrial membrane potential

4T1 cells (2.5×10^5 cells per well in a 6-well plate) were incubated with overnight and added 200 μg/mL of L, LD, LDG, LDGM+Glu, LDGM+Lac, LDGM+Glu+Lac for 24 hours of incubation. Then the cells were incubated with JC-1 probe for 20 minutes and observed the treated cells under a multifunctional microscope. The JC-1 monomers and aggregates were detected in the fluorescence isothiocyanate fluorescein (FITC) and red fluorescent protein (RFP) channels.

4.8.7 Detect intracellular oxygen content

Oxygen-sensitive fluorescent probe $[Ru(dpp)_3]Cl_2$ was used to detect the intracellular oxygen level. The fluorescence of this probe can be dynamically quenched by oxygen molecules: the higher the oxygen concentration, the lower the fluorescence intensity; conversely, under hypoxic conditions, the fluorescence is enhanced. 4T1 cells were seeded in a 6-well plate at a density of 2×10^5 cells per well and cultured in a 37°C, 5% CO_2 incubator for 24 hours until cells adhered and reached 80% confluence. The original culture medium was discarded, and 1 mL per well of $[Ru(dpp)_3]Cl_2$ probe solution (final concentration 5 μM) was added, followed by incubation for 4 hours to allow sufficient probe uptake by the cells. After probe incubation, the medium containing the probe was removed, and the cells were gently washed twice with pre-warmed PBS to remove unbound probe. Using a reverse sequential method (150, 120, 90, 60, 30 min), the following treatment solutions were added (1 mL per well, concentration 125 μg/mL): L group, LD group, LDG group, LDGM Glu group, LDGM Lac group (containing 20 mM lactate), LDGM Glu Lac group (containing 20 mM glucose and 20 mM lactate). The cell plate was returned to the incubator for cultivation. At the designated time points, the treatment solutions were removed, and the cells were gently washed three times with PBS, 2 minutes each time. Then, 1 mL PBS was added per well, and the fluorescence images were immediately observed and captured using an inverted fluorescence microscope. The excitation wavelength of $[Ru(dpp)_3]Cl_2$ is 450-490 nm, and the emission wavelength is 610-620 nm, presenting red fluorescence under the fluorescence microscope. The higher the degree of hypoxia, the stronger the fluorescence signal.

4.8.8 Co-localization of lysosomes

4T1 cells in a confocal culture dish, were respectively treated with L/Cy5.5 for 0, 2, 4 and 6 hours, then stained with LysoTracker Green for 50 minutes, fixing the cells with 4% paraformaldehyde solution (1 ml) for 10 minutes, and staining with DAPI for 15 minutes. Take pictures of the cells using a confocal laser scanning microscope.

4.8.9 Flow cytometry endocytosis

4T1 cells in a 6-well plate for 24 hours, were respectively treat with L/Cy5.5 for 0, 2, 4 and 6 hours, and then detect using a flow cytometer.

4.8.10 Lactic acid metabolism

To evaluate the ability of LDGM to metabolize lactic acid. 4T1 cells were cultivated in a 6-well plate for 24 hours, adding 200 μg/mL of LDG+Glu, LDGM, LDGM+Glu and incubated for 24 hours, then collecting the cells, washing the cell suspension with PBS. Cells is broke by cell homogenizer, centrifuging to obtain the supernatant and measuring the lactic acid concentration via lactic acid detection kit.

4.8.11 Western blot analysis

The cell samples were collected after treated, using a RIPA lysis buffer containing 1% protease inhibitor and performed ice-cold lysis for 30 minutes. Also, the protein samples were collected from the supernatant and quantified them using the BCA assay. Then, equal amounts of protein was mixed with protein loading buffer and boiled at 100°C for 15 minutes. Each sample was loaded equally into 12.5% PAGE gel lanes and performed electrophoresis. The protein was transferred to a polyvinylidene fluoride (PVDF) membrane and incubated at room temperature for 1 hour in 10% skimmed milk buffer. Subsequently, the PVDF membrane was incubated with the primary antibodies (Cas-3, GPX4, Ferritin) at 4°C overnight, and then the secondary antibody was added in accordance with the instructions. Finally, the chemiluminescence image was obtained using a chemiluminescence imaging system.

4.8.12 LPO Fluorescence Detection

In brief, 4T1 cells were cultured in 24-well plates (7×10^4 cells per well) for 24 hours, then the cells were washed with PBS and 200 μg/mL of PBS, LDGM+Glu, LDGM+ Lac, and LDGM+Glu+Lac were added and incubated for 24 hours at 37°C. The cells were incubated with the LPO probe for 30 minutes and fluorescence imaging was recorded using a multifunctional fluorescence microscope.

4.8.13 DNA Damage Immunofluorescence Assay

Cells were plated in confocal dishes overnight, with the treatment groups as follows: Control, DOX (10 μM), LDGM (200 μg/mL), LGM (LDGM not including DOX) (200 μg/mL). After 12 hours of treatment, DNA damage in 4T1 cells was assessed using Beyotime's DNA damage detection kit (γ-H2AX immunofluorescence) according to the manufacturer's instructions. Observation and data recording were performed using a confocal fluorescence microscope (DMI3000B, Leica, Germany). (doi: 10.1038/s41419-024-06688-5.)

4.8.14 Fe^{3+} Reduction Experiment

Riboflavin Competition Experiment .Aerobically culture MR-1 to the logarithmic phase (OD_{600} = 0.5-0.8), centrifuge to collect the cells, and wash three times with PBS to remove endogenous riboflavin. Resuspend the cells in PBS (OD_{600} = 0.5-1.0) and purge with N_2 for 10 minutes. The groups are as follows: ① Blank control group (PBS, 20 mM sodium lactate, 10 mM ferric citrate); ② Wash group (washed MR-1, sodium lactate, ferric citrate); ③ Riboflavin supplementation group (washed MR-1, sodium lactate, ferric citrate, 10 μM riboflavin). After anaerobic culture at 30°C for 24 h, take 0.5 mL of the culture and add 0.5 mL of 0.5 M HCl for acidification, then centrifuge at

4,000 g for 10 min. React 100 μL of the supernatant with 1 mL of 0.5% o-phenanthroline coloring solution, adjust pH to about 5 with sodium acetate buffer, and measure absorbance at 510 nm. Calculate Fe^{2+} concentration using a standard curve.

4.8.15 LDGM Multidurg Resistance Experiment

Lactic acid supplementation/LDHA inhibitor experiment: Refer to the method of Chen et al. (Nature, 2024, DOI: 10.1038/s41586-024-07620-9). Seed 4T1 cells at 1×10^4 cells/well in confocal dishes and culture overnight, then pretreat in groups for 12 hours: control group (regular culture), lactic acid group (20 mM lactic acid), inhibitor group (1 μM Diacomit). After pretreatment, discard the medium and perform combined treatment for 12 hours: the control group receives fresh medium; The DOX group was cultured in medium containing 10 μM DOX; the DOX lactate group was cultured in medium containing 20 mM lactate and 10 μM DOX; the DOX lactate inhibitor group was cultured in medium containing 1 μM Diacomit, 20 mM lactate, and 10 μM DOX; the LDGM group was cultured in medium containing 200 μg/mL LDGM; the LDGM lactate group was cultured in medium containing 200 μg/mL LDGM and 20 mM lactate. DNA damage was detected using γ-H2AX immunofluorescence staining: fixed with 4% paraformaldehyde, permeabilized with 0.3% Triton X-100, blocked with 5% BSA, incubated overnight at 4°C with anti-γH2AX antibody (1:200), stained with Alexa Fluor 488 secondary antibody (1:200), counterstained with DAPI, and observed under a confocal microscope.

4.9 Animal Experiment

The tumor-targeting, anti-tumor efficacy and safety of LDGM were evaluated through in vivo imaging, tumor growth curve, and histological section analysis.

4.9.1 Animal Model: BALB/c mice (female, 6 weeks old, 18 ± 2 grams) were purchased from Rat Expo (Yichang, China). A 4T1 xenograft tumor model was established by inoculating 2×10^6 4T1 cells (100 μL PBS) into the back of each BALB/c female mouse. Then, treatment was initiated when the 4T1 tumor reached approximately 100-150 mm^3.

4.9.2 Evaluation of LDGM's Tumor Targeting

In vivo and in vitro fluorescence imaging. First, L, MR-1, and L/MR-1 were labeled with Cy5.5, and then the labeled drugs were injected into the blood of 4T1 tumor-bearing female BALB/c mice through the tail vein (Cy5.5 0.2 mg/kg). The in vivo fluorescence imaging was monitored at predetermined intervals (0, 2, 4, 8 h) using a near-infrared imaging instrument. After 8 hours of fluorescence imaging, the mice were sacrificed and their major organs (heart, liver, spleen, lung, kidney) and tumors were dissected and collected for in vitro fluorescence imaging analysis.

4.9.3 Evaluation of LDGM's Metabolism In Vivo

First, L, MR-1, and L/MR-1 were labeled with Cy5.5, and then the labeled drugs were injected into the blood of 4T1 tumor-bearing female BALB/c mice through the tail vein (Cy5.5 0.2 mg/kg). The in vivo fluorescence imaging was monitored at predetermined intervals (0, 6, 24, 30, 48, 54 h) using a near-infrared imaging instrument. After 54 hours of fluorescence imaging, the mice were sacrificed and their major organs (heart, liver, spleen, lung, kidney) and tumors were dissected and collected for in vitro fluorescence imaging analysis.

4.9.4 Anti-Tumor Evaluation In Vivo

4T1 tumor-bearing mice were divided into 6 groups for various treatments, as follows: (1) intravenous injection of 100 μL PBS solution, (2) intravenous injection of 100 μL L solution, (3) intravenous injection of free DOX solution, (4) intravenous injection of LD suspension, (5) intravenous injection of LDG suspension, (6) intravenous injection of LDGM suspension. Groups 4 - 6 all had the same 5 μg/mL DOX dose. The weight and tumor size of each mouse were monitored for 14 days. The tumor volume (V) was calculated as (width ^{2} × length) / 2.

4.9.5 Tumor Immunofluorescence Analysis and Pathological Evaluation

After 14 days of various treatments, the BALB/c mice carrying 4T1 tumors were sacrificed, and their tumors were removed for immunofluorescence analysis. The tumors of the Control, L, DOX, LD, LDG, and LDGM groups were stained for TUNEL, Ki67, and HE staining detection. The major organs (heart, liver, spleen, lung, kidney) of the Control, L, DOX, LD, LDG, and LDGM groups were stained for HE staining detection. Six groups of mice, each with 270 μL of ocular blood, were taken and placed in anticoagulation tubes for blood routine tests (WBC, RBC, PLT). For each group of mice, one full blood sample was left at room temperature for 2 hours, then centrifuged at 3000 rpm in 2-8℃ environment for 15 minutes, and the supernatant was collected for blood biochemical tests (ALT, AST, BUN, CR).