

## Supporting Information

### **A functionalized cell membrane biomimetic nanoformulation based on layered double hydroxide for combined tumor chemotherapy and sonodynamic therapy**

Gaoming Li,<sup>a</sup> Yunqi Guo,<sup>a</sup> Cheng Ni,<sup>a</sup> Zhiqiang Wang,<sup>a</sup> Mengsi Zhan,<sup>a</sup> Huxiao Sun,<sup>a</sup> Goeun Choi,<sup>c</sup> Jin-Ho Choy,<sup>c</sup> Xiangyang Shi<sup>\*a,b</sup> and Mingwu Shen<sup>\*a</sup>

<sup>a</sup> State Key Laboratory for Modification of Chemical Fibers and Polymer Materials, Shanghai Engineering Research Center of Nano-Biomaterials and Regenerative Medicine, College of Biological Science and Medical Engineering, Donghua University, Shanghai 201620, China

<sup>b</sup> CQM-Centro de Química da Madeira, Universidade da Madeira, Campus Universitário da Penteada, 9020-105 Funchal, Portugal

<sup>c</sup> Intelligent Nanohybrid Materials Laboratory (INML), Institute of Tissue Regeneration Engineering (ITREN), Dankook University, Cheonan 31116, Republic of Korea

---

\* Corresponding author. E-mail: xshi@dhu.edu.cn (X.Y. Shi) and mwshen@dhu.edu.cn (M.W. Shen)

## Experimental

### Materials

Magnesium nitrate hexahydrate ( $\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ ), aluminum nitrate nonahydrate ( $\text{Al}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ ), sodium hydroxide (NaOH), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), and N-hydroxysuccinimide (NHS) were purchased from Sinopharm Chemical Reagent, Ltd. (Shanghai, China). Methotrexate hydrate (MTX) and dimethyl sulfoxide (DMSO, 99.9%, SuperDry) were from J&K Chemical, Ltd. (Shanghai, China). 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[(polyethylene glycol)-2000]-amine (DSPE-PEG2000-NH<sub>2</sub>) was from Xi'an Qiyue Biotechnology Co., Ltd. (Xi'an, China). Chlorin e6 (Ce6) was from Bide Pharmatech Co., Ltd. (Shanghai, China). 2,2,6,6-Tetramethylpiperidine (TEMP) was from Shanghai Macklin Biochemical Co., Ltd. (Shanghai, China). MCF-7 cells (a human breast cancer cell line), B16-F10 cells (a murine melanoma cell line), and RAW 264.7 cells (a mouse macrophage cell line) were acquired from Institute of Biochemistry and Cell Biology (the Chinese Academy of Sciences, Shanghai, China). Sodium dodecyl sulfate (SDS) sample loading buffer and SDS-polyacrylamide gel were from Tanon Science & Technology Co., Ltd. (Shanghai, China). Roswell Park Memorial Institute (RPMI)-1640 medium, Dulbecco's Modified Eagle Medium (DMEM), penicillin-streptomycin and trypsin (0.25%) were from Gino Biomedical Technology Co., Ltd. (Hangzhou, China). Fetal bovine serum (FBS) was from Gibco (Carlsbad, CA). 4',6-Diamidino-2-phenylindole (DAPI) and BBoxiProbe were from BestBio Biotechnology Co., Ltd. (Shanghai, China). Phenylmethanesulfonyl fluoride (PMSF), cell lysis buffer, bicinchoninic acid (BCA) protein assay kit, cell counting kit-8 (CCK-8), 2, 7-dichlorofluorescein diacetate (DCFH-DA), annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit, and calcein/PI live/dead cytotoxicity assay kit were from Beyotime Biotechnology Co., Ltd. (Shanghai, China). C11-BODIPY<sup>581/591</sup> was from Shanghai Maokang

Biotechnology Co., Ltd. (Shanghai, China). All chemicals were used as received. Regenerated cellulose dialysis membranes with a molecular weight cut-off (MWCO) of 3000 Da was from Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China). Water used in all experiments was purified using a PURIST UV Ultrapure Water system (RephiLe Bioscience, Ltd., Shanghai, China) with a resistivity higher than 18.2 M $\Omega$ ·cm.

### **Synthesis of LDH and LDH-MTX Nanoparticles (NPs)**

A colloidal LDH-MTX was prepared by the direct coprecipitation method. In brief, MTX was dispersed in water (20 mL) and titrated with a NaOH (0.5 M) solution to give a 0.032 M solution of MTX at pH 7.0, which was added with Mg(NO<sub>3</sub>)<sub>2</sub>·6H<sub>2</sub>O (0.032 M) and Al(NO<sub>3</sub>)<sub>3</sub>·9H<sub>2</sub>O (0.016 M) to achieve their respective concentrations. The pH of the mixture was adjusted to 9.5  $\pm$  0.2 by NaOH (0.5 M). The mixture solution was stirred at room temperature for 48 h under a nitrogen atmosphere and centrifuged (3800 g, 10 min) to collect the precipitate (LDH-MTX NPs), which was purified by washing with water for three times to remove excess ions. The obtained LDH-MTX NPs were resuspended in water and stored at 4 °C before use. The drug-free LDH NPs were also synthesized under the same experimental conditions without the addition of MTX.

### **Preparation of LDH-MTX@CM<sub>M</sub> NPs and LDH-MTX@CM<sub>B</sub> NPs**

MCF-7 cell membranes (CM<sub>M</sub>) and B16-F10 cell membranes (CM<sub>B</sub>) were first harvested from MCF-7 cells and B16-F10 cells, respectively according to the literature protocols.<sup>1, 2</sup> For CM<sub>M</sub> collecting, MCF-7 cells were regularly cultured in RPMI-1640 medium containing 10% FBS and 1% penicillin-streptomycin in a Thermo Scientific cell incubator (Waltham, MA) at 5% CO<sub>2</sub> and 37 °C. Then, the cells were digested to collect cell precipitate and the precipitate was mixed with cell lysis buffer (containing 1% PMSF) under ice bath for 15 min. Afterwards, the mixture solution was repetitively frozen and thawed to completely lyse the cells. Subsequently, the mixture solution was centrifuged at 700 g for 10 min at 4 °C to remove the precipitate, and the supernatant was further centrifuged at

14000 g for 30 min at 4 °C to obtain the CM<sub>M</sub> in precipitate. The CM<sub>M</sub> precipitate was resuspended in phosphate buffered saline (PBS) and stored at 4 °C for further use. The CM<sub>B</sub> precipitate was prepared using the same protocol as that of CM<sub>M</sub>.

For the synthesis of LDH-MTX@CM<sub>M</sub> NPs, the prepared LDH-MTX NPs (200 µg) were dispersed in 1 mL of CM<sub>M</sub> suspension (harvested from 5 × 10<sup>6</sup> MCF-7 cells) and the dispersion was extruded for 11 times using an Avanti mini extruder (Avanti Polar Lipids, Inc., Alabaster, AL). Subsequently, the dispersion was centrifuged at 3800 g for 10 min at 4 °C to obtain the LDH-MTX@CM<sub>M</sub> NPs. LDH-MTX@CM<sub>B</sub> NPs were also prepared under the same experimental conditions using CM<sub>B</sub> suspension (harvested from 5 × 10<sup>6</sup> B16-F10 cells).

### **Synthesis of DSPE-PEG-Ce6**

DSPE-PEG2000-NH<sub>2</sub> was reacted with Ce6 with a molar feeding ratio of PEG/Ce6 at 1: 1.5 through an EDC/NHS-mediated covalent reaction. Briefly, NHS (8.69 mg, in 5 mL DMSO) and EDC (14.38 mg, in 5 mL DMSO) were added to the Ce6 solution (8.95 mg, in 5 mL DMSO) under stirring for 3 h at room temperature in the dark. After that, DSPE-PEG2000-NH<sub>2</sub> (27.91 mg, in 5 mL DMSO) were slowly dropped into the above mixture under stirring for 24 h at room temperature in the dark. The mixture solution was then dialyzed in a dialysis bag (MWCO = 3000 Da) against water (9 times, 2 L) for 3 days to remove the solvent and unconjugated reactants, and lyophilized to get the DSPE-PEG-Ce6 as a green solid and stored at -20 °C.

### **Preparation of LDH-MTX@CM<sub>M</sub>-Ce6 and LDH-MTX@CM<sub>B</sub>-Ce6 NPs**

LDH-MTX@CM<sub>M</sub>-Ce6 and LDH-MTX@CM<sub>B</sub>-Ce6 NPs were formed by a lipid-insertion method according to the literature.<sup>3</sup> Briefly, a solution of DSPE-PEG-Ce6 was added into the preformed LDH-MTX@CM<sub>M</sub> NPs solution and the mixture was incubated in PBS (pH 7.4) at 37 °C for 1 h. Then, the mixture solution was centrifuged at 3800 g for 10 min at 4 °C to obtain the LDH-MTX@CM<sub>M</sub> NPs.

The LDH-MTX@CM<sub>B</sub>-Ce6 NPs were prepared using the same protocol as the LDH-MTX@CM<sub>M</sub>-Ce6. The encapsulation efficiency (EE) and loading content (LC) of DSPE-PEG-Ce6 (quantified through UV-vis spectrometry, see below) in the LDH-MTX@CM<sub>M</sub>-Ce6 NPs were calculated according to the following equations:

$$EE (\%) = (M_E/M_0) \times 100\% \quad (1)$$

$$LC (\%) = (M_E/M_N) \times 100\% \quad (2)$$

where  $M_E$ ,  $M_0$ , and  $M_N$  represent the masses of encapsulated DSPE-PEG-Ce6, initial DSPE-PEG-Ce6, and LDH-MTX@CM<sub>M</sub>-Ce6 NPs, respectively.

### Characterization Techniques

The content of MTX in the LDH-MTX NPs was analyzed by elemental analyzer (Vario ELIII, Elementar, Langensfeld, Germany). The size and morphology of the obtained LDH materials were observed by transmission electron microscopy (TEM, JEM-2100, JEOL Ltd., Tokyo, Japan) operating under an accelerating voltage of 200 kV. Each sample dispersed in ethanol (0.1 mg/mL) was deposited onto a carbon-coated copper grid and air dried before measurements. The hydrodynamic sizes and zeta potentials of the obtained LDH, LDH-MTX, LDH-MTX@CM<sub>M</sub>, LDH-MTX@CM<sub>M</sub>-Ce6, and LDH-MTX@CM<sub>B</sub>-Ce6 were measured using a Malvern Zetasizer Nano ZS model ZEN3600 (Worcestershire, UK) equipped with a standard 633-nm laser. Each sample was dispersed in water at a concentration of 1 mg/mL before measurements. To confirm the stability of the LDH-MTX@CM<sub>M</sub>-Ce6 NPs, the NPs were dispersed in water, PBS, or cell culture medium (RPMI-1640 containing 10% FBS) to test their hydrodynamic size in a period of one week. UV-vis spectrometry was carried out using a Lambda 25 UV-vis spectrophotometer (Perkin Elmer, Boston, MA) to determine the loading of MTX and Ce6. Each sample was dispersed in water before measurements. X-ray diffraction (XRD) was performed using an X-ray diffractometer (Rigaku Cop., Tokyo, Japan) with a Cu K $\alpha$  radiation ( $\lambda = 0.154056$  nm) at 40 kV to confirm the formation of LDH and LDH-MTX.

The membrane protein retention on the surface of the LDH-MTX@CM<sub>M</sub>-Ce6 or LDH-MTX@CM<sub>B</sub>-Ce6 NPs was examined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The membrane proteins of the CM<sub>M</sub>, LDH-MTX@CM<sub>M</sub>-Ce6, CM<sub>B</sub>, or LDH-MTX@CM<sub>B</sub>-Ce6 were firstly quantified by a BCA assay kit and adjusted to the same protein concentration (0.8 mg/mL) before SDS-PAGE analysis according to the standard protocols.<sup>4</sup> For comparison, the LDH-MTX NPs without cell membrane coating were also tested as the negative control under the same experimental conditions.

### ***In Vitro* MTX Release Kinetics**

*In vitro* release of MTX from the LDH-MTX@CM<sub>M</sub>-Ce6 NPs was studied under both pH 6.5 and pH 7.4. Typically, an aqueous solution of LDH-MTX@CM<sub>M</sub>-Ce6 (1 mg/mL, 1 mL) was placed in a dialysis bag (MWCO = 3000), and then exposed to 9 mL of phosphate buffer with pH 7.4 or pH 6.5. The entire system was kept in a constant temperature vibrator at 37 °C. At different time intervals, 1 mL of solution was taken out from the outer phase and the volume of outer phase was maintained constant by adding 1 mL of the corresponding buffer solution. The released MTX was quantified using UV-vis spectroscopy at 302 nm. The experiment was performed in triplicate for each sample.

### **Detection of Singlet Oxygen**

A BBoxiProbe fluorescence probe was used to investigate the singlet oxygen (<sup>1</sup>O<sub>2</sub>) generation ability of LDH-MTX@CM<sub>M</sub>-Ce6. In brief, a solution of LDH-MTX@CM<sub>M</sub>-Ce6 ([Ce6] = 2.5 μg/mL) mixed with the probe (1 μL) was irradiated under ultrasound (US, 1.0 MHz, 1.0 W·cm<sup>-2</sup>) for 0, 2, 4, 5, 6, 8, and 10 min, respectively. Then, fluorescence spectra of the solution were recorded by a QuantMaster-40 fluorescence spectrometer (Protein Technologies Inc., Tucson, AZ) using 488 and 525 nm as the excitation and emission wavelength, respectively (the excitation and emission slit openings were set as 5 mm). Each sample was tested in triplicate.

Electron spin resonance (ESR) spectrometer (Bruker EMXnano, Karlsruhe, Germany) was also used to detect  $^1\text{O}_2$  generation after US treatment of LDH-MTX@CM<sub>M</sub>-Ce6 ([Ce6] = 2.5  $\mu\text{g}/\text{mL}$ ). TEMP, an  $^1\text{O}_2$  entrapment agent, was added to each sample to detect the ESR signals of TEMP + US, TEMP + LDH-MTX@CM<sub>M</sub>-Ce6, and TEMP + LDH-MTX@CM<sub>M</sub>-Ce6 + US (1.0 MHz, 1.0  $\text{W}\cdot\text{cm}^{-2}$ , 2 min), respectively. The following instrument settings were used to collect ESR spectra: 0.1 G field modulation, 100 G scan range, and 30 s sweep time.

### **Cell Culture and US Treatment**

MCF-7, B16-F10, and RAW 264.7 cells were employed in this work. MCF-7 Cells were cultured in RPMI-1640 medium with 1% penicillin-streptomycin and 10% FBS at 37 °C in a Thermo cell incubator (5% CO<sub>2</sub>). B16-F10 and RAW 264.7 cells were cultured under the same above conditions except the medium, which is DMEM. Unless otherwise specified, the US conditions in all cell experiments were kept at 1.0 MHz, 1.0  $\text{W}\cdot\text{cm}^{-2}$ , and 2 min.

### ***In Vitro* Cytotoxicity Assay**

CCK-8 assay was performed to analyze the cytotoxicity of different materials. Firstly, MCF-7 cells were seeded in a 96-well plate at a density of  $1 \times 10^4$  cells per well with 100  $\mu\text{L}$  of medium for each well and incubated overnight. The next day, medium of each well was replaced with LDH, free Ce6, free MTX, free Ce6 + US, LDH-MTX@CM<sub>M</sub>, LDH-MTX@CM<sub>M</sub>-Ce6, LDH-MTX@CM<sub>B</sub>-Ce6 + US, or LDH-MTX@CM<sub>M</sub>-Ce6 + US ([MTX] = 10  $\mu\text{g}/\text{mL}$  for all MTX-related groups, and the concentration of LDH or free Ce6 corresponded to the LDH-MTX@CM<sub>M</sub>-Ce6 group), and cells were incubated for 24 h. Cells treated with PBS or PBS + US were used as control, and the US-treated groups received US treatment after incubation of materials for 4 h, followed by incubation for another 18 h. Subsequently, the medium in each well was taken out and the cells were washed with PBS for three times, incubated with 100  $\mu\text{L}$  serum-free medium containing 10% CCK-8 for additional 3 h. The

absorbance of each well was recorded by a Thermo Scientific Multiskan MK3 ELISA reader (Waltham, MA) at 450 nm. For each sample, 6 parallel wells were tested to give a mean value and standard deviation (SD).

### Cellular Uptake Assays

The cellular uptake of LDH-MTX@CM<sub>M</sub>, free Ce6, LDH-MTX@CM<sub>B</sub>-Ce6, and LDH-MTX@CM<sub>M</sub>-Ce6 by MCF-7 cells was detected through confocal laser scanning microscopy (CLSM, Carl Zeiss LSM 700, Jena, Germany). In brief, MCF-7 cells were seeded in confocal dishes at a density of  $2 \times 10^5$  cells per dish with 1 mL medium and incubated for 24 h. Then, the medium was replaced with 1 mL of medium containing LDH-MTX@CM<sub>M</sub>, free Ce6, LDH-MTX@CM<sub>B</sub>-Ce6, or LDH-MTX@CM<sub>M</sub>-Ce6 ([MTX] = 10  $\mu\text{g}/\text{mL}$  for all MTX-related groups, and the concentration of free Ce6 corresponded to the LDH-MTX@CM<sub>M</sub>-Ce6 group) and the cells were incubated for 4 h. Subsequently, the cells were washed, fixed with 2.5% glutaraldehyde for 15 min and stained with DAPI for 15 min at 37 °C before CLSM observation.

The cellular uptake of different materials by MCF-7 cells were also quantified by inductively coupled plasma-optical emission spectroscopy (ICP-OES) measurements (Leeman Prodigy, Hudson, NH) of Mg contents in cells. In brief, MCF-7 cells were seeded in 12-well plates at a density of  $1 \times 10^5$  cells per well with 1 mL of medium and cultured overnight. Afterwards, the medium was replaced with 1 mL of medium containing LDH-MTX@CM<sub>M</sub>-Ce6 NPs or LDH-MTX@CM<sub>B</sub>-Ce6 NPs at the same Mg concentration (12 mM), and cells were incubated for additional 6 h. For the US groups, MCF-7 cells were first incubated with LDH-MTX, LDH-MTX@CMM-Ce6 or LDH-MTX@CMB-Ce6 NPs for 4 h, US irradiated, and continuously incubated for 2 h. Then, the cells were washed with PBS, trypsinized, collected, counted, and digested by *aqua regia* solution (nitric acid/hydrochloric

acid, v/v = 1:3) for 4 h. The digestion solution was diluted with water, and analyzed by ICP-OES to determine the cellular Mg content. Triplicated experiments were performed for each sample.

To evaluate the homotypic targeting and immune escape ability endowed by CM<sub>M</sub> or CM<sub>B</sub> coating, the intracellular Mg contents in B16-F10 or RAW 264.7 cells were also quantified by ICP-OES after the cells were incubated with LDH-MTX, LDH-MTX@CMM-Ce6 or LDH-MTX@CMB-Ce6 NPs ([Mg] = 12 mM) for 6 h (n = 3).

### **Oxidative Stress Assays**

To investigate the level of oxidative stress inside MCF-7 cells after different treatments, the intracellular reactive oxygen species (ROS) and lipid peroxidation (LPO) levels were detected, respectively. The generation of ROS inside MCF-7 cells was tested using DCFH-DA as a fluorescence probe through both flow cytometry assay and CLSM observation. For flow cytometry, MCF-7 cells were seeded in 6-well plates at a density of  $2 \times 10^5$  cells per well with 1 mL medium and incubated overnight. After that, MCF-7 cells were treated with different materials including (1) PBS, (2) PBS + US, (3) LDH-MTX@CM<sub>M</sub> + US, (4) free Ce6, (5) free Ce6 + US, (6) LDH-MTX@CM<sub>M</sub>-Ce6, (7) LDH-MTX@CM<sub>B</sub>-Ce6 + US, and (8) LDH-MTX@CM<sub>M</sub>-Ce6 + US ([MTX] = 10  $\mu$ g/mL for all MTX-related groups, and the concentration of free Ce6 corresponded to the LDH-MTX@CM<sub>M</sub>-Ce6 group) for 6 h. The US groups received US irradiation right after the incubation of different materials for 6 h. Then, the cells in each well were washed with PBS for 3 times, treated with 1 mL DCFH-DA probe (1 mL, 10  $\mu$ M, dispersed in serum-free medium) for additional 30 min in the dark, washed, trypsinized, centrifuged, and resuspended in 1 mL of PBS. Next, the stained cells were analyzed using a FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ). For each sample,  $1 \times 10^4$  cells were counted and each measurement was repeated for 3 times.

For CLSM observation, MCF-7 cells were seeded in confocal dishes at a density of  $2 \times 10^5$  cells per dish with 1 mL medium and incubated overnight. Cells were treated with different materials as described above, stained with DCFH-DA probe, washed, fixed with 2.5% glutaraldehyde for 15 min, and stained with DAPI for 15 min at 37 °C before CLSM observation.

For LPO accumulation assay through CLSM observation, MCF-7 cells were similarly seeded in confocal dishes ( $2 \times 10^5$  cells per dish), incubated and treated with different materials for 6 h according to the above protocols related to intracellular ROS detection. After that, the culture medium was removed and the cells were washed with PBS for three times, treated with 1.0 mL of C11-BODIPY<sup>581/591</sup> probe (50  $\mu$ M, dispersed in serum-free DMEM medium) for 20 min in the dark, washed with PBS for three times, fixed with glutaraldehyde (2.5%) for 15 min, and stained with DAPI for 15 min at 37 °C before CLSM observation.

### **Cell Cycle Analysis**

MCF-7 cells were seeded into 6-well plates at a density of  $2 \times 10^5$  cells per well and cultured overnight.

Then, cells were treated with (1) PBS, (2) PBS + US, (3) free Ce6, (4) free MTX, (5) free Ce6 + US, (6) LDH-MTX@CM<sub>M</sub>-Ce6, (7) LDH-MTX@CM<sub>B</sub>-Ce6 + US, or (8) LDH-MTX@CM<sub>M</sub>-Ce6 + US ([MTX] = 10  $\mu$ g/mL for all MTX-related groups, and the concentration of Ce6 corresponded to the Ce6 concentration in LDH-MTX@CM<sub>M</sub>-Ce6 group) for 24 h. Note that the US groups received US irradiation right after the incubation of different materials for 4 h, followed by incubation for another 18 h. After that, cells in each well were washed with PBS for three times, trypsinized, collected through centrifugation (1000 rpm, 5 min), and fixed with 70% precooled ethanol at 4 °C for 12 h. The cells were then washed with PBS for 3 times and stained with a mixture solution containing 1% Triton X-100, 0.01% RNase and 0.05% propidium iodide (PI) for 30 min at 37 °C in the dark. Flow cytometry

was employed to quantify the DNA content to estimate the percentages of the cell population in different phases of each cell cycle. Modfit software (Verity Software House, Topsham, ME) was used for fitting analysis.

### **Cell Apoptosis Assay**

The cell apoptosis assay was performed using an Annexin V-FITC/PI apoptosis detection kit by flow cytometry. Typically, MCF-7 cells were seeded and cultured in 6-well plates at a density of  $2 \times 10^5$  cells per well over night, treated with (1) PBS, (2) PBS + US, (3) free Ce6, (4) free MTX, (5) free Ce6 + US, (6) LDH-MTX@CM<sub>M</sub>-Ce6, (7) LDH-MTX@CM<sub>B</sub>-Ce6 + US, or (8) LDH-MTX@CM<sub>M</sub>-Ce6 + US ([MTX] = 10  $\mu\text{g}/\text{mL}$  for all MTX-related groups, and the concentration of Ce6 corresponded to the Ce6 concentration in LDH-MTX@CM<sub>M</sub>-Ce6 group) for 12 h. Cells in US groups were US irradiated after treated with different materials for 4 h, then incubated for another 8 h. Subsequently, cells in each well were washed, collected, and resuspended in 195  $\mu\text{L}$  of binding buffer, added with 5  $\mu\text{L}$  of annexin V-FITC and 5  $\mu\text{L}$  of PI, and incubated for 15 min at room temperature in the dark before flow cytometry analysis. For each sample,  $1 \times 10^4$  cells were counted and each measurement was repeated for 3 times.

### **Live/Dead Costaining Assay**

To further validate the therapeutic efficacy of the materials, MCF-7 cells were seeded in 12-well plates at a density of  $1 \times 10^5$  cells per well and cultured overnight. Then, cells were treated with (1) PBS, (2) PBS + US, (3) free Ce6, (4) free MTX, (5) free Ce6 + US, (6) LDH-MTX@CM<sub>M</sub>-Ce6, (7) LDH-MTX@CM<sub>B</sub>-Ce6 + US, or (8) LDH-MTX@CM<sub>M</sub>-Ce6 + US ([MTX] = 10  $\mu\text{g}/\text{mL}$  for all MTX-related groups, and the concentration of Ce6 corresponded to the Ce6 concentration in LDH-MTX@CM<sub>M</sub>-Ce6 group) for 24 h. Cells in US groups were US irradiated after treated with different materials for 4

h, followed by incubation for another 18 h. Subsequently, a calcein/PI live/dead cytotoxicity assay kit was applied to stain the cells, which were observed by CLSM.

### **Hemolysis Assay**

The hemocompatibility of the prepared LDH-MTX@CM<sub>M</sub>-Ce6 NPs was evaluated *via* hemolysis assay according to protocols described in our previous work.<sup>5</sup> Mouse whole blood was obtained from healthy female BALB/c nude mice (15-20 g, 5 weeks old) purchased from Shanghai Slac Laboratory Animal Center (Shanghai, China). All animal experiments were carried out after approval by the ethical committee for animal care and use of Donghua University (approval # DHUEC-NSFC-2023-02) and also according to the policy of the National Ministry of Health of China. In brief, mouse red blood cells (RBCs) were obtained from fresh whole blood (2 mL) by centrifugation and washed with PBS, followed by diluting 50 times with PBS to establish a negative control (0.5 mL RBC suspension, 0.5 mL PBS) and a positive control (0.5 mL RBC suspension, 0.5 mL water), respectively. Meanwhile, the obtained RBC suspension was mixed with LDH-MTX@CM<sub>M</sub>-Ce6 with different MTX concentrations ([MTX] = 1, 2.5, 5, 10 and 25 μg·mL<sup>-1</sup>, respectively) and incubated at 37 °C for 2 h. For each concentration, three parallel samples were analyzed. Afterwards, the control groups and the RBC suspensions containing LDH-MTX@CM<sub>M</sub>-Ce6 were centrifuged at 10000 rpm/min for 10 min, and photographed after centrifugation. The absorbance of the supernatant at 540 nm was measured using UV-vis spectrophotometer. Hemolysis rate was calculated according to the following equation:

$$\text{Hemolysis rate (\%)} = \frac{D_t - D_{nc}}{D_{pc} - D_{nc}} \times 100\% \quad (3)$$

where  $D_t$ ,  $D_{pc}$  and  $D_{nc}$  are the OD values (at 540 nm) of the tested samples, positive control and negative control, respectively.

### ***In Vivo* Pharmacokinetics**

To explore the pharmacokinetics of LDH-MTX@CM<sub>B</sub>-Ce6 NPs or LDH-MTX@CM<sub>M</sub>-Ce6 NPs, healthy BALB/c nude mice were intravenously injected with LDH-MTX@CM<sub>B</sub>-Ce6 NPs or LDH-MTX@CM<sub>M</sub>-Ce6 NPs ([Mg] = 10 mM, in 0.1 mL PBS for each mouse) through tail vein. At different

time points (0.5 h, 1 h, 2 h, 4 h, 8 h, 12 h, 24 h, or 48 h) postinjection, blood was collected from the orbital sinus of each mouse, weighed, and digested by *aqua regia* for 24 h. Each sample was diluted with water, and the Mg content was quantified by ICP-OES. The blood circulation half-decay time ( $t_{1/2}$ ) was determined using a one-phase decay exponential model by Graphpad prism<sup>®</sup> 8.0.2 software (San Diego, CA). The experiment was performed in triplicate for each sample.

### ***In Vivo* and *Ex Vivo* Fluorescence Imaging**

BALB/c nude mice were subcutaneously injected with  $2 \times 10^6$  B16-F10 cells (in 0.1 mL of PBS for each mouse) into the left hind leg and  $2 \times 10^6$  MCF-7 cells (in 0.1 mL of PBS for each mouse) into the right hind leg, respectively to establish a xenografted dual-tumor model. When the tumor volume reached about 300 mm<sup>3</sup>, dual tumor-bearing mice were divided into two groups of LDH-MTX@CM<sub>M</sub>-Ce6 NPs and LDH-MTX@CM<sub>B</sub>-Ce6 NPs for fluorescence imaging (n = 3 for each group). Each tumor-bearing mouse was intravenously injected with LDH-MTX@CM<sub>M</sub>-Ce6 NPs or LDH-MTX@CM<sub>B</sub>-Ce6 NPs ([MTX] = 2 mg/mL, in 0.1 mL PBS) *via* tail vein. The fluorescence images of the mice were recorded at 2 h, 4 h, and 6 h postinjection using an *in vivo* fluorescence imaging system (IVIS Lumina series III, Caliper Lifescience Co., Hopkinton, MA). Then, the tumor-bearing mice were sacrificed at 6 h postinjection, and the B16-F10 tumor/MCF-7 tumor and major organs were collected and imaged as well.

### ***In Vivo* Antitumor Efficacy**

BALB/c nude mice were subcutaneously injected with  $2 \times 10^6$  MCF-7 cells (in 0.1 mL of PBS for each mouse) to establish a xenografted MCF-7 tumor model. When the tumor volume reached about 100 mm<sup>3</sup>, the tumor-bearing mice were divided into six groups (n = 5 in each group) and intravenously administrated with 0.1 mL PBS, or 0.1 mL PBS containing free MTX, free Ce6, LDH-MTX@CM<sub>M</sub>-Ce6 NPs or LDH-MTX@CM<sub>B</sub>-Ce6 ([MTX] = 2 mg/mL for all MTX-related groups, and the

concentration of free Ce6 corresponded to the Ce6 concentration in LDH-MTX@CM<sub>M</sub>-Ce6 groups) every 2 days for 4 times, respectively. The tumors of the US-treated groups were exposed to an external US irradiation (1.0 MHz, 1.0 W·cm<sup>-2</sup>, and 4 min) at 4 h after each injection for total 4 times. The tumor volume and body weight of each mouse were recorded every other day for 14 days, and the tumor volume was calculated as  $\text{Volume} = \text{Length} \times \text{Width}^2/2$ . The relative tumor volumes and relative body weights were calculated based on the tumor volume and body weight of mice before different treatments.

To verify the therapeutic effect of different NPs, one tumor-bearing mouse from each group was sacrificed at 14 days post-treatments to collect the tumor, which was photographed, weighted, fixed, embedded in paraffin, and sectioned for hematoxylin and eosin (H&E), TdT-mediated dUTP Nick-End Labeling (TUNEL), and Ki67 staining according to the standard protocols.<sup>6</sup>

### **Biosafety Examinations**

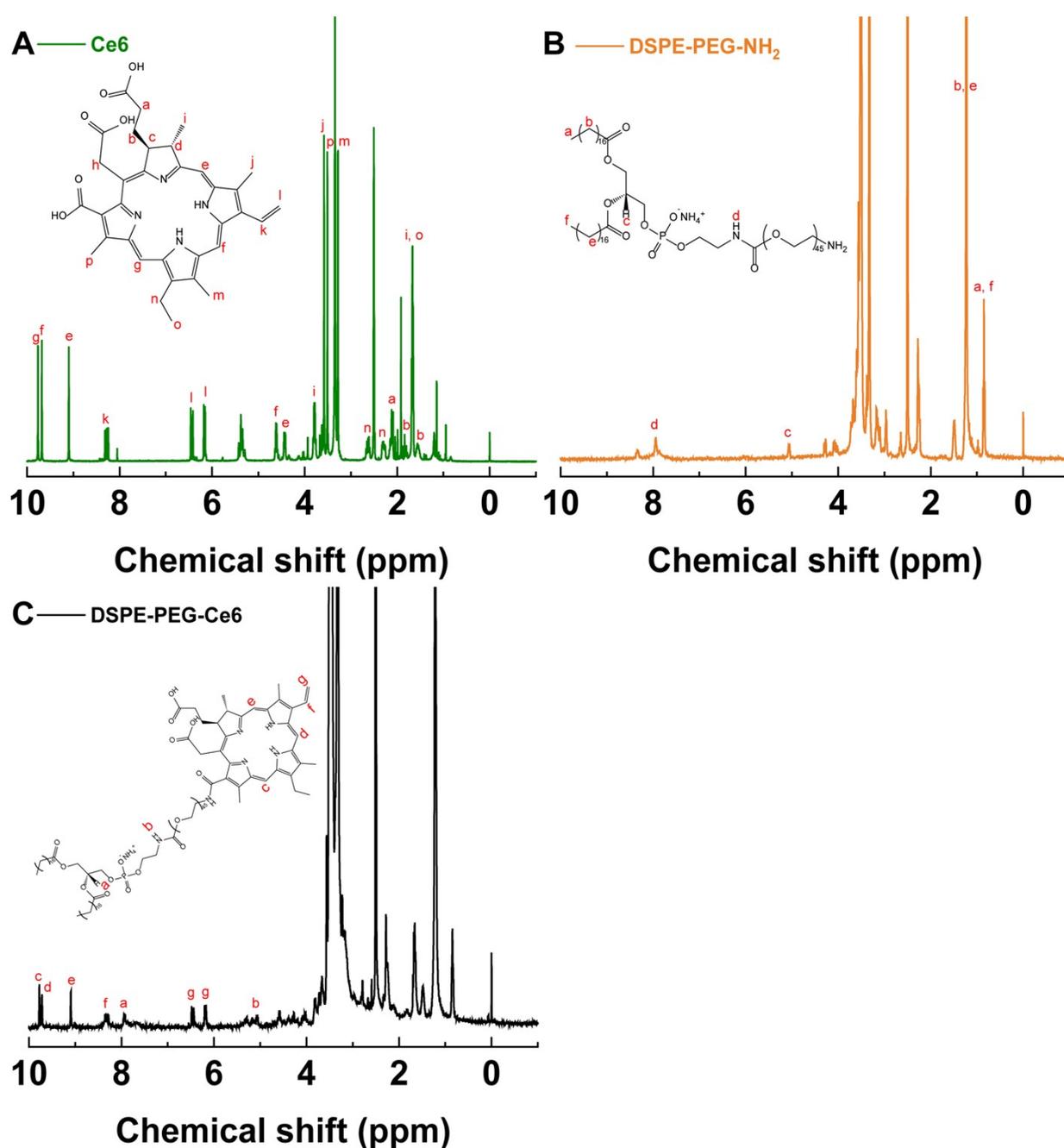
To evaluate the biosafety of the prepared LDH-MTX@CM<sub>M</sub>-Ce6 NPs, the major organs (heart, liver, spleen, lungs, and kidneys) of mice in different groups were collected at 14 days post treatment, fixed, embedded in paraffin, and sectioned for H&E staining to observe the histological changes.

Furthermore, blood of healthy mice after different treatments was also analyzed to confirm the biosafety of the developed LDH-MTX@CM<sub>M</sub>-Ce6 NPs. Briefly, each healthy mouse was intravenously administrated with 0.1 mL PBS, or 0.1 mL PBS containing free MTX, free Ce6, LDH-MTX@CM<sub>B</sub>-Ce6, or LDH-MTX@CM<sub>M</sub>-Ce6 ([MTX] = 2 mg/mL for all MTX-related groups, and the concentration of free Ce6 corresponded to the Ce6 concentration in LDH-MTX@CM<sub>M</sub>-Ce6 groups), n = 3 in each group) *via* tail vein. At 7 days postinjection, the mice were sacrificed, and the blood was collected and stabilized with heparin for blood routine and blood biochemical analysis. The blood cell counts were performed on an automated blood cell counter (BC-2800 Vet Analyzers, Mindray, Shenzhen, China) for blood routine analysis, including white blood cell (WBC), RBC, hemoglobin (HGB), and platelet (PLT). Then, the blood samples were centrifugated at 2000 rpm for

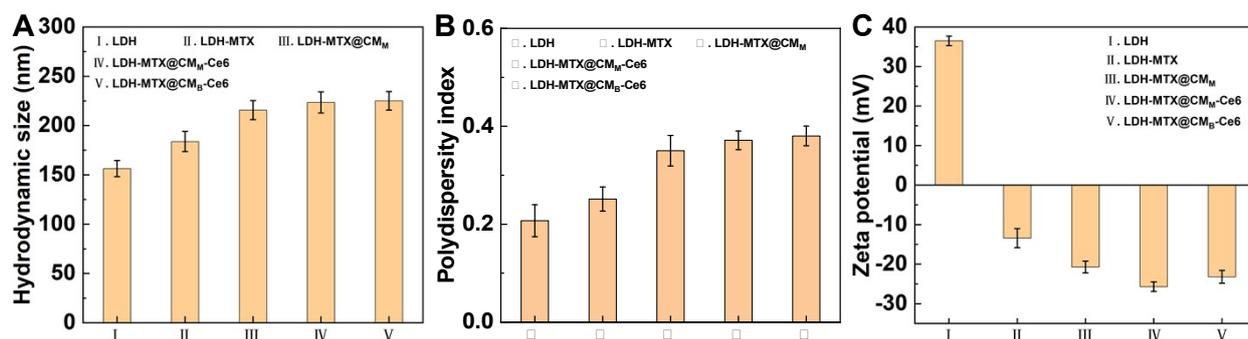
3 min to obtain the serum, and the serum biochemistry markers including alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine (CR), and uric acid (UA) were analyzed by Servicebio Technology Co., Ltd. (Wuhan, China).

### Statistical Analysis

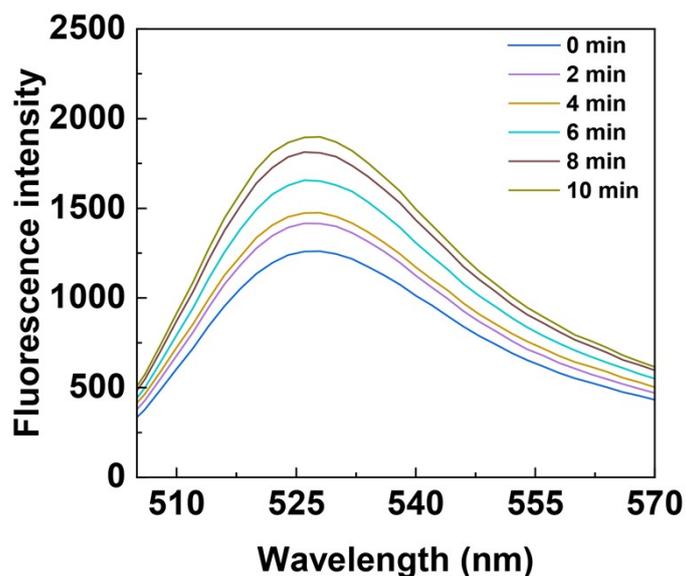
All experimental data were represented as the mean  $\pm$  SD through at least three experiments. One-way analysis of variance statistical method was used to analyze the experimental results through IBM SPSS Statistic 25 software (IBM, Armonk, NY). A p value of 0.05 was selected as a significance level, and the data were indicated with (\*) for  $p < 0.05$ , (\*\*) for  $p < 0.01$ , and (\*\*\*) for  $p < 0.001$ , respectively.



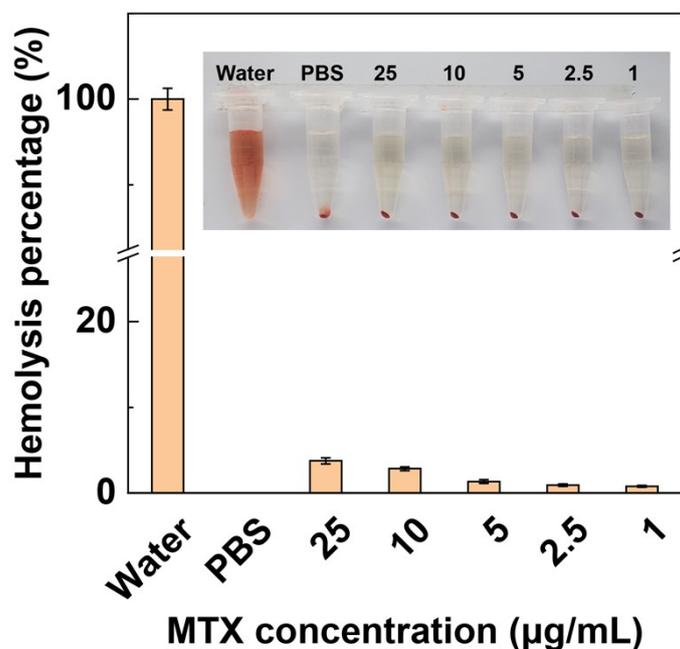
**Figure S1.**  $^1\text{H}$  NMR spectra of (A) Ce6, (B) DSPE-PEG-NH<sub>2</sub>, and (C) DSPE-PEG-Ce6.



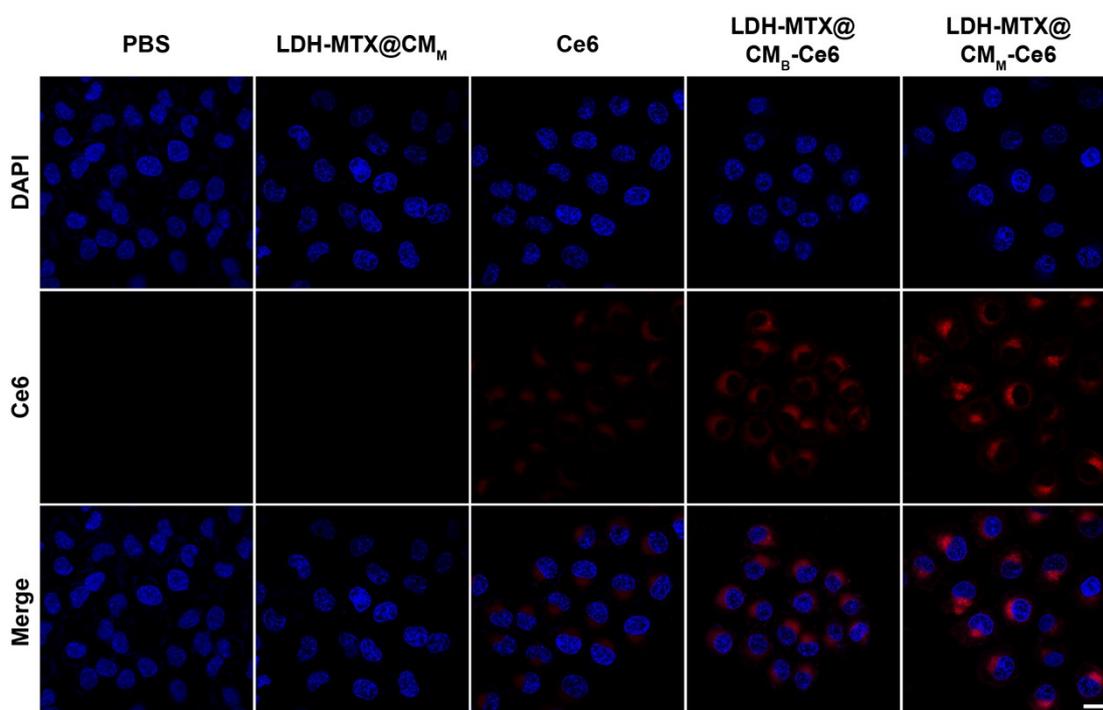
**Figure S2.** (A) Hydrodynamic sizes, (B) polydispersity indexes, and (C) zeta potentials of LDH, LDH-MTX, LDH-MTX@CM<sub>M</sub>, LDH-MTX@CM<sub>M</sub>-Ce6, and LDH-MTX@CM<sub>B</sub>-Ce6 (n = 3).



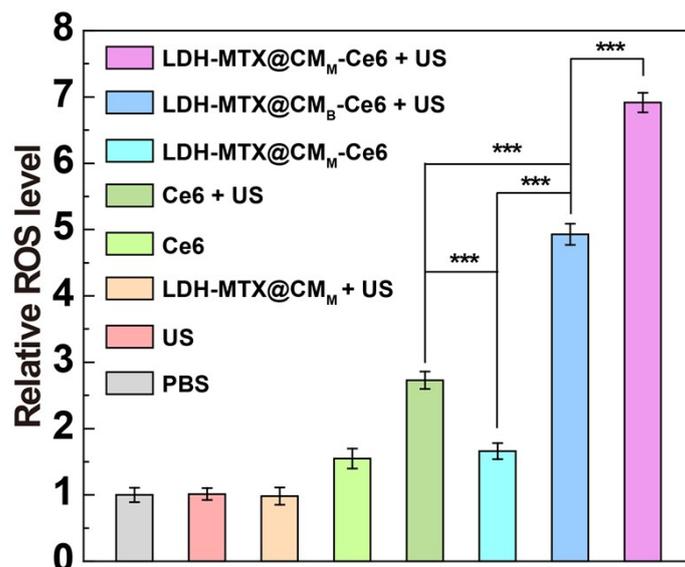
**Figure S3.** Time-dependent  $^1\text{O}_2$  generation of LDH-MTX@CM<sub>M</sub>-Ce6 after US irradiation (1.0 MHz, 1 W cm<sup>-2</sup>) for different time periods.



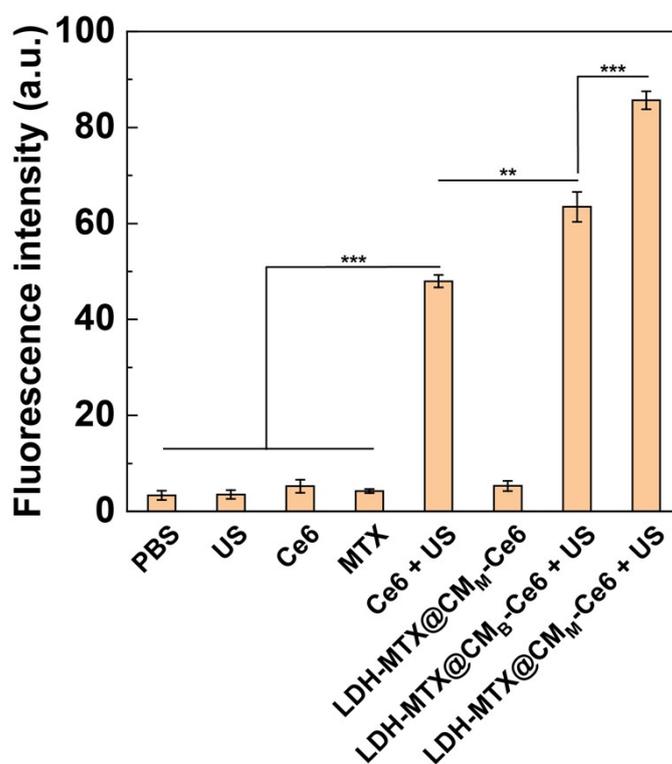
**Figure S4.** Hemolysis percentage of red blood cells (RBCs) treated with LDH-MTX@CM<sub>M</sub>-Ce6 NPs at various MTX concentrations for 2 h (n = 3). Inset shows the photograph of RBCs treated with the NPs at different MTX concentrations, followed by centrifugation. Water and PBS were used as positive and negative controls, respectively.



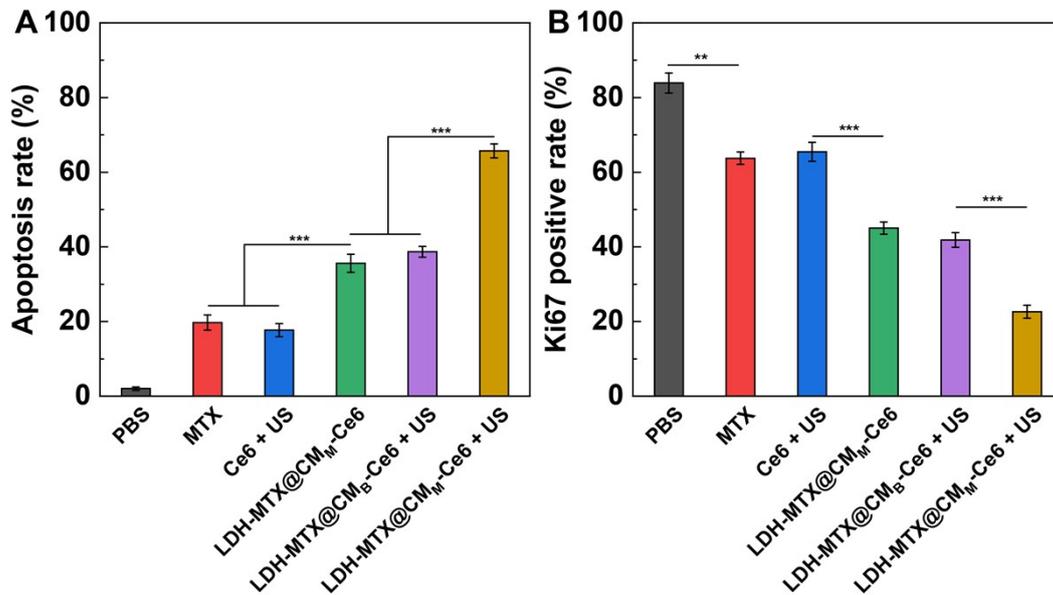
**Figure S5.** CLSM images of MCF-7 cells treated with PBS, LDH-MTX@CM<sub>M</sub>, free Ce6, LDH-MTX@CM<sub>B</sub>-Ce6 or LDH-MTX@CM<sub>M</sub>-Ce6 for 4 h at the same Ce6 concentration (2.5 µg/mL, scale bar = 20 µm for each panel).



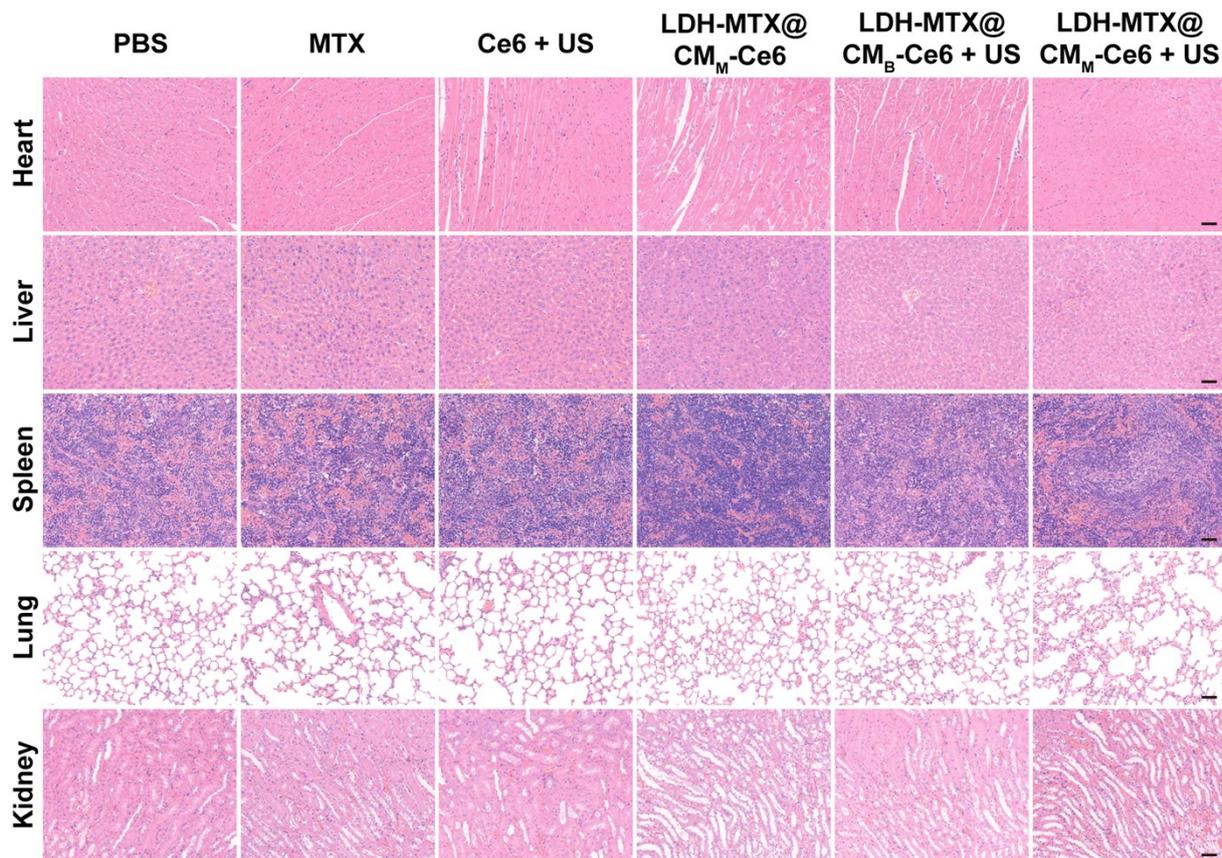
**Figure S6.** The relative fluorescence intensity of intracellular ROS level in MCF-7 cells after incubation with PBS, PBS + US, LDH-MTX@CM<sub>M</sub> + US, free Ce6, Ce6 + US, LDH-MTX@CM<sub>M</sub>-Ce6, LDH-MTX@CM<sub>B</sub>-Ce6 + US or LDH-MTX@CM<sub>M</sub>-Ce6 + US for 6 h as analyzed by flow cytometry (n = 3, and \*\*\* is for p < 0.001).



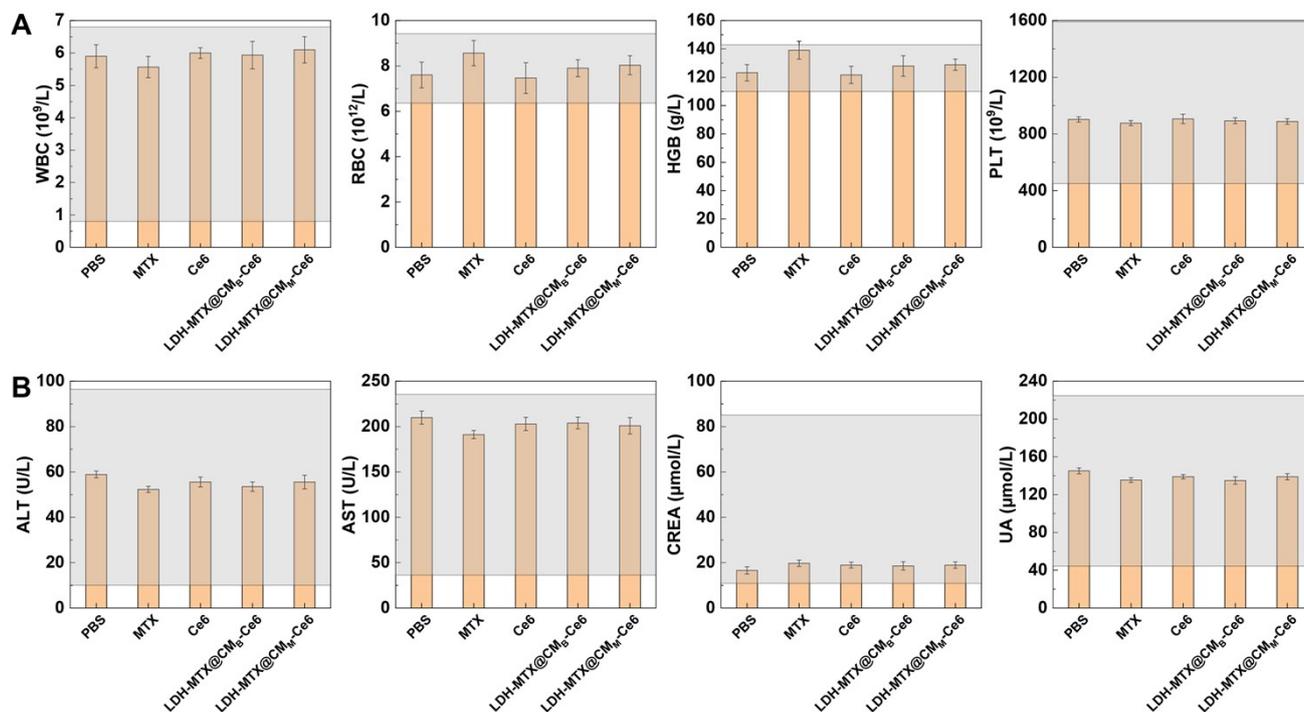
**Figure S7.** Quantitative analysis of average green fluorescence intensity of LPO in cells through CLSM images in Figure 2E (n = 3, \*\* is for p < 0.01 and \*\*\* is for p < 0.001, respectively).



**Figure S8.** Quantitative analysis of (A) the apoptosis rate and (B) Ki67-positive tumor cells after different treatments for 14 days (n = 3, \*\* is for p < 0.01 and \*\*\* is for p < 0.001, respectively).



**Figure S9.** Representative H&E-stained sections of major organs of tumor-bearing mice after different treatments for 14 days. Scale bar for each panel represents 50 μm.



**Figure S10.** (A) The blood routine analysis including levels of WBC, RBC, HGB, PLT and (B) blood biochemistry analysis including liver function parameters (ALT, AST), and kidney function parameters (CR, UA) of healthy mice at 7 days post different treatments. PBS-treated healthy mice were used as control, and n = 3 for each sample.

## References

1. M. Gao, C. Liang, X. Song, Q. Chen, Q. Jin, C. Wang and Z. Liu, *Adv. Mater.*, 2017, **29**, 1701429.
2. W. Xie, W.-W. Deng, M. Zan, L. Rao, G.-T. Yu, D.-M. Zhu, W.-T. Wu, B. Chen, L.-W. Ji, L. Chen, K. Liu, S.-S. Guo, H.-M. Huang, W.-F. Zhang, X. Zhao, Y. Yuan, W. Dong, Z.-J. Sun and W. Liu, *ACS Nano*, 2019, **13**, 2849-2857.
3. C. Gao, Y. Wang, J. Sun, Y. Han, W. Gong, Y. Li, Y. Feng, H. Wang, M. Yang, Z. Li, Y. Yang and C. Gao, *Acta Biomater.*, 2020, **108**, 285-299.
4. Y. Guo, Y. Fan, G. Li, Z. Wang, X. Shi and M. Shen, *ACS Appl. Mater. Interfaces*, 2021, **13**, 55815-55826.
5. C. Zhang, W. Tu, X. Chen, B. Xu, X. Li, C. Hu, M. Shen, S. Song, C. Jiang, S. Yao, A. Pich, Y. Liu and X. Shi, *Theranostics*, 2022, **12**, 3420-3437.
6. C. Yang, Y. Gao, Y. Fan, L. Cao, J. Li, Y. Ge, W. Tu, Y. Liu, X. Cao and X. Shi, *Theranostics*, 2021, **11**, 1721-1731.