Electronic supplementary information

Intelligent design of iron-doped LDH nanosheets for cooperative chemo-chemodynamic therapy of tumors

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Part of experimental details

Materials

Ferrous chloride tetrahydrate ($FeCl_2$•$4H_2O$), aluminum chloride hexahydrate ($AlCl_3$•$6H_2O$) and sodium hydroxide (NaOH) were purchased from Sinopharm Chemical Ltd (Shanghai, China). The tea polyphenol epigallocatechin gallate (EGCG), methylene blue (MB) and 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) were obtained from MACKLIN (Shanghai, China). Hyaluronic acid (HA, Mw = 5830) was from Zhenjiang Dong Yuan Biotechnology Corporation (Zhenjiang, China). 3-aminophenylboronic acid (3-PBA·HCl) was obtained from Sigma-Aldrich (St. Louis, MO). Regenerated cellulose dialysis membrane with a molecular weight cut-off (MWCO) of 1000 was acquired from Fisher Scientific (Pittsburgh, PA). Mouse melanoma cell line B16 was from Institute of Biochemistry and Cell Biology (the Chinese Academy of Sciences, Shanghai, China). RPMI-1640 medium, fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Hangzhou Jinuo Biomedical Technology (Hangzhou, China). Cell counting kit-8 (CCK-8) and 4',6-diamidino-2-phenylindole (DAPI) were supplied by 7Sea Pharmatech Co., Ltd. (Shanghai, China). Annexin V-FITC (fluorescein isothiocyanate)/PI apoptosis detection kit was acquired from BestBio 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 MΩ·cm.

Characterization

SEM images were taken via a field emission scanning electron microscope (S-4800, Hitachi, Ltd., Tokyo, Japan). TEM images were obtained by transmission electron microscopy (JEM 2100F, JEOL Ltd., Tokyo, Japan) operated at 200 kV. Zeta potential and dynamic light scattering (DLS) measurements were carried out using a Malvern Zetasizer Nano ZS system (model ZEN3600, Worcestershire, UK) equipped with a standard 633-nm laser. UV-Vis spectrophotometer was carried out using a Lambda 25 UV-Vis spectrophotometer (Perkin Elmer, Boston, MA). Fourier transform infrared (FTIR) spectra was recorded on a Nicolet 6700 FTIR spectrophotometer (Thermo Electron Corporation, Madison, WI). Samples were mixed with milled KBr crystals and pressed to form 13-

mm diameter disks before measurements. X-ray diffraction (XRD) analysis was carried out using a D/max 2550 VB+/PC X-ray diffractometer (Rigaku Cop., Tokyo, Japan) with Cu Kα radiation (λ = 0.154056 nm) at 40 kV and 200 mA and a 2θ scan range of 5-90°. A TG 209 F1 (NETZSCH Instruments Co., Ltd., Bavaria, Germany) thermogravimetric analyzer was used for thermogravimetric analysis (TGA) of the samples under nitrogen atmosphere in a temperature range of 50-1000 °C. ^{1}H NMR measurements were performed using a Bruker DRX 500 NMR (400 MHz) spectrometer. All samples were dissolved in D_2O before measurements. The concentration of Fe in LDH-EGCG-HA was analyzed by Leeman Prodigy inductively coupled plasma-optical emission spectroscopy (ICP-OES, Hudson, NH).

Synthesis of HA-PBA

The HA-PBA polymer conjugates were prepared by conjugating the 3-aminomethyl PBA to HA using DMTMM as the coupling agent.[1] 6.3 mL of a 2.2 mg/mL 3-PBA·HCl solution and 25.0 mL of HA solution were mixed for 10 min in an orbital shaker at 25 ℃. 6.3 mL of a freshly prepared 17.9 mg/mL solution of DMTMM were then added to the reaction mixture. The reaction was allowed to proceed overnight under gentle shaking. The reaction products were precipitated in cold ethanol (0 - 4 ℃, ≈15–20 times the reaction volume), left overnight in ethanol, isolated via centrifugation (4500 rpm, 5 min, 4 ℃), dissolved in deionized water. After that, the mixtures were transferred to 1 KDa molecular weight cut-off (MWCO) dialysis bags and dialyzed against water for at least 3 days at room temperature, with the water changed three times every day. The dialyzed solutions were freeze-dried using a benchtop lyophilizer to obtain the HA-PBA polymer conjugates.

Drug loading

The EGCG encapsulation efficiency and percentage can be calculated by measuring the concentration of free EGCG in the collected supernatants after 3 times of centrifugation using a UV-Vis spectrophotometer at 276 nm and a standard EGCG absorbance-concentration calibration curve. The drug loading content (DL %) and entrapment efficiency (EE %) of nanodisks can be calculated using equation (1) and (2), respectively.

$$DL\ \% = M_t / M_0 \times 100\ \% \qquad (1)$$

$$EE\ \% = M_t / M_L \times 100\ \% \qquad (2)$$

Where M_t, M_0 and M_L stand for the masses of the encapsulated EGCG, the initial EGCG, and the drug loaded nanocomplexes, respectively.[2]

***In vitro* drug and iron ion responsive release**

In vitro drug release profile of EGCG and iron ion release profile from LDH-EGCG-HA were investigated at pH 5.0, pH 6.5 and pH 7.4 buffer solutions. LDH-EGCG-HA were dispersed in 1 mL corresponding buffer solution at the concentration of 1 mg/mL, and then sealed in a dialysis bag (cutoff Mw = 1000). The dialysis bag was immersed in 9 mL buffer solution (pH 5.0, pH 6.5 or pH 7.4) and placed in shaker at 37 °C for 48 h. Within a specified time interval (0.5, 1, 2, 4, 6, 8, 12, 24, 48 h), 1 mL sample solution was taken out from buffer solution to measure the released amount of EGCG (λ_{EGCG} = 276 nm) by UV-Vis spectrophotometer and to measure the released amount of iron ion by ICP-OES, and then an equivalent amount of fresh buffer solution was added.

Evaluation of •OH generation

The production of •OH was detected by UV-Vis spectrophotometer with methylene blue (MB) served as a sensor. In brief, LDH-EGCG-HA solution containing 10 μg/mL MB, 10 mM H_2O_2 was allowed to stand at 37 °C for 2 h, the absorbance of the above solutions at 664 nm was measured to record the degradation of MB. Similarly, pure LDH and EGCG were used for comparison. In addition, the degradation of MB by LDH-EGCG-HA was performed in the presence of 0.5 mM or 10 mM GSH.

Cytotoxicity assay

Mouse melanoma cells B16 with CD44 receptors over-expressed and fibrosarcoma cells L929 were cultured in 1640 medium supplemented with 10 % FBS, 100 U/mL penicillin, and 100 U/mL streptomycin in a Thermo Scientific cell incubator at 37 °C and 5 % CO_2. For pre-HA group, B16 cells were pre-treated with HA solution (2.0 mM) for 2 h before the addition of materials in order to block the CD44 receptors on cell surface.

The cytotoxicity of LDH and LDH-EGCG-HA were evaluated by CCK-8 assay. Typically, L929 cells were grown in 96-well plate with a density of 1×10^4 cells per well. When the cells were

completely adhered, different EGCG concentrations (5, 10, 20, 40 and 60 μg/mL) of LDH and LDH-EGCG-HA medium solution were added into the plate (n = 6). After being incubated for 24 h, the medium was poured out and the cells were washed with PBS. Finally, the cells were incubated with 100 μL of serum free medium containing 10 μL of CCK-8 solution at 37 °C for another 4 h in a dark environment. The absorption value at 450 nm was determined by a microplate reader.

In order to evaluate the inhibition effect of LDH-EGCG-HA, B16 cells (1×10^4 cells/well) were cultured in 96-well plate overnight. Next, B16 cells were incubated with fresh medium containing free EGCG, LDH, LDH-EGCG, LDH-EGCG-HA and LDH-EGCG-HA + pre-HA at different EGCG concentrations (5, 10, 20, 40 and 60 μg/mL) for 24 h or 48 h. Finally, the absorbance values of different groups were determined by CCK-8 assay, and the cell survival rate was calculated.

Cellular uptake

The cellular uptake of LDH-EGCG-HA by B16 *in vitro* was quantitatively evaluated by measuring the Fe concentration in cells using ICP-OES. B16 cells were seeded into 12-well plates at a density of 2×10^5 cells per well and cultured overnight to lead cells to adherence. Then, the medium was replaced with fresh medium containing LDH-EGCG or LDH-EGCG-HA at different EGCG concentrations (5, 10, 20, 40, 60 μg/mL) and cells were further incubated for 4 h. Afterwards, the medium was removed carefully and cells were washed 3 times with PBS, treated with trypsin, suspended in fresh medium. The remaining cells were collected by centrifugation (104 g, 5 min), and treated with 1.0 mL aqua regia solution for 24 h. Finally, the samples were diluted in PBS and measured the concentration of phagocytic segregants by ICP-OES.

Cell apoptosis

Flow cytometry was applied to analyze the cell apoptosis. The cells were seeded in 6-well plates for 24 h cultivation. Then, the culture medium was replaced by fresh culture medium containing LDH, Free EGCG, LDH-EGCG or LDH-EGCG-HA (with an equivalent EGCG concentration of 40 μg/mL). After incubating for 24 h, the medium was removed carefully and cells were washed 3 times with PBS, the cells were trypsinizated and collected in the centrifuge tube. When redispersed

in Annexin V-FITC binding buffer, these cells were stained with Annexin V-FITC/PI at room temperature for 15 min, and the cells of each group were finally subjected to flow cytometry analysis.

GSH detection

The intracellular GSH was measured by the GSH and GSSG Assay Kit. B16 cells were seeded into a 6-well plate at a density of 5×10^4 cells per well and incubated for 24 h. Then, the culture medium was replaced by fresh culture medium containing LDH, Free EGCG, LDH-EGCG or LDH-EGCG-HA (with an equivalent EGCG concentration of 40 μg/mL). After incubating for 12 h, the cells were collected with centrifugation, and the supernatant was discarded. The cell precipitates were resuspended in protein remover M (10 mg/30 μL), subjected to 3 cycles of freezing−thawing, and then centrifugated at 1000 g for 10 min at 4 °C. The supernatant was reserved for GSH and GSSG assay according to the manufacturer's protocol.

Determination of intracellular reactive oxygen species (ROS) and lipid peroxidation (LPO)

The intracellular ROS inside B16 cells was measured with 20,70-dichlorofluorescein diacetate (DCFH-DA). First, B16 cells were seeded into a 6-well plate at a density of 5×10^4 cells per well and incubated for 24 h. Then, the culture medium was replaced by fresh culture medium containing LDH, Free EGCG, LDH-EGCG or LDH-EGCG-HA (with an equivalent EGCG concentration of 40 μg/mL). After a further incubation for 4 h, the cells were washed 3 times with PBS, followed by a treatment with DCFH-DA solution (10 mM) for another 15 min at 37 °C. Finally, the cells were washed thoroughly with PBS, and the production of intracellular ROS was detected quantitatively by using a flow cytometry and qualitatively with confocal laser scanning microscopy (CLSM).

The intracellular LPO inside B16 cells was evaluated by CLSM with BODIPY$^{581/591}$-C11. First, B16 cells were seeded into a 6-well plate at a density of 5×10^4 cells per well and incubated for 24 h. Then, the culture medium was replaced by fresh culture medium containing LDH, free EGCG, LDH-EGCG or LDH-EGCG-HA (with an equivalent EGCG concentration of 40 μg/mL). After a further incubation for 4 h, the cells were washed 3 times with PBS, followed by a treatment with BODIPY$^{581/591}$-C11 solution for another 15 min at 37 °C. Finally, the cells were washed thoroughly

with PBS, and the LPO level was subsequently analyzed by CLSM.

Western blot analysis

To perform Western blot analysis, the B16 cells were seeded in 6-well plates and incubated for 24 h. Next, the cells were subjected to different treatments: LDH, Free EGCG, LDH-EGCG, LDH-EGCG-HA (with an equivalent EGCG concentration of 40 μg/mL). After incubation for 24 h, the treated cells were harvested and washed 3 times with PBS, then 200 μL of lysis buffer containing phenylmethanesulfonyl fluoride was added into each tube under an ice-bath for 30 min. The lysates were analyzed via Western blotting to detect the GPX-4 (glutathione peroxidase 4), xCT (the cystine/glutamate antiporter), p53 and caspase-3 protein expression according to the literature protocols.[3] The expression of GAPDH in the cells was also measured as control.

Bio-distribution *in vivo*

All animal experiments were conducted under the direction of the Ethical Committee of Shanghai Songjiang District Central Hospital, and according to the policy of the National Ministry of Health. To set-up tumor xenograft model, about 2×10^6 B16 cells suspended in saline (100 μL) were subcutaneous injected in the right leg of C57BL/6 mice (20-25 g, 6 weeks, Shanghai Slac Laboratory Animal Center, Shanghai, China). When the volume of the tumor reached 200 mm^3, mice were randomly divided into 2 groups. B16 tumor-bearing mice were intravenously injected with LDH-EGCG or LDH-EGCG-HA ([EGCG] = 5 mg/kg, 100 μL). After the mice were sacrificed at the given time points (1 h, 2 h, 4 h, 8 h, 10 h and 24 h, respectively), the heart, liver, spleen, lung, kidney and tumor were extracted. These organ and tumor samples were weighed and digested by aqua regia solution for a week. After complete tissue digestion, each sample was diluted by water to 4 mL. Then, the Fe content in these samples were measured by ICP-OES for 3 times and the data were expressed as mean ± SD (n = 3).

***In vivo* antitumor activity**

B16 tumor-bearing mice were randomly divided into 5 groups and intravenously injected with PBS (100 μL), LDH (13.5 mg/kg, 100 μL), Free EGCG ([EGCG] = 5 mg/kg, 100 μL), LDH-EGCG ([EGCG] = 5 mg/kg, 100 μL), LDH-EGCG-HA ([EGCG] = 5 mg/kg, 100 μL) on day 1, 4, and 7.

The tumor volumes and body weights were recorded every two days. The tumor volume was calculated according to a formula of $V = W^2 \times L/2$, where W and L represent the width and length of tumor. The relative tumor volume was calculated based on the tumor volume of the first day. After 14 days, tumors and major organs were excised from euthanized mice, photographed and weighed, followed by hematoxylin and eosin (H&E) staining, and tumor also followed by GPX-4, Ki67, CD31 staining. Standard TdT mediated dUTP nick-end labeling (TUNEL) staining tests were performed according to our previous work to confirm the tumor cell apoptosis efficacy.[4]

Statistical analysis

All statistical results were presented as the mean ± standard deviation (SD). The differences between two groups were calculated using unpaired Student's t-test. *$P < 0.05$ was considered as statistically significant, **$P < 0.01$ was considered as moderately significant, ***$P < 0.001$ was considered as highly significant.

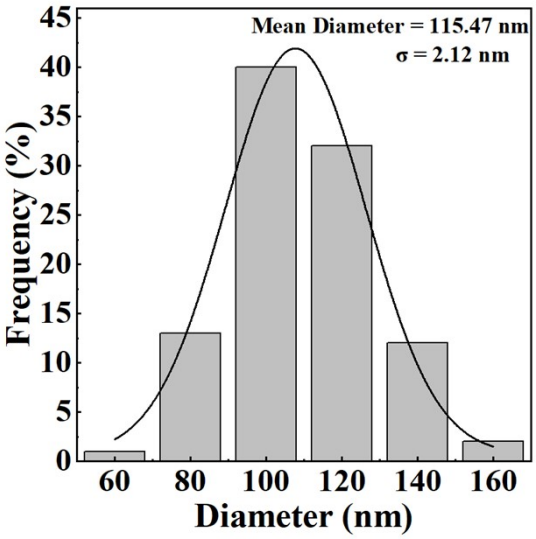

Fig. S1 Size distribution histograms of LDH.

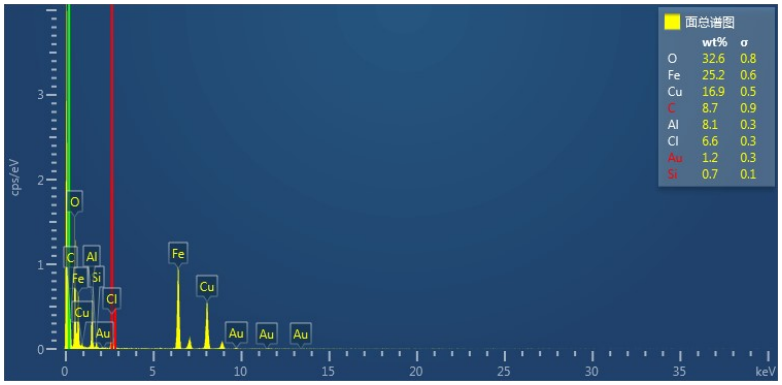

Fig. S2 EDX result of LDH.

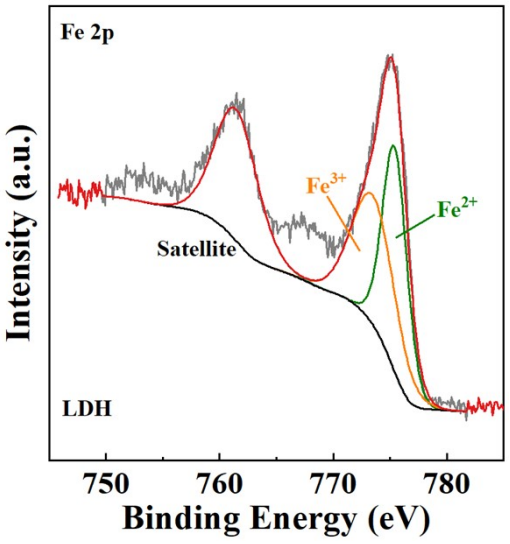

Fig. S3 XPS result of LDH.

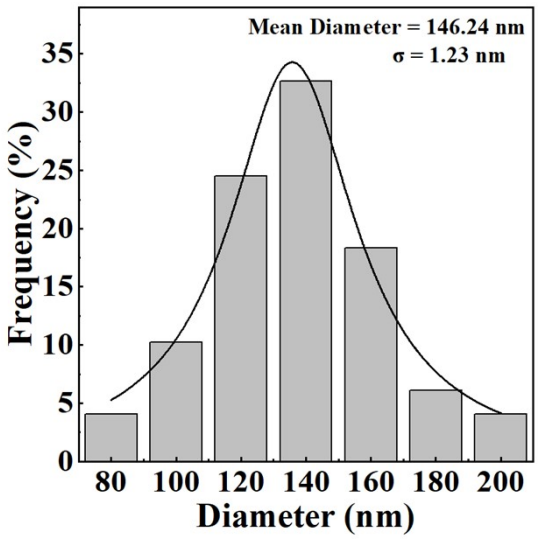

Fig. S4 Size distribution histograms of LDH-EGCG.

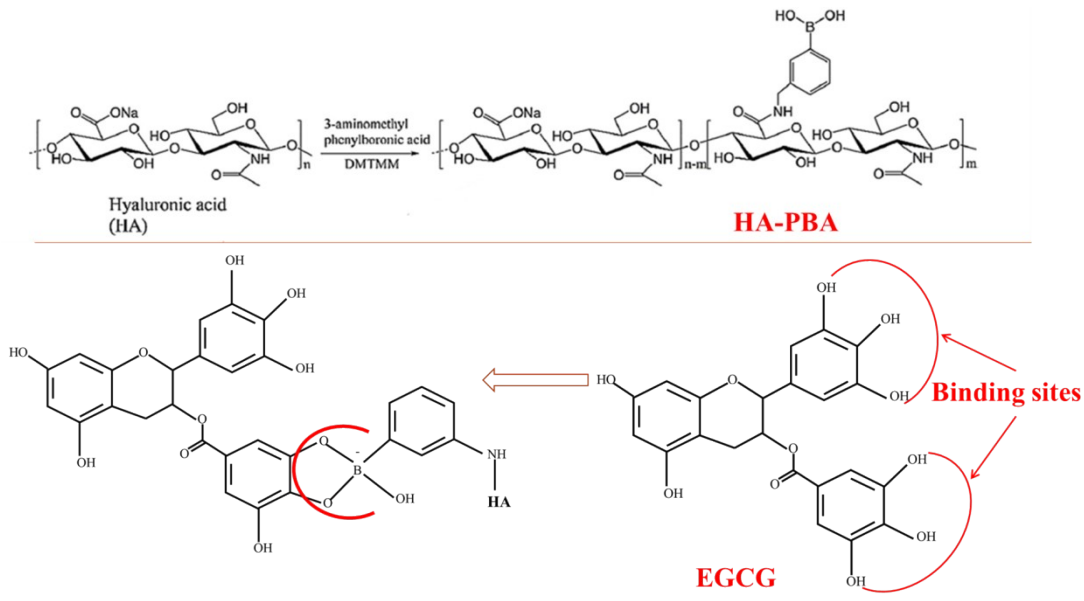

Fig. S5 Scheme for the synthesis of HA-PBA and the fabrication of LDH-EGCG-HA.

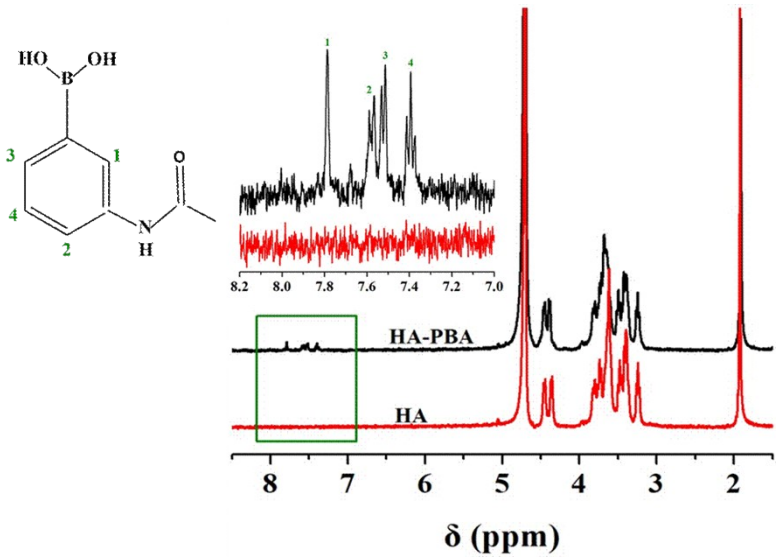

Fig. S6 ^{1}H NMR spectra of HA and HA-PBA.

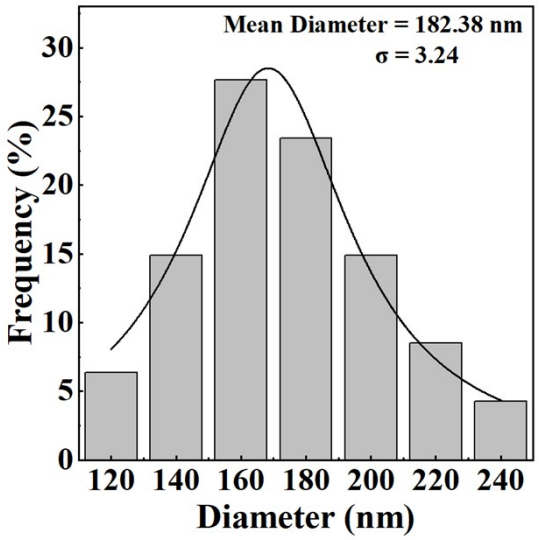

Fig. S7 Size distribution histograms of LDH-EGCG-HA.

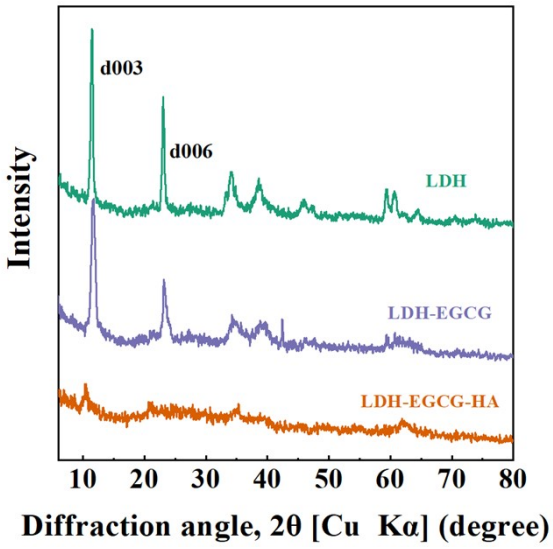

Fig. S8 XRD spectra of LDH, LDH-EGCG, and LDH-EGCG-HA.

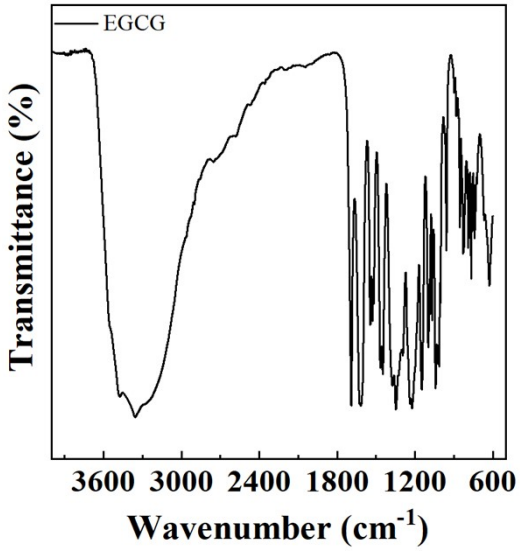

Fig. S9 FT-IR spectra of EGCG.

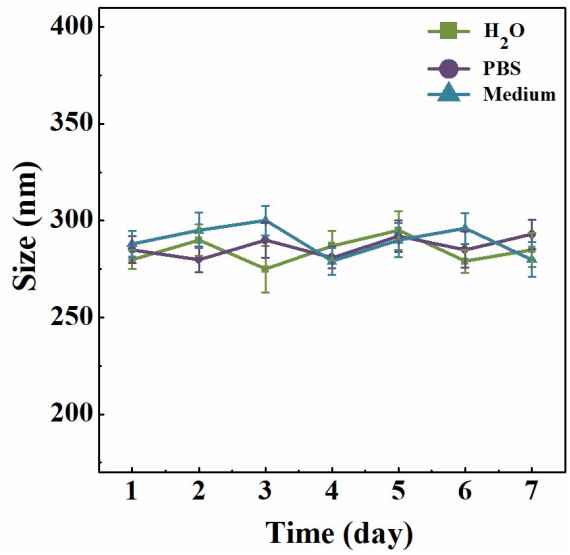

Fig. S10 The hydrodynamic diameters of LDH-EGCG-HA in water, phosphate buffer saline (PBS), and cell culture medium (RPMI 1640) for 7 days.

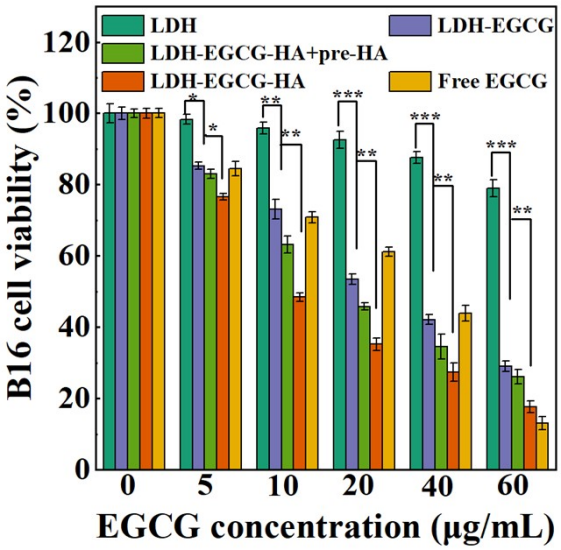

Fig. S11 Viability of B16 cells treated with free EGCG, LDH, LDH-EGCG, LDH-EGCG-HA and LDH-EGCG-HA + pre-HA at different concentrations of EGCG for 48 h.

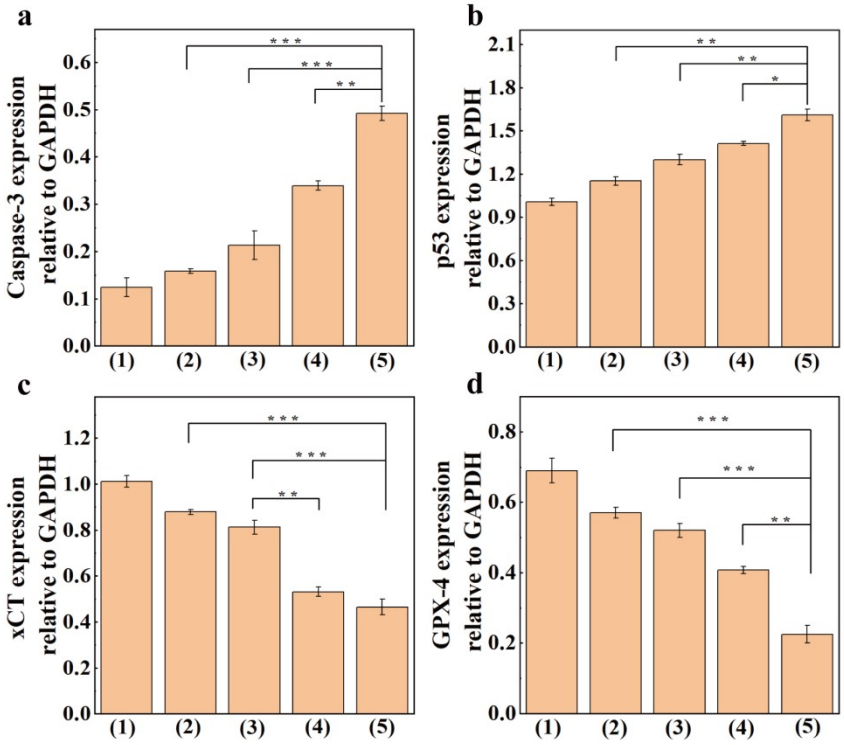

Fig. S12 The corresponding statistical data of the (a) caspase-3, (b) p53, (c) xCT and (d) GPX-4 expressions in the B16 cells that received different treatments with (1) PBS, (2) LDH, (3) free EGCG, (4) LDH-EGCG and (5) LDH-EGCG-HA.

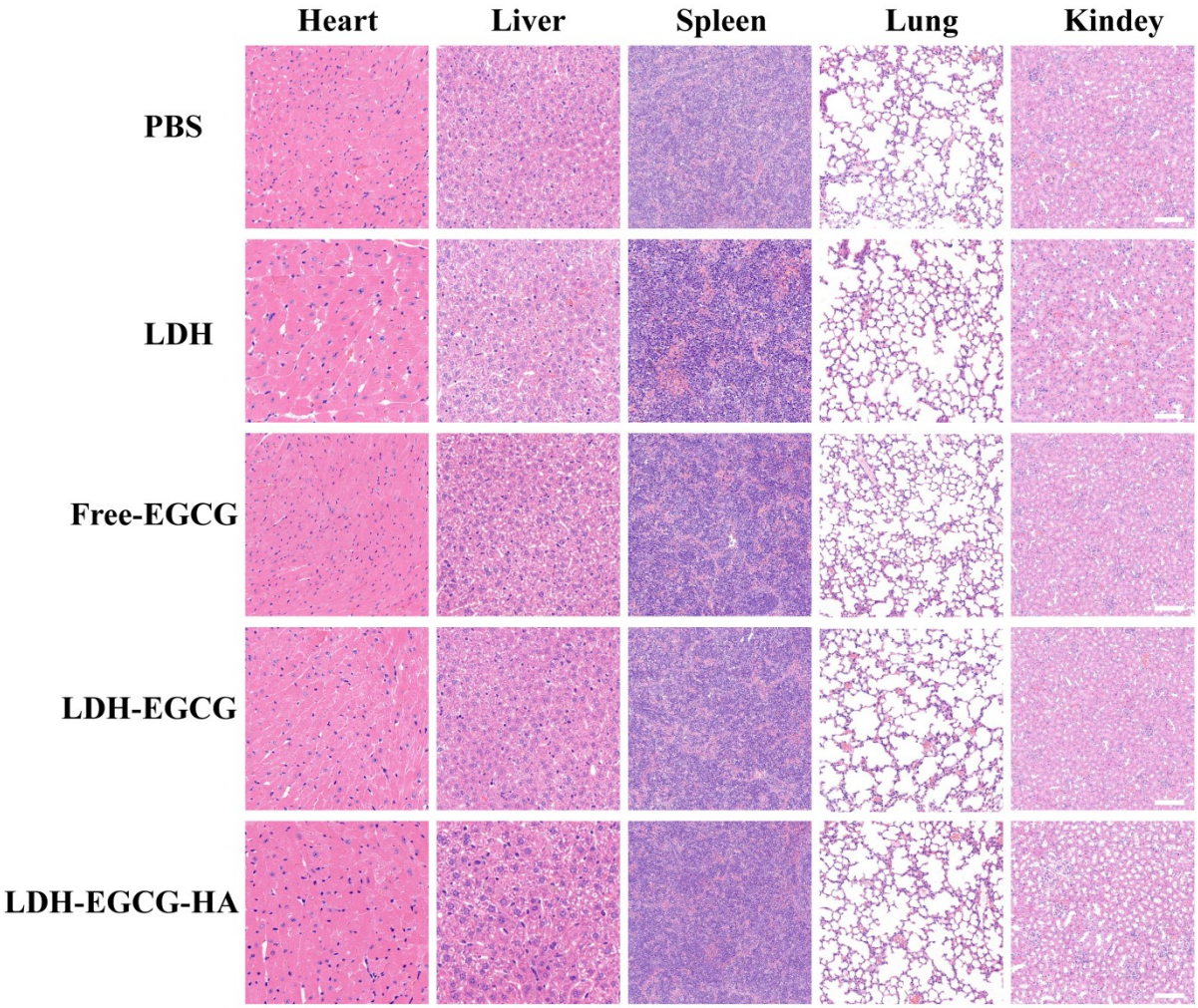

Fig. S13 H&E-stained histological images of major organs after different formulations (scale bar: 100 μm).

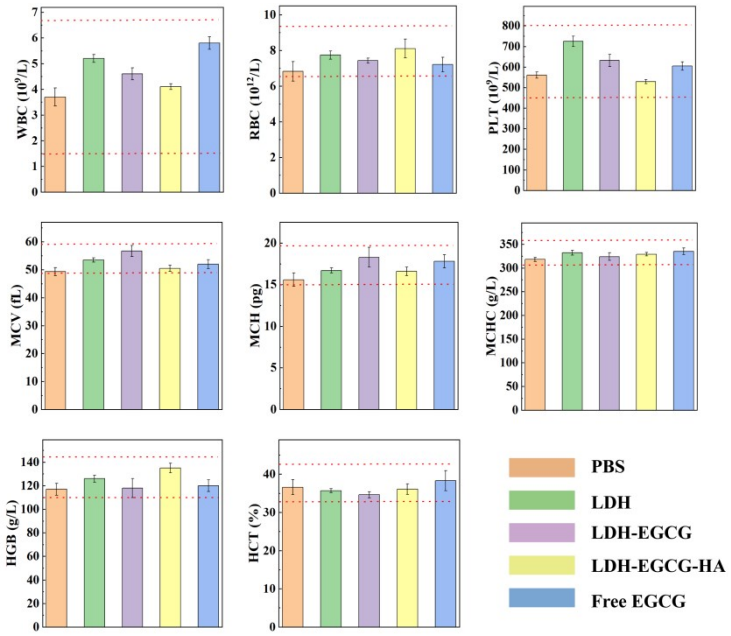

Fig. S14 Routine blood analysis results of the mice collected on the 14th day after the intravenous injection of PBS (control), LDH, LDH-EGCG, LDH-EGCG-HA, or Free EGCG. The blood indexes including WBC, RBC, PLT, HGB, MCV, MCH, HCT and MCHC indicate the numbers of white blood cells, red blood cells, and platelets, concentration of hemoglobin, mean corpuscular volume, mean corpuscular hemoglobin, hematocrit, and mean corpuscular hemoglobin concentration, respectively.

References

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