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Supporting Information

GalNAc-modified $CaCO_3$ nano-immunomodulator for targeted and responsive immunotherapy against orthotopic liver cancer

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Table of Contents

EXPERIMENTAL SECTION

Materials and reagents. Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$) and ammonia bicarbonate (NH_4HCO_3) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). (2S,3R,4R,5R,6R)-3-Acetamido-6-(Acetoxymethyl) Tetrahydro-2H-Pyran-2,4,5-Triyl Triacetate (GalNAc-5OAc), 2-Azidoethanol, trimethylsilyl trifluoromethanesulfonate (TMSOTf) and triethylamine (TEA) were purchased from Shanghai Adamas Reagent Co., Ltd. 1,2-Distearoyl -sn-glycero-3-phosphate (DSPA), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000 (DSPE-PEG2000) and 1,2-dihexadecanoyl-sn-glycero-3-phosphocholine (DPPC) were purchased from Xi'an Ruixi Biological Technology Co., Ltd. Cholesterol was purchased from Heowns Biochem Technologies, LLC, Tianjin. 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[Alkyne(polyethylene glycol)]-2000 (DSPE-PEG2000-ALK) was purchased from Shanghai Ponsure BIOTECH INC. CpG ODNs-N_3 (5' to 3': TCCATGACGTTCCTGACGTT) was synthesized by Sangon Biotechnology Co. Ltd. (Shanghai, China). 3-(4,5-dimethyl-thiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT), was purchased from Beijing Solarbio Science & Technology Co., Ltd. Hoechst 33342, propidium iodide (PI), calcein acetoxymethyl ester (Calcein-AM) and calcium colorimetric assay kit were purchased from Beyotime Biotech. Inc. Anti-CALR antibody, anti-HMGN1 antibody and DyLight 594 conjugated affinipure goat anti-rabbit IgG (H+L) were purchased from Boster Biological Technology Co., Ltd. The mouse Hepatocarcinoma cell line (Hepa 1-6) was purchased from Ubigene Biosciences (Guangzhou, China). Female C57BL/6J mice (6-8 weeks, 20 g) were obtained from SiPeiFu Beijing Biotechnology Co., Ltd. Fetal bovine

serum (FBS) in cell culture medium was purchased from Gibco. The experimental water used was Mill-Q secondary ultrapure water (18.2 MΩ·cm^{-1}). All the other chemical reagents were of analytical grade and used without further purification.

Instruments. TEM imaging was carried out on a HT7700 electron microscope (HITACHI, Japan). Zeta potential was performed on a Malvern Zeta Sizer Nano (Malvern Instruments). Absorption spectra was measured on a U-4100 UV-visible spectrophotometer (HITACHI, Japan). All pH measurements were performed with a pH-3c digital pH-meter (Shanghai LeiCi Device Works, Shanghai, China) with a combined glass-calomel electrode. Absorbance in MTT assay was measured in a microplate reader (RT 6000, Rayto, USA). Confocal fluorescence imaging experiments were performed with TCS SP8 confocal laser scanning microscopy (Leica Co., Ltd. Germany) with a 20× objective. Live animal imaging system (IVIS Lumina III, US) was applied *in vivo* imaging. Fluorescence spectra were obtained with FLS-980 Edinburgh.

Synthesis of $CaCO_3$ nanoparticles. Prepare two 500 mL beakers covered with aluminum foils, which were punctured with dense pores. Add 220 mg of $CaCl_2 \cdot 2H_2O$ and 100 mL of anhydrous EtOH together to each large beaker. Then prepare four 50 mL beakers and add 2 g of dry NH_4HCO_3 to each small beaker. All of these beakers were put into a vacuum drying chamber and the whole system were kept in a vacuum environment at 25 ℃ or 37 ℃ for 24 h. Then $CaCO_3$ nanoparticles were separated by centrifugation at 12000 rpm. For each beaker, the yield of $CaCO_3$ nanoparticles synthesized under 25 °C was 12.67±1.05 mg, and the yield under 37 °C was 25.33±2.20 mg. The yield of $CaCO_3$ nanoparticles at 37 ℃ is about 200% of that at 25 ℃. Therefore, the temperature of 37 ℃ was chosen for subsequent experiments.

The prepared $CaCO_3$ nanoparticles were dried in the vacuum drying chamber and finally stored in -20 ℃.

Synthesis of $CaCO_3$(DOX) nanoparticles. DOX·HCl (1 eq) was dissolved in CH_2Cl_2 and then TEA (3 eq) was added to the solution. After stirring under room temperature in dark for 24 h, the CH_2Cl_2 and Et_3N·HCl was evaporated. Next, 20 mg of $CaCO_3$ nanoparticles and 5 mg of DOX were dissolved in 1 mL of anhydrous DMSO. After stirring under room temperature in dark for 24 h, $CaCO_3$(DOX) nanoparticles were separated by centrifugation at 12000 rpm and washed with EtOH for 3 times.

Synthesis of $CaCO_3$(DOX)@liposome. The above prepared 20 mg of nanoparticles were dissolved in 0.4 mL of EtOH and mixed with 16 mg of DSPA (dissolved in 6 mL of $CHCl_3$). Then the mixture was treated by ultrasonic for 20 min and stirred under room temperature in dark overnight. The obtained solution was centrifuged to remove free DSPA, and then nanoparticles were redispersed into $CHCl_3$ (named as solution A). Then, DPPC, cholesterol, and DSPE-PEG2000-ALK in $CHCl_3$ (at 4:4:2 M ratio) were added to solution A and stirred overnight. Afterwards, the $CHCl_3$ was evaporated, and the obtained $CaCO_3$(DOX)@liposome nanoparticles were dissolved in PBS for further use. $CaCO_3$@liposome nanoparticles were prepared in a similar way.

Synthesis of GalNAc-N_3.

Step 1: 1.17 g of GalNAc-5OAc (3 mmol, 1 eq) was dissolved in 1,2-dichloroethane (DCE, 30 mL) under 0 ℃, then 667 μL of TMSOTf (3.9 mmol, 1.3 eq) was added dropwise. After stirring at 0 ℃ for 10 min, the reaction system was moved to a 50 ℃ oil bath and refluxed for 5 h. Then the reaction mixture was diluted with DCE and quenched with TEA.

The mixture was washed with saturated $NaHCO_3$ solution and saturated NaCl solution. Finally, the organic phase was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The product (named as product A, yield 80%) of this step was used directly without further purification.

Step 2: Product A (2.4 mmol, 1.2 eq) and 140 μL of 2-azidoethanol (2 mmol, 1 eq) was dissolved in DCM (18 mL) under 0 ℃, and 103 μL of TMSOTf (0.6 mmol, 0.3 eq) was added dropwise. After stirring at 0 ℃ for 10 min, the reaction system was moved to a 50 ℃ oil bath and refluxed for 17 h. Then the reaction system was quenched with TEA and purified by silica gel chromatography with CH_2Cl_2/CH_3OH (v/v, 20:1) as eluent, affording GalNAc-5OAc-N_3 as a pale-yellow solid.

Step 3: 416 mg of GalNAc-5OAc-N_3 (1 mmol, 1.0 eq) and 167 mg of MeONa (0.31 mmol, 0.31 eq) was dissolved in 10 mL of MeOH with the pH at about 9. The system was allowed to stir at 23 ℃ for 3 h. Then ion-exchange resin was added into the solution until the pH was about 5 or 6. Finally, the resin was filtered, and the filtrate was dried to obtain GalNAc-N_3 as a pale-yellow solid.

Synthesis of $CaCO_3$(DOX)@liposome-CpG-GalNAc. Firstly, 15 mg of $CaCO_3$(DOX)@liposome nanoparticles (2 mg/mL in PBS), 1 OD of CpG ODNs-N_3 and 3 mg of GalNAc-N_3 were dissolved in PBS (named as solution B). Then, 3 mg of L-ascorbic acid sodium salt and 0.4 mg of $CuSO_4 \cdot 5H_2O$ were mixed in water (named as solution C). Subsequently, solution C was added to solution B and stirred at room temperature in dark for 4 h. Finally, the obtained $CaCO_3$(DOX)@liposome-CPG-GalNAc nanoparticles (denoted as CaDLCG) were centrifuged and washed with PBS for 3 times. $CaCO_3$@liposome-GalNAc,

$CaCO_3$@liposome-CPG-GalNAc, $CaCO_3$(DOX)@liposome-CPG and $CaCO_3$(DOX)@liposome-GalNAc (denoted as CaLG, CaLCG, CaDLC and CaDLG, respectively) were prepared in a similar way.

Determination of the loading of DOX and CpG ODNs. The loading amount of DOX was calculated by UV absorption spectra of the supernatant before and after loading. The linkage amount of CpG was calculated by Nanodrop of the dialysate of the supernatant.

DOX, Ca^{2+} and CpG ODNs release assay. CaDLCG (CpG-Cy3) nanoparticles were dissolved in PBS (pH 6.5 or pH 7.4) and stirred gently. Then, solutions taken out at different time points (0, 0.15, 0.5, 1, 2, 3, 6, 9, 12, 24 h) was centrifuged to obtain supernatant for further detection. DOX release was measured by UV-Vis absorption spectrum and fluorescence spectrum, Ca^{2+} release was measured by calcium colorimetric assay kit, and CpG-Cy3 release was measured by fluorescence spectrum.

Carbazole experiment. CaLG was prepared and then centrifugated to separate the supernatant and precipitate. Then, the supernatant of CaLG, the precipitate of CaLG, the solution of GalNAc-N_3 and the precipitate of CaL were firstly treated with 0.025mol/L of borax-sulfuric acid solution in ice-water bath and secondly heated on water bath for 30 min. After the solutions were cooled to room temperature, 0.125% of carbazole-ethanol solutions was added for color reaction.

Cell lines and animals. Hepa 1-6 cells were cultured in DMEM medium containing 10% FBS, 100 U mL^{-1} of 1% antibiotics (penicillin/streptomycin) and were maintained at 37 ℃ in a 5% CO_2/95% air humidified incubator (Panasonic). Animal experiments were reviewed and approved by the Ethics Committee of Shandong Normal University, Jinan, P. R. China

(approval number AEECSDNU2023047). All animal experiments were conducted and obeyed the Principles of Laboratory Animal Care (People's Republic of China) and the Guidelines of the Animal Investigation Committee, Biology Institute of Shandong Academy of Science, China.

MTT assay. Cells were planted into the 96-well plate and incubated for 24 h. Then, fresh medium containing different concentrations of CaDLCG (0, 0.1, 0.2, 0.4, and 0.8 $mg \cdot mL^{-1}$) or different kinds of nanoparticles (0.8 $mg \cdot mL^{-1}$) were added. After being incubated for another 24 h or 48 h, the cell culture medium was removed, and the cells were washed with PBS buffer for three times. Then 200 μL of MTT (0.5 $mg\ mL^{-1}$) was added and DMSO was used to dissolve formazan crystals 4 h later. The absorbance at 490 nm was measured to calculate survival rate.

Live/dead cell staining assay. Hepa 1-6 cells were planted into confocal dishes incubated for 24 h. Then fresh medium containing different concentrations of CaDLCG nanoparticles or different kinds of nanoparticles were added. After being incubated for another 24 h or 48 h, the cell culture medium was removed and the cells were washed with PBS buffer for three times. Then the cells were stained with Calcein-AM and PI for 20 min. Finally, the cells were washed twice with PBS, and confocal imaging was performed using a TCS SP8 confocal laser scanning microscopy. Green fluorescence of Calcein-AM was excited at 488 nm and red fluorescence of PI was excited at 633 nm.

Immunofluorescent staining assay. Hepa 1-6 cells were planted into confocal dishes incubated for 4 h. Then fresh medium containing different kinds of nanoparticles were added. After being incubated for another 6 h, the cell culture medium was removed and the cells

were washed with PBS buffer for three times. Then, cells were treated with paraformaldehyde for 20 min, 0.1% Triton-100 for 3 min and 2% BSA for 1h successively. Next, anti-CRT or anti-HMGB1 antibody was added into confocal dishes and cells were incubated under 4 ℃ for 24h in dark. After washed with PBS for three times, DyLight 594 conjugated affinipure goat anti-rabbit IgG (H+L) was added and cells were incubated under 37 ℃ for 1h in dark. Cell nucleus was stained by Hoechst 33342 for 15 min in dark. Confocal imaging was performed using a TCS SP8 confocal laser scanning microscopy. Blue fluorescence of Hoechst 33342 was excited at 405 nm and red fluorescence of goat anti-rabbit IgG (H+L) was excited at 561 nm.

Flow cytometry. Bone marrow dendritic cells were extracted from the femur and tibia of the male C57 mice and cultured in 1640 medium containing IL-4 (20 ng/mL) and GM-CSF (20 ng/mL). Seven days later, the dendritic cells were treated with PBS, CpG ODNs, CaL or CaLC for 24h. Then the cells were washed with PBS, resuspended, and stained with anti-CD11c-FITC and anti-SIINFEKL/H-2Kb(25-D1.16)-PE-Cyanine7 for flow cytometry.

Cell wound healing experiment. Hepa 1-6 cells was seeded in 6-well plate and the then a pipette tip was used to lightly scratch on the bottom of the plate. Serum-free cell culture medium containing different concentrations of CaDLCG was added and photos were taken at 0,6,12,24 hours, respectively.

Cell cloning experiment. 3000 of Hepa 1-6 cells was seeded in 6-well plate and incubate with different nanoparticles. One week later, the cells were treated with paraformaldehyde for 20 min and then stained with crystal violet.

Establishment of orthotopic transplantation tumor model of liver cancer. Hepa 1-6 cells

were digested by trypsin, and then washed with PBS for three times. About every 1×10^7 of Hepa 1-6 cells were dispersed in 50 μL of serum-free DMEM medium and the cell suspension was injected to mice liver to form an orthotopic transplantation tumor model.

In vivo therapy. After growing for one weeks in the liver, mice were randomly divided into six groups, and were intravenously injected with PBS, CaLG, CaLCG, CaDLC, CaDLG and CaDLCG at day 7, 9, 11, 13 and 15 (50 mg kg^{-1}). Mice were euthanized at day 21, and the tumor-bearing livers were photographed. The animal body weight was also recorded every 2 days.

Ex vivo **immunofluorescence staining assays.** Mice with orthotopic liver cancer were randomly divided into seven groups, and were intravenously injected with PBS, DOX, CaLG, CaLCG, CaDLC, CaDLG and CaDLCG (1.875 mg kg^{-1} of DOX and 50 mg kg^{-1} of others). Mice were euthanized and the orthotopic liver cancer tissues were carefully isolated and analyzed by immunofluorescence staining for CRT, HMGB1, CD3, CD4 and CD8.

SUPPORTING FIGURES

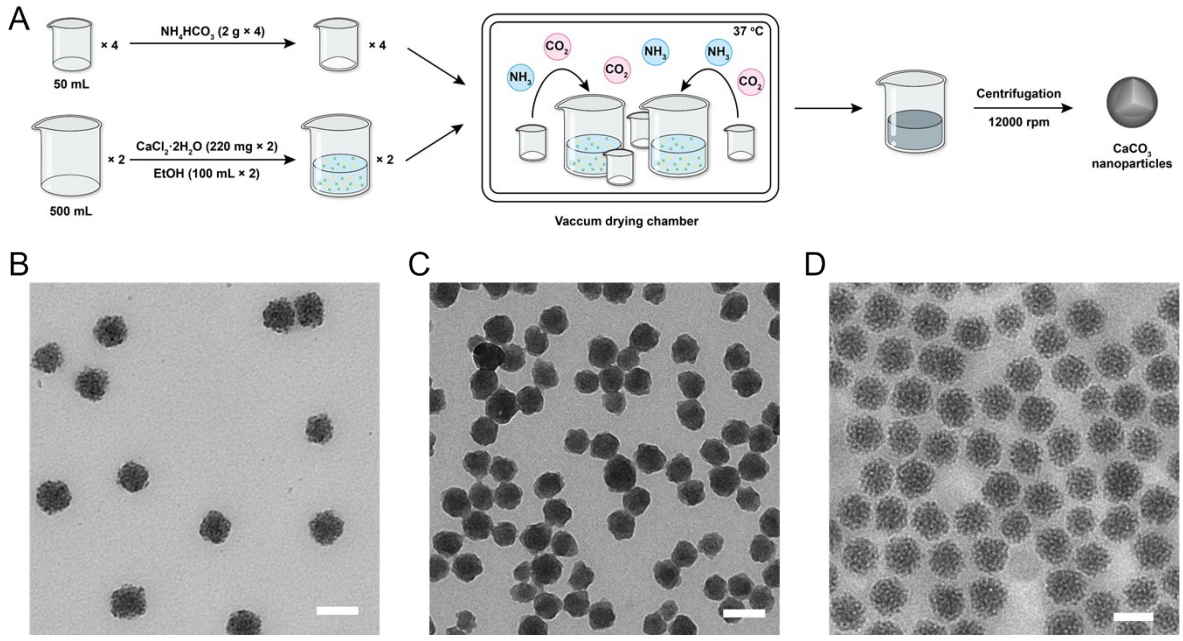


Figure S1. (A) The synthesis steps of $CaCO_3$ nanoparticles. The TEM images of (B) $CaCO_3$, (C) $CaCO_3$(DOX) and (D) CaDL. Scale bars: 100 nm.

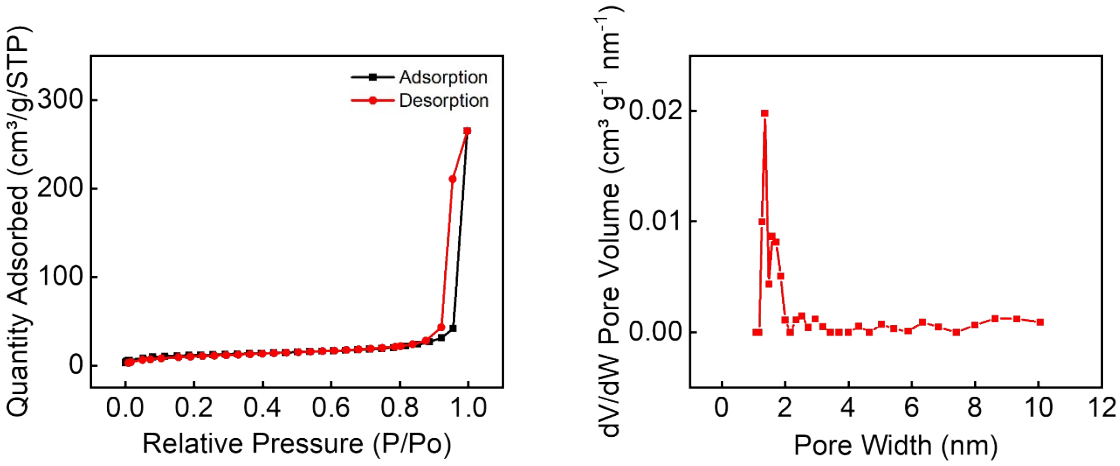
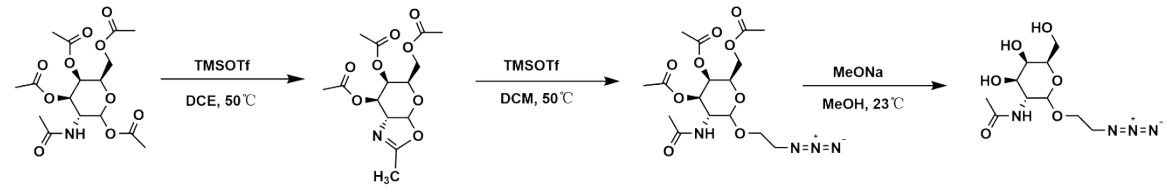
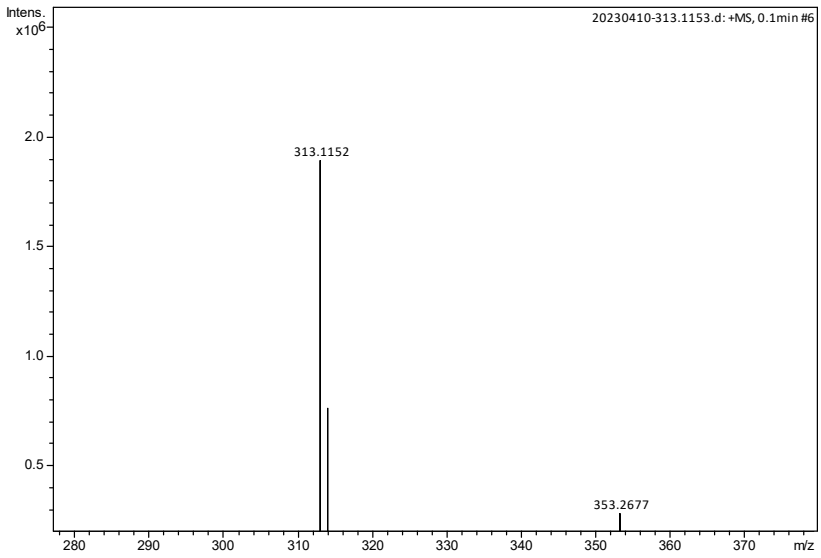


Figure S2. The N_2 adsorption and desorption isothermal curve and pore width of $CaCO_3$ nanoparticles.





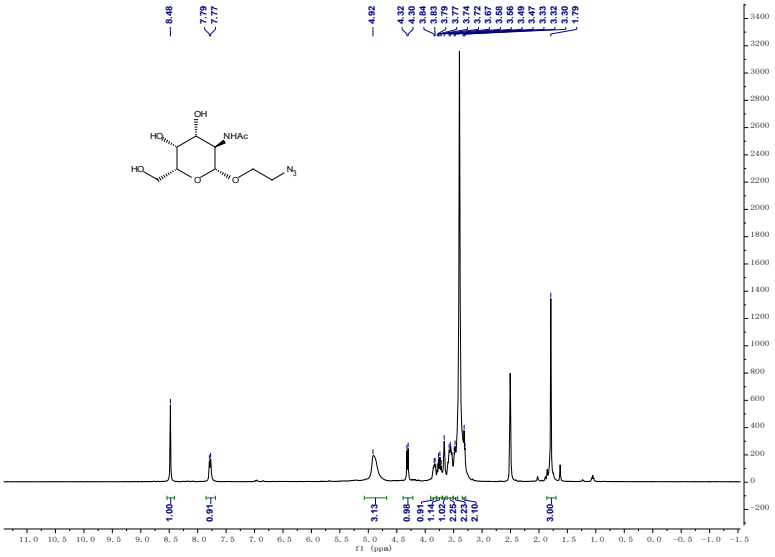


Figure S3. Synthesis steps, mass spectrometry and ^{1}H NMR Spectroscopy of GalNAc-N_3.

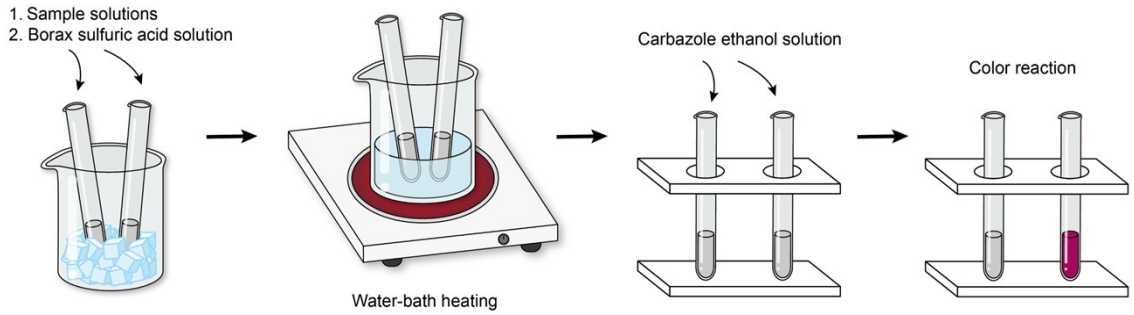


Figure S4. The experimental steps of carbazole analysis.

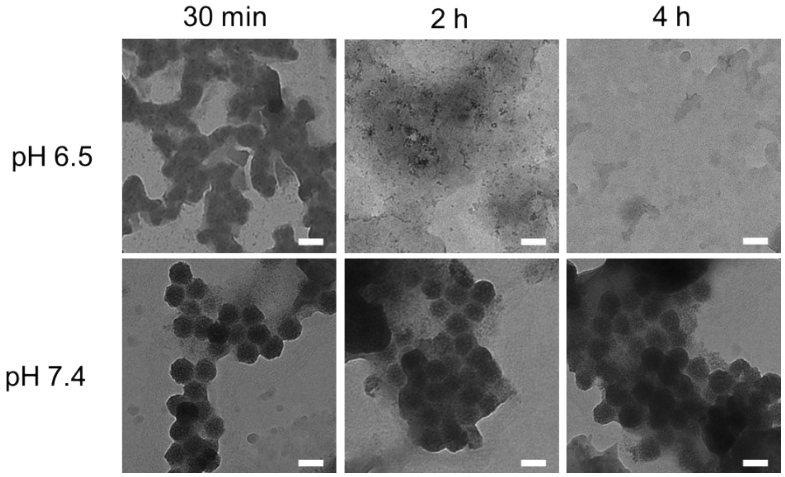


Figure S5. The TEM images of CaDLCG dispersed in the buffer solutions of different pH (6.5 and 7.4). Scale bar: 100 nm.

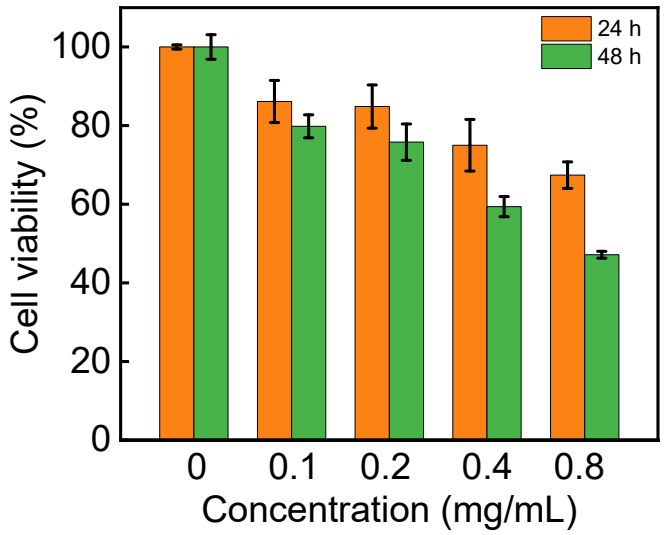


Figure S6. The viability of Hepa1-6 cells after incubated with different concentrations of CaDLCG for 24 and 48 h.

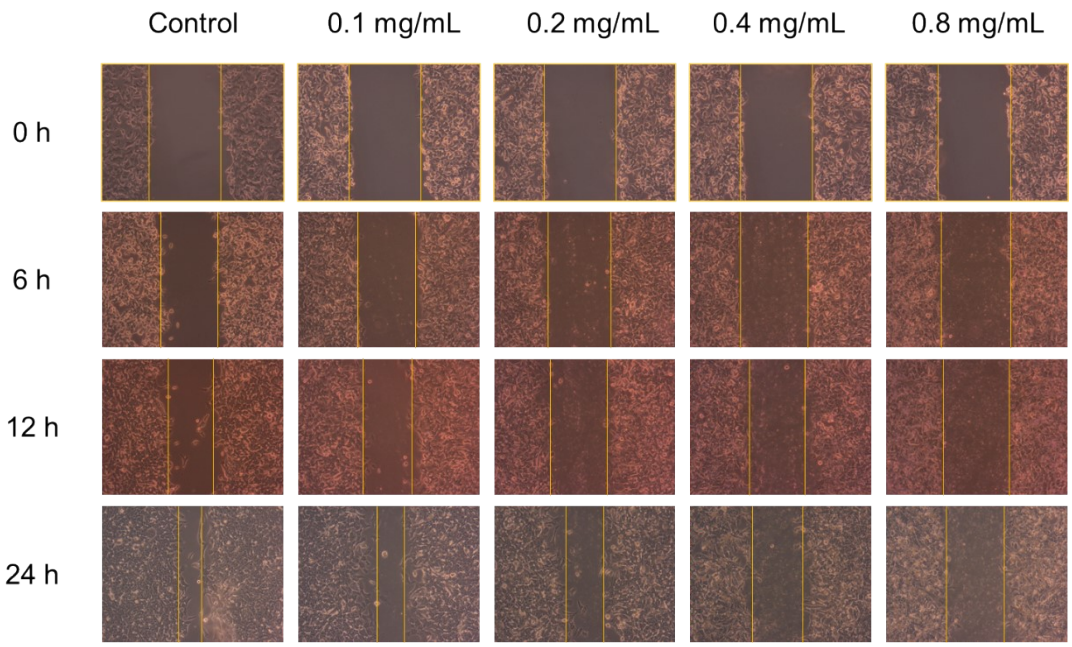


Figure S7. The wound-healing assays of Hepa1-6 cells after incubated with different concentrations of CaDLCG.

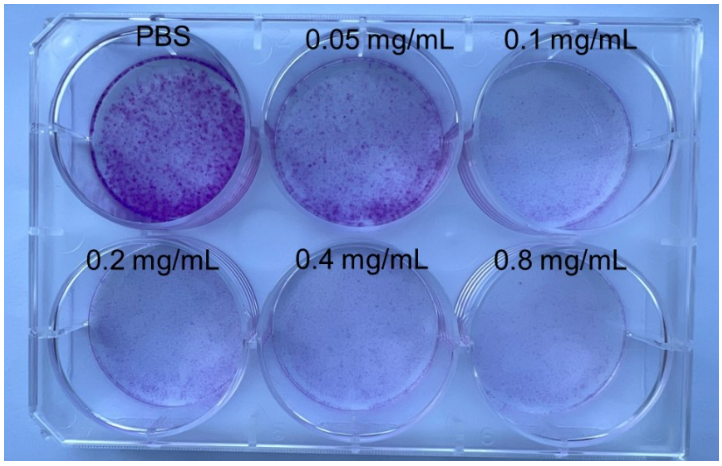


Figure S8. The colony formation assays of Hepa1-6 cells after incubated with different concentrations of CaDLCG.

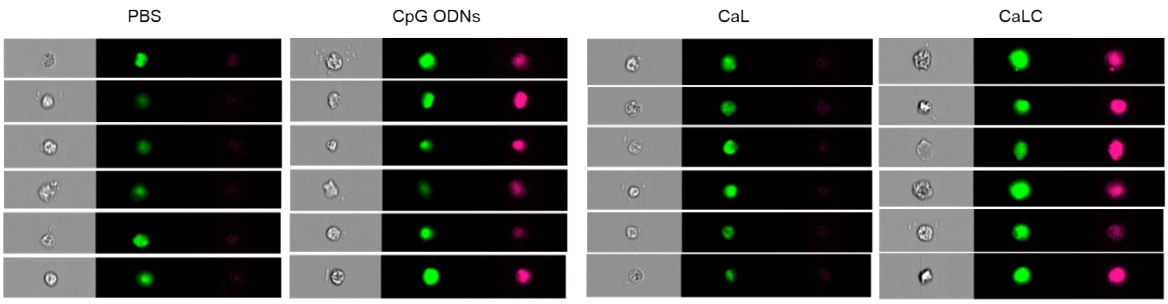


Figure S9. The representative cells of flow cytometry in different groups. Green channel: FITC-anti mouse CD11c. Red channel: anti-SIINFEKL/H-2Kb(25-D1.16)-PE-Cyanine7.

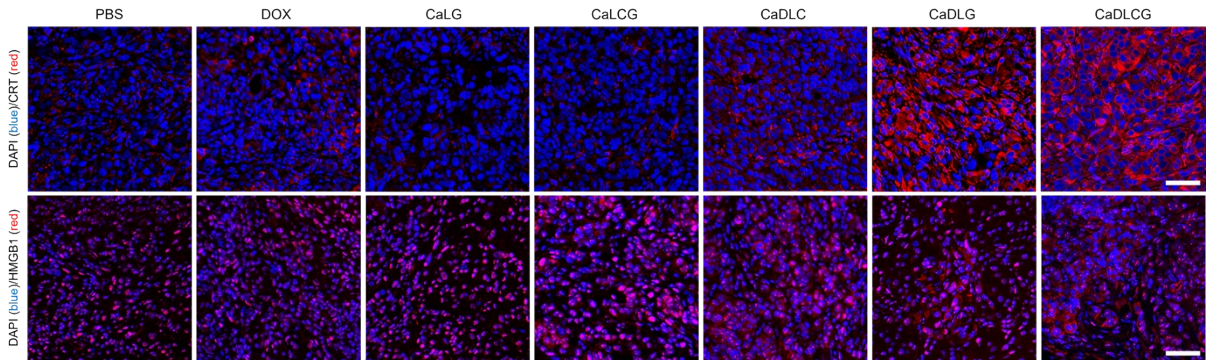


Figure S10. Immunofluorescence staining images of CRT and HMGB1 on liver tumor sections of mice. Scale bars: 50 μm.

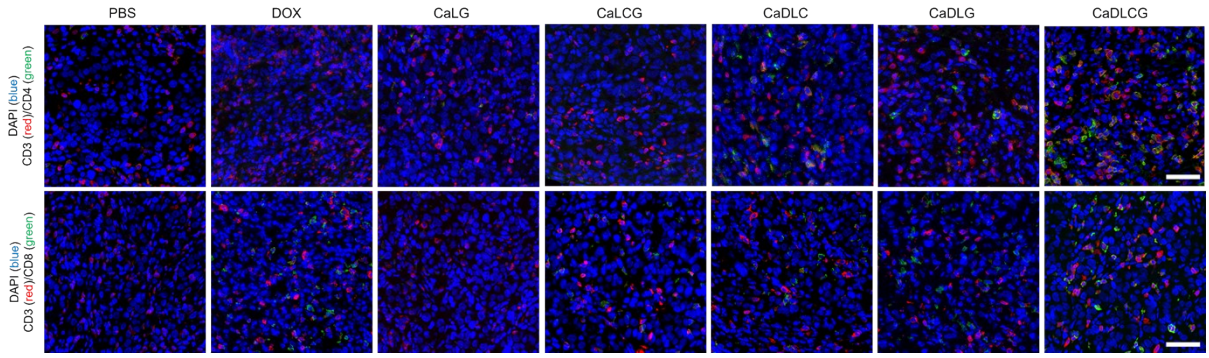


Figure S11. Immunofluorescence images of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells on liver tumor sections of mice. Scale bars: 50 μm.

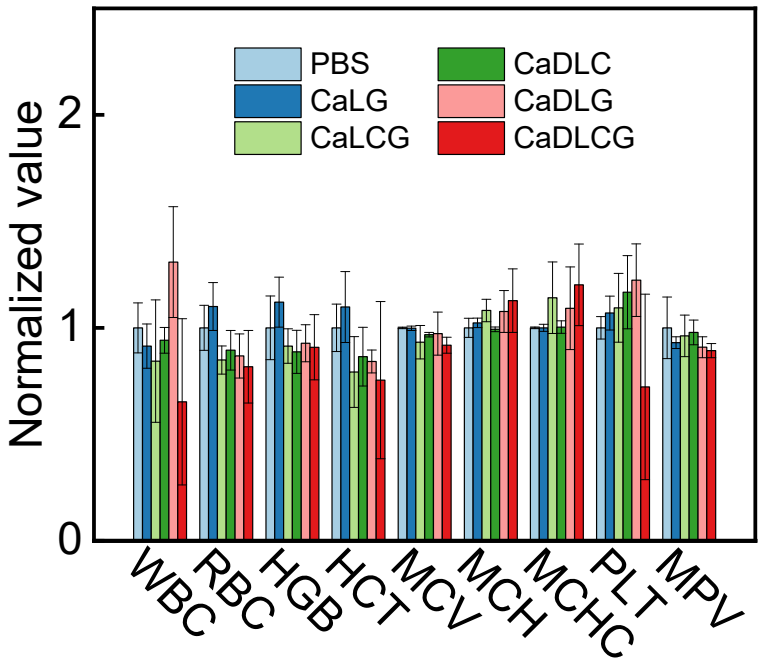


Figure S12. The normalized value of routine blood indicators. WBC: white blood cell count; RBC: red blood cell count; HGB: hemoglobin; HCT: hematocrit, MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; PLT: platelet count; MPV: mean platelet volume.

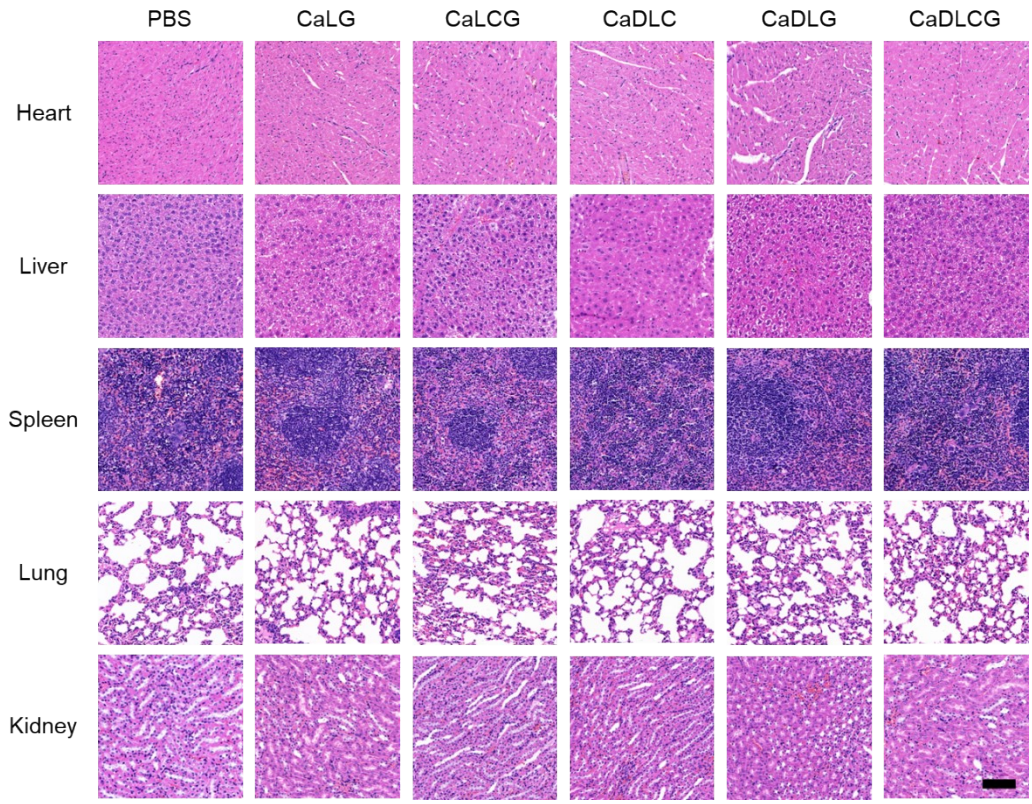


Figure S13. H&E-stained images of major organs of mice after different treatments. Scale bar: 100 μm.