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Artificial cell system for biocompatible gene delivery in cancer therapy

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Experimental Section

***Materials*:** DNase I, ethidium bromide (EB), Lipofectamine™ 2000, Cy7 and DAPI were offered by Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). YOYO-1 was purchased from Invitrogen Co., Ltd. (Carlsbad, CA, USA) and All other chemicals and reagents otherwise stated were from Sinopharm Chemical reagent Co., LTD and of analytical grade.

The reporter plasmid (pEGFP-C3, 4.7 kb) encoding enhanced green fluorescent protein (GFP) and plasmid pCMV-Neo-Bam-p53wt (wt-p53, 8.4 kb) encoding wild type p53 (p53) driven by CMV promoter purchased from Addgene were propagated in DH-5α *Escherichia coli* and purified by Endo Free Plasmid Maxi Kit (Qiagen, Germany). All other reagents were of analytical grade and used without further purification. [1]

Cell culture: HeLa (human cervical carcinoma) was a gift from Dr. Pengfei Cui (China Pharmaceutical University), A549 (human lung carcinoma), HepG2 (human hepatic carcinoma), MCF-7 (human breast carcinoma) and NIH3T3 (mouse embryonic fibroblast) cell lines were purchased from Institute of Biochemistry and Cell Biology (Shanghai, China). All cells were cultured in dulbecco's modified eagle medium (DMEM, Sigma, USA) supplemented with 10% (v/v) fetal bovine serum (FBS, Hyclone, USA), 100 U/mL penicillin and 100 μg/mL streptomycin (Beyotime Biotechnology, China) in a humidified atmosphere of 95% air/5% CO_2 incubator (Thermo Forma 311, Thermo Scientific, USA) at 37 °C. All experiments were performed on cells in the logarithmic phase of growth. [2]

Multi-cellular tumor spheroid model (MCTS): A 96-well plate (Corning, USA) was firstly covered with autoclaved agarose solution (1.5%, w/v) at 50 μL/well and then cooled to room temperature. Mixed MCF-7 and NIH3T3 cells (1:1) were seeded at a density of 2×10^3 cells per well and incubated for 4 days to grow into MCTS. The formation of MCTS was monitored using optical microscope (TE2000-S, Nikon, Japan). [3]

Animal model: Female BALB/c nude mice (4-5 weeks, 16 g) and New Zealand rabbit were purchased from Shanghai Laboratory Animal Center (SLAC, China) and maintained at 22 ± 2 °C with access to food and water ad libitum. MCF-7 or HepG2 tumor bearing tumor xenograft model was established according to previous report with minor modifications. [3] Briefly, the suspensions of MCF-7 or HepG2 cells (2×10^6) in 100 μL of phosphate buffer saline (PBS, 0.01 M, pH 7.4) were inoculated subcutaneously in the flank of nude mice. Tumor sizes were measured using a Vernier caliper, and tumor volumes were calculated as $V = a^2 \times b/2$ mm^3 (a: minor axis; b: major axis).

Isolation of cell membrane (CM): The CM was obtained from corresponding cells using previous reported protocol. [4] In brief, cells were cultured in 100 mm culture dish and allowed to grow for 90% confluence. Afterwards, cells were collected and added into a hypotonic medium in an ice bath for 30 min. The large aggregates were removed by low speed centrifugation (Allegra 64R, Beckman Coulter, USA) at 600g for 10 min, and the supernatant were subjected to high speed centrifugation (10000g) for another

30 min. The collected pellets were sonicated for 5 min using a bath sonicator to prepare CM vesicles.

Preparation of artificial cell (AC): Cells were cultured in 100 mm culture dish and allowed to grow for 90% confluence. Afterwards, cells were collected and washed twice with PBS (containing 5 mmol/L of sodium butyrate). Afterwards, PBS containing 0.5% (v/v) of Triton X-100, 2 mmol/L phenyl methyl sulfonyl fluoride and 0.02% NaN_3 were added to resuspend the cells on ice at the concentration of 10^7 cells/mL. After 10 min of extraction, the mixture was centrifuged (2,000 rpm, 10 min, 4 °C) to collect the sediment. The sediment was washed twice using the same solution and then incubated with 0.2mol/L HCl at 4 °C overnight. The mixture was centrifuged to obtain supernatant and stored in -80°C until further usage. The histone content in the supernatant was determined using the BCA protein quantification kit (Solarbio) according to the manufacturer's instructions.

To prepare the binary complex (BC) of DNA and histone, the DNA was firstly dissolved in HEPES buffer (HEPES 20 mM, pH 7.4) to obtain a final concentration of 0.1 mg/mL. Afterwards, the DNA solution was dropwise added into HEPES buffer containing histone at the various weight/weight (w/w) ratios (histone/DNA, 0.5 to 10) under vigorous vortex. The mixed solution was allowed to stay at room temperature for 30 min for the formation of BC. Lipofectamine ™ 2000/DNA (Lipo) complexes prepared according to manufacturer's instructions were used as controls.

The CM vesicles were then dropwise added into the BC solution at various w/w ratios (CM/BC, 0.01 to 2) under vigorous vortex. The mixture was subsequently extruded through a 100 nm polycarbonate porous membrane on a mini extruder (Avanti Polar Lipids, USA) and then allowed to stay at room temperature for 30 min for the formation of AC.

Characterizations of nanoparticles: The particle size and zeta potential were further determined by Particle/Zeta Analyzer (Litesizer 500, Anton Paar, Austria). Moreover, the morphology and particle size of different nanoparticles were observed by transmission electron microscopy (TEM, JEM-1200, JEOL, Japan) at an accelerating voltage of 80 kV.

To determine the DNA loading capacity and encapsulation efficiency of AC, the as-prepared AC was firstly subjected to centrifugation at 100000 rpm under 4 °C for 1 h (Optima MAX-XP, Beckman Coulter, USA). Afterwards, the supernatant was collected and incubated with equal volume of EB soluetion (5 μg/mL) at 37 °C for 30 min. Afterwards, the fluorescence absorption of the solution was assessed using fluorescence spectrophotometer (F-2500, Hitachi, Japan) at the Ex of 546 nm and Em of 590 nm.

The agarose gel electrophoresis was employed to assess the DNA binding and protecting capacity. In brief, BC or AC at various w/w ratios were loaded onto a 1 % agarose gel (containing equal amount of 0.2 μg DNA) with naked DNA as control. The samples were run on the gel in 0.5 × TBE buffer at 90 V for 60 min and photographed using a Gel analyzer (Gel Logic 200, Kodak, USA). [1]

DNase I (3 μL, 1 U/μL in 10× reaction buffer containing 100 mM Tris-HCl, 1 mM $CaCl_2$, 25 mM $MgCl_2$) was added to AC and allowed to incubated for different time

intervals. The DNase I was inactivated with EDTA (3 μL, 50 mM) for 20 min at 37 °C. After that, the samples were treated with polyanion heparin (12 μL, 5 μg/μL) for 1 h at 37 °C to dissociate pDNA from complexes. The results were examined by agarose gel electrophoresis as mentioned above. Naked DNA with (incubation with DNase I for 30 min) or without treatment was used as controls, respectively. The stability of AC was also conducted by challenging AC with different ingredients (0.5 mg/mL BSA, 1 M NaCl, 0.5 mg/mL heparin, 0.5 mg/mL protein CM) for 1 h. The released DNA was also detected by agarose gel electrophoresis. [1]

CM and AC were incubated of RIPA lysis buffer (Solarbio) to isolate the whole proteins. Afterward, equal amounts of protein samples quantified by BCA kit were separated on a 10% SDS-PAGE and then subjected to coomassie blue staining as reported previously. [5]

The freshly prepared AC was diluted with phosphate buffered saline (PBS, pH 7.4, 1:10, v/v) and mouse plasma Afterwards, the change in particle size was monitored for 48 h to estimate the colloidal stability.

For hemolysis assay, red blood cells (RBCs) obtained from New Zealand rabbit were diluted in saline to obtain 2% suspension. The Lipo and AC were added into RBCs suspension to achieve the designated concentrations and the mixture was incubated at 37 °C for 1 h. After that, samples were centrifuged (3000 rpm × 10 min) and supernatants were subjected to UV-vis measurement at 545 nm. RBCs suspension incubated with distilled water and saline under the same condition were employed as positive controls (100% hemolysis) and negative (0% hemolysis), respectively. [6]

In vitro cancer targeting ability： The DNA was labeled with fluorescent dye YOYO-1 as described previously [7] and then used to prepare nanoparticles. A549, HeLa, HepG2 and MCF-7 cells were seeded at a density of 3×10^5 cells per dish in 35 mm confocal dishes (Φ = 15 mm) and cultured overnight. Afterwards, cells were treated with AC for 8 h, followed by confocal laser scanning microscopy (CLSM, BX61W1-FV1000, Olympus, Japan) observation.

In vivo tumor targeting ability: The DNA was labeled with fluorescent dye Cy7 as described previously [8] then used to prepare nanoparticles. Afterwards, MCF-7 tumor bearing mice were administered intravenously with Cy7-labeled AC at a dose of 5 μg Cy7 per mouse. The in vivo tumor targeting efficacy and biodistribution of Cy7 labeled polyplexes were evaluated using In Vivo Imaging System (Maestro *In-vivo* Imaging System, USA) equipped with Cy7 filter sets (excitation/emission, 720/790 nm). After living imaging, the mice were sacrificed, and the tumor tissues as well as major organs (heart, liver, spleen, lung and kidney) were excised for ex vivo imaging using the same imaging system.

Cell transfection: Transfection of different formulations mediated DNA in MCF-7 cells and tumor tissue were investigated. MCF-7 cells were seeded at a beginning cell density of 3×10^5 cells per well of 6-well plates and grown overnight. The freshly prepared polyplexes were diluted with free medium with/without serum and added to the wells after the primary growth medium was discarded. The amount of DNA was fixed at 1 μg/well. After the cells were co-incubated with polyplexes at 37 °C for 4 h, cells were washed twice with PBS and treated with fresh medium, followed by an

additional 48 h of incubation. The expression of green fluorescent protein (GFP) in cells was observed under the inverted fluorescent microscope (Axio Observer A1, Zeiss, Germany).

Moreover, the expression of p53 in cells was further evaluated by western blotting at protein level. In brief, proteins of cells were isolated RIPA lysis buffer as mentioned above. After quantified by BCA kit, equal amount of samples were separated on a 10% SDS-PAGE and transferred to the PVDF membranes (Merck Millipore, USA). Immune complexes were formed by incubation of the proteins with mouse anti-wt-p53 (Abcam, UK) and mouse anti-β-actin (Abcam, UK) primary antibodies overnight at 4 °C. The membranes were rinsed and probed with HRP-conjugated anti-mouse secondary antibodies (Abcam, UK). Immunoreactive protein blots were visualized by ECL immunoblotting detection reagents (Pierce, USA) using scotography.

To further evaluate the expression of DNA in MCF-7 tumors, MCF-7 tumor bearing mice were intravenously injected with a single dose of Lipo and AC (2.5 mg DNA/kg). At 48 h post injection, the mice were sacrificed to harvest the tumor tissues and then subjected to cryotomy. The expression of GFP in cells was reflected as fluorescent intensity and the expression of p53 was revealed by immunohistochemical staining, which were observed by inverted fluorescent microscope.

In vitro anticancer assay: The cell viability of the different formulations was assessed using the MTT assay. For cell viability assay of blank carriers, MCF-7 cells were seeded at 1.0×10^4 cells/well in 96-well plates and then cultured overnight for 70-80% cell confluence. The primary growth medium was removed and replaced with 200 μL of medium, to which Lipo, BC or AC (containing non-therapeutic pEGFP-C3) were added to achieve final concentration of 2, 5, 10, 20, 50 and 100 μg/mL. The plates were returned to the incubator for another 48 h incubating. After that, 20 μL of 5 mg/mL MTT solution in PBS was added to each well for additional 4 h incubation. The medium was carefully removed and replaced by 150 μL of DMSO to dissolve the formazan crystals. The absorbance was measured at 570 nm using Microplate Reader (Bio-Rad, model 680, USA) and untreated cells were taken as a control with 100% viability. The cell viability (%) was calculated according to the following equation:

$$\text{Cell viability (\%)} = (\text{Asample} / \text{Acontrol}) \times 100\%$$

For cell viability assay of therapeutic formulations, therapeutic p53-contained Lipo, BC or AC were incubated with cells according to the above-mentioned procedure with slight modification. In the experiment, the concentration of p53 in samples was fixed at 2 μg/mL. The variations in expression level of apoptosis related proteins (activated Caspase-3, Bcl-2 and Cytochrome C) after incubation with different formulations were determined using western blot as described above.

MCTS with diameters of 300-400 μm were divided into four groups (n = 3). The selected spheroids were treated with fresh medium containing Lipo, BC or AC (p53 concentration of 2 μg/mL). Untreated MCTS was employed as control. The spheroids were allowed to continue to incubate at 37 °C for 5 days. The diameter of the spheroids was recorded every day using an optical microscope.

In vivo tumor growth inhibition: MCF-7 or HepG2 tumor-bearing mice with tumor volume between 50-100 mm^3 were used to assess the therapeutic efficacy of AC.

The mice were divided into three groups (n = 6 per group) and treated with saline (the control group), Lipo, BC and AC at p53 dosage of 2.5 mg/kg. Each sample was intravenously injected via tail vein every 2 days. The measurement of tumor size and mice body weight was repeated once every 2 days before injection over a 14-day therapeutic period and the tumor volume was calculated by the formula: $(W^2 \times L)/2$, where W and L is the shortest and longest diameter, respectively. Afterwards, The rate of survival was calculated based on Kaplan–Meier plot for up to 60 days. At Day 14, mice in each group were randomly selected and euthanized. The main organs (heart, liver, spleen, lung and kidney) and tumor were collected, weighed, washed with saline thrice and fixed in 10% formalin. Formalin-fixed main organs and tumors were embedded in paraffin blocks to prepare sections and then subjected to TUNEL, Ki67 or collagen (COL) staining, respectively. Tissue images were captured at 200× magnification with the optical microscope.

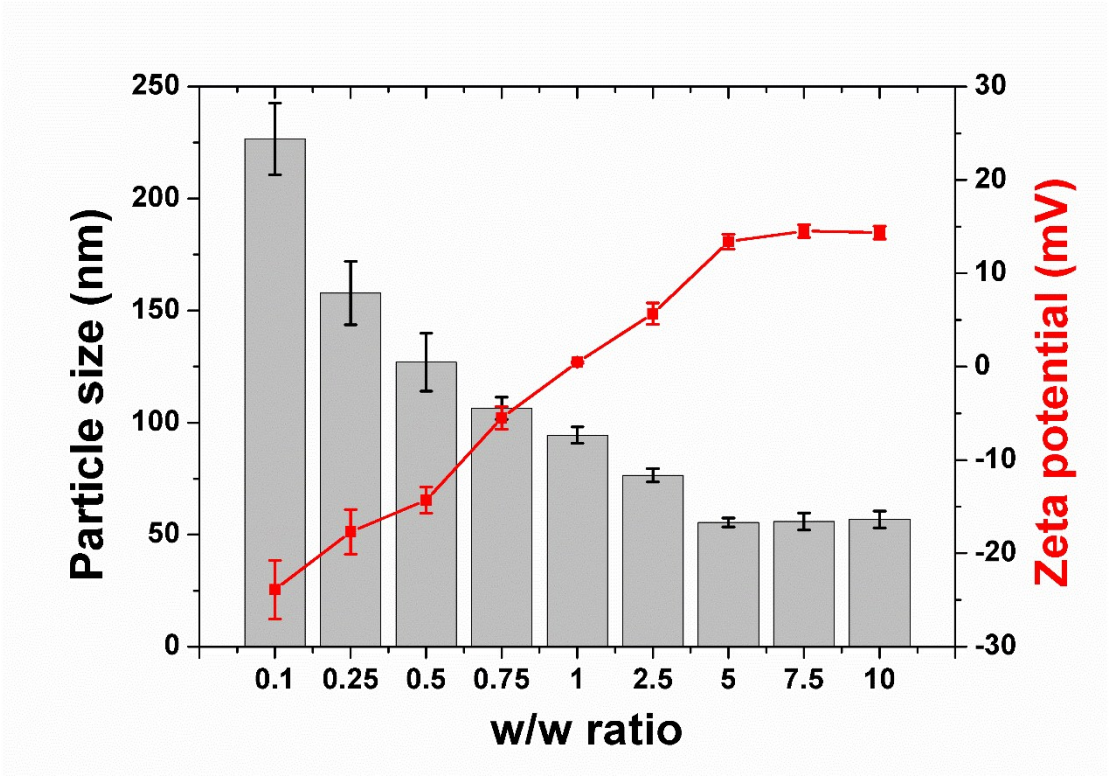


Fig. S1. The size and zeta potential variations of BC at different w/w ratios of histone to DNA. Results were expressed as mean ± S.D. (n = 3).

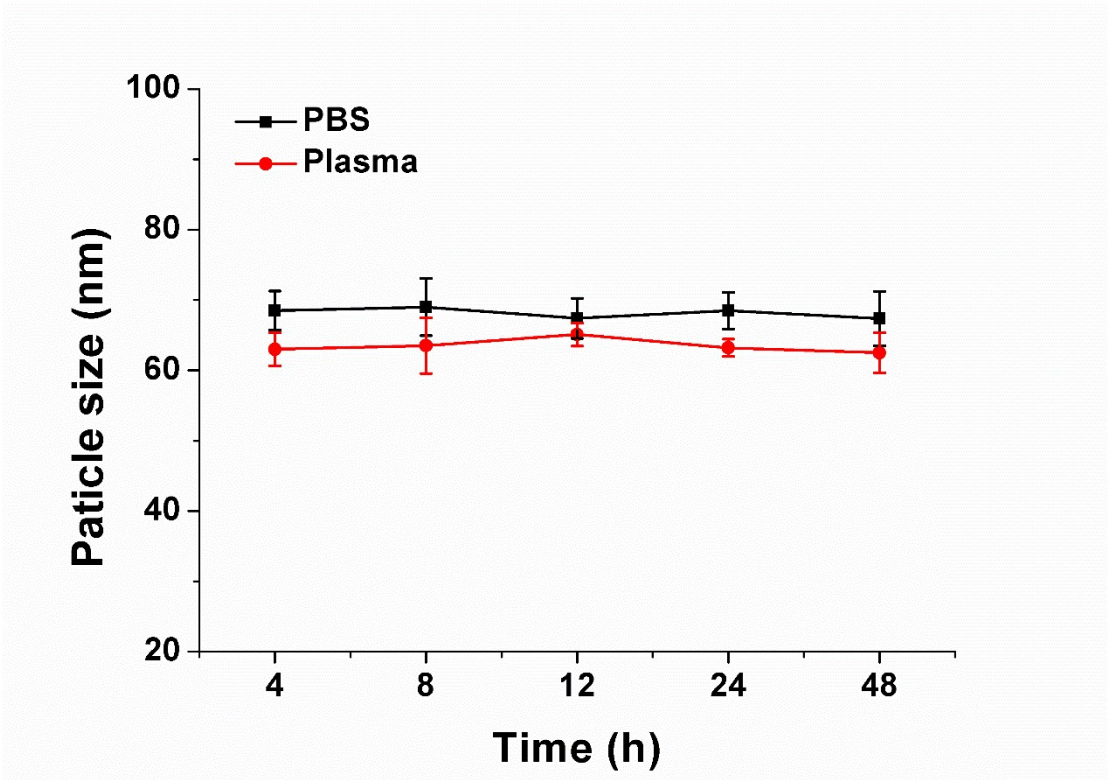


Fig. S2. The storage stability of AC in PBS and plasma, respectively. Results were expressed as mean ± S.D. (n = 3).

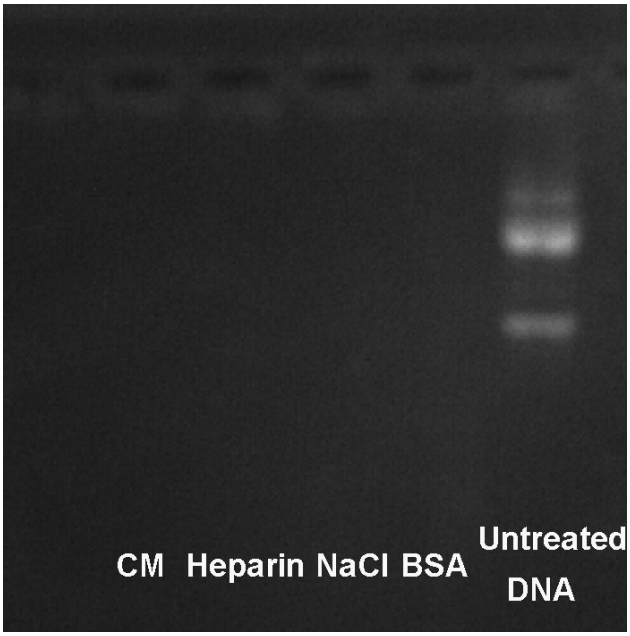


Fig. S3. The stability of AC under the challenge of BSA, NaCl, heparin and CM.

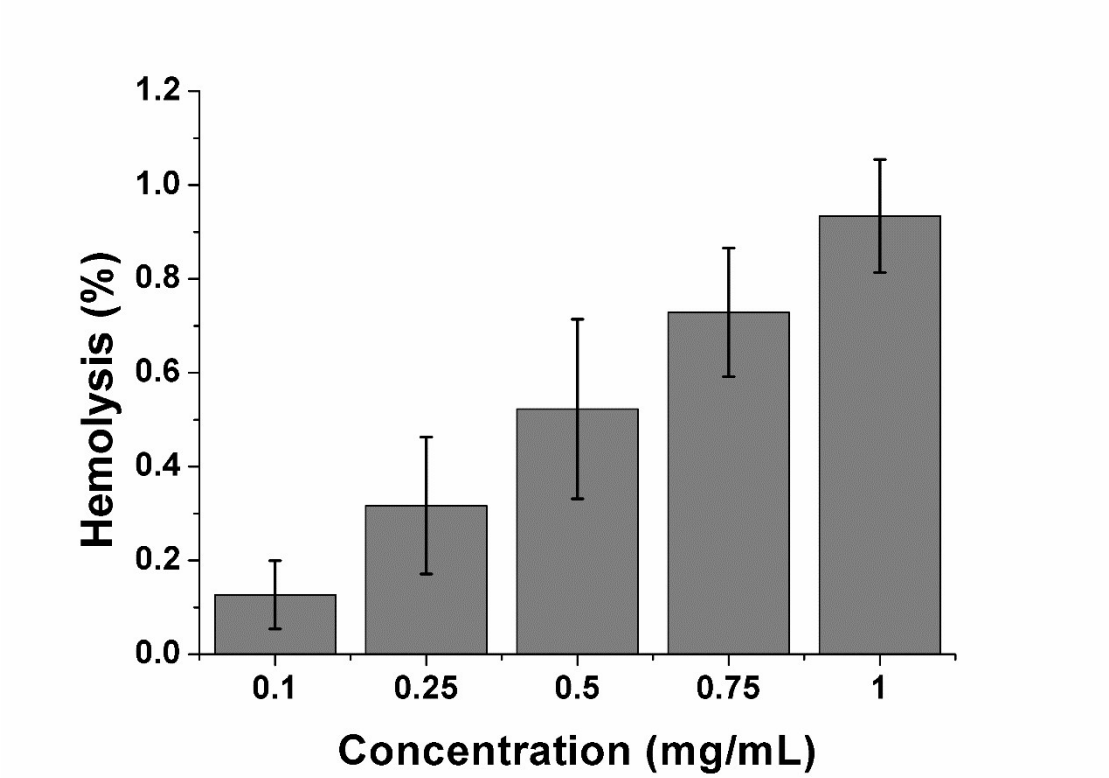


Fig. S4. Hemolysis of AC at different concentrations. Results were expressed as mean ± S.D. (n = 3).

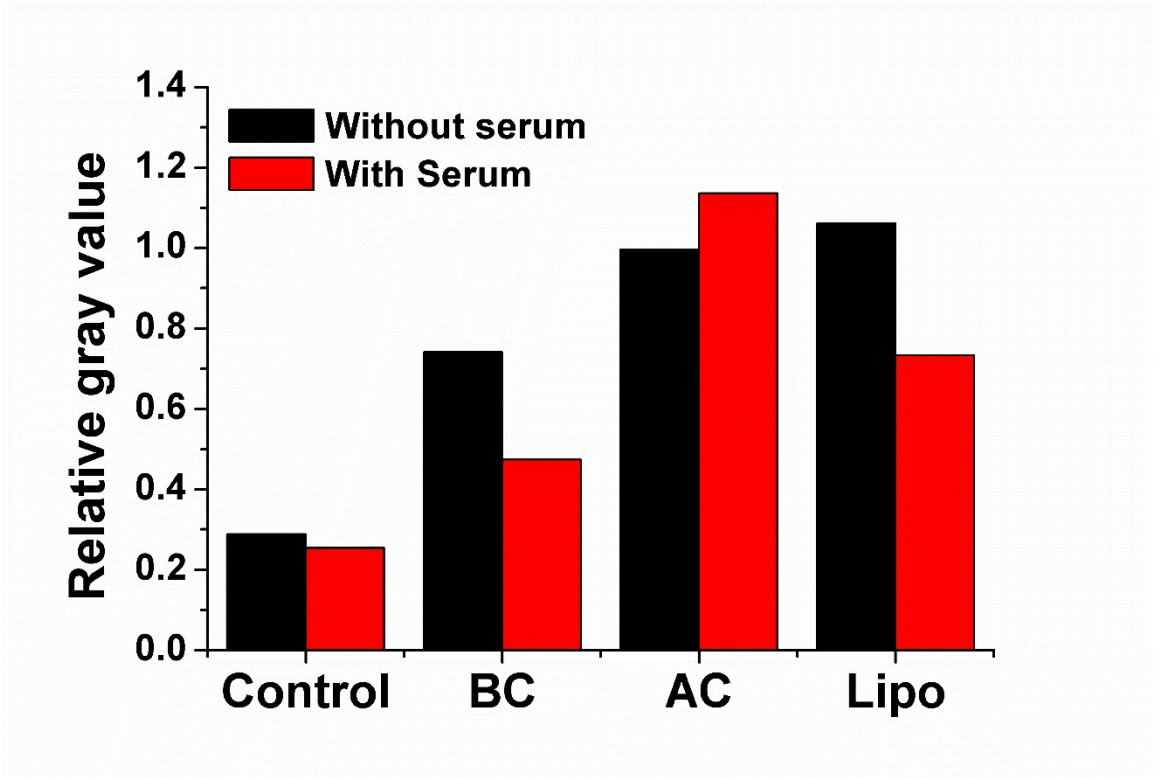


Fig. S5. The semi-quantitative analysis of gray value in Fig. 3C.

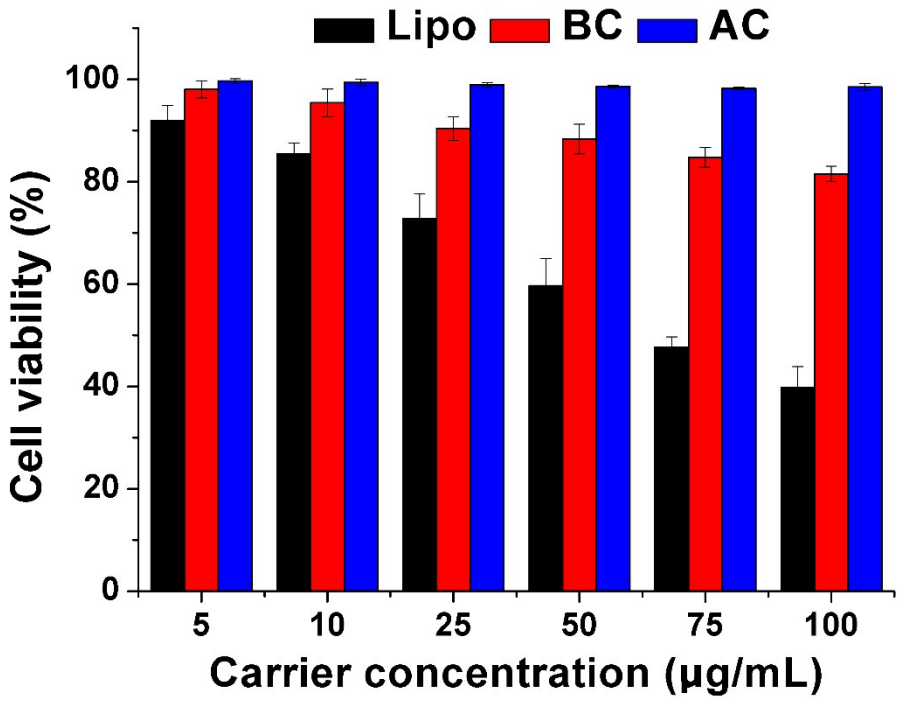
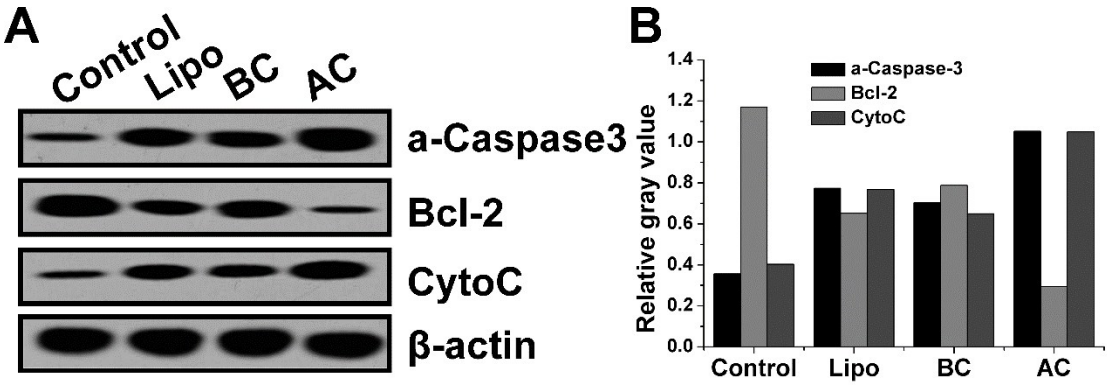


Fig. S6. Cell viabilities of MCF-7 cells incubated with different formulations containing non-therapeuti



c pEGFP-C3 at different nanoparticle concentrations (5-100 μg/mL) for 48 h, respectively. Results were expressed as mean ± S.D. (n = 3).

Fig. S7. The expression level of apoptosis related proteins (activated Caspase3,Bcl-2 and Cytochrome C) after treated with different formulations. (A) Optical image and (B) semi-quantitative analysis of gray value.

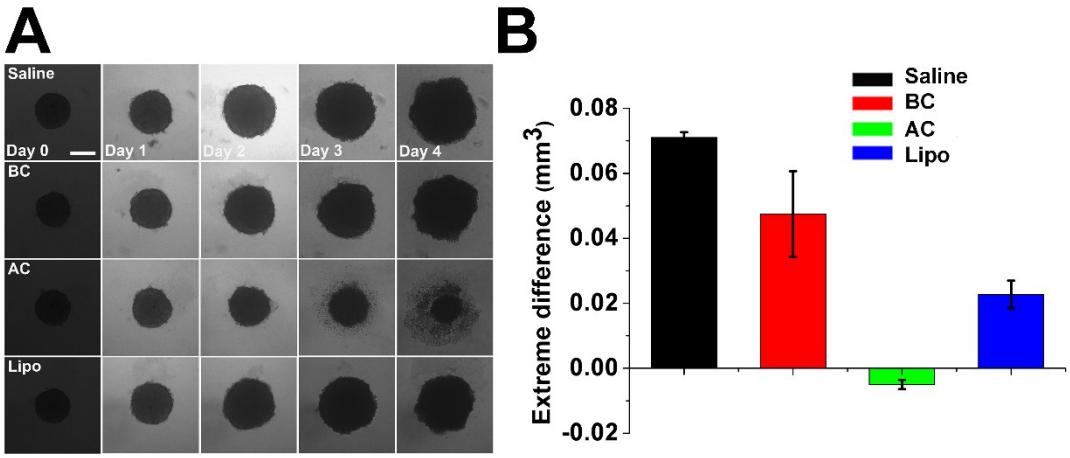


Fig. S8. (A) Optical images of MCTS after treated with saline, BC, AC or Lipo. (B) Extreme difference of MCTS (between Day 0 and Day 4) treated with different formulations. Scale bar: 200 μm. Results were expressed as mean ± S.D. (n = 3).

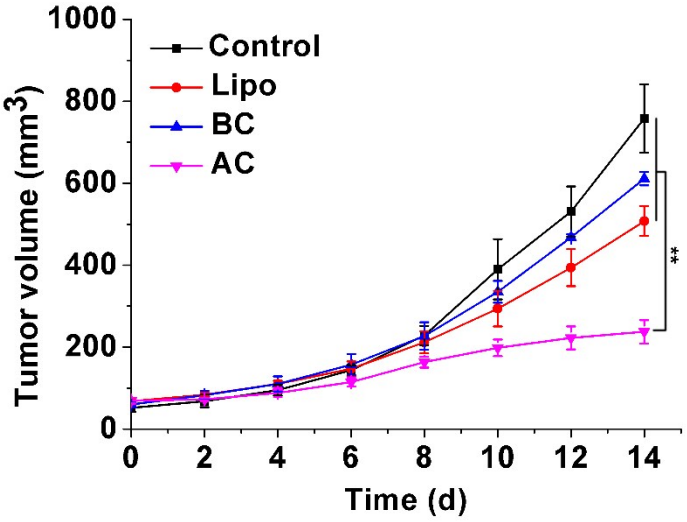


Fig. S9. The tumor volume analyses of HepG2 tumor-bearing BALB/c nude mice after intravenous administration of different formulations. The measurement of tumor volumes was repeated every 2 days for two weeks. Dose: 2.5 mg DNA/kg (n = 3). Results were represented as mean ± S.D. $^{**}P<0.01$.

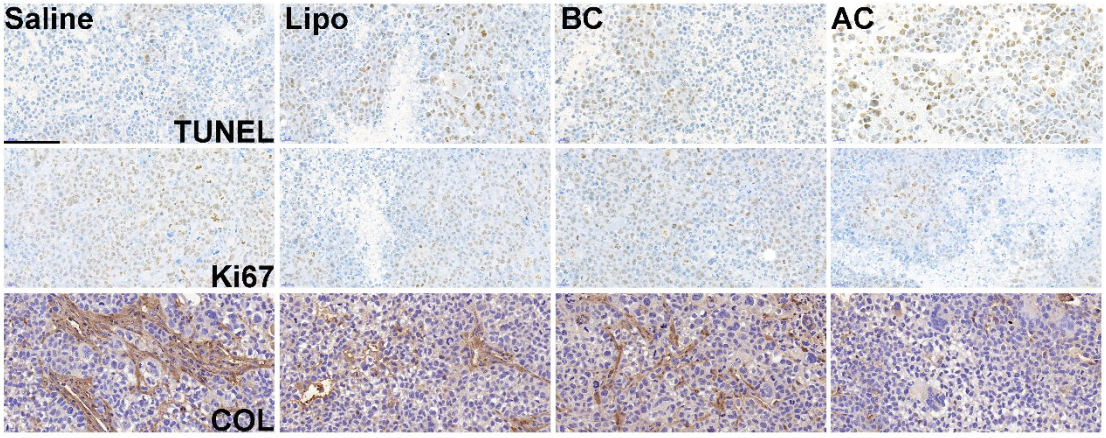


Fig. S10. Representative images of *ex vivo* tumor sections (from HepG2 tumors) assayed by immunohistology using TUNEL, Ki67 and COL antibodies. Scale bar: 100 μm.

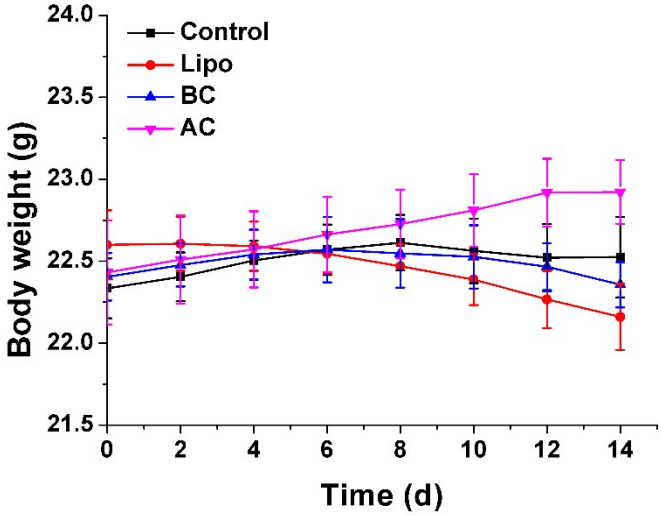


Fig. S11. The body weight analysis of MCF-7 tumor-bearing BALB/c nude mice after intravenous administration of saline, Lipo, BC or AC, respectively. The measurement of body weight was repeated every 2 days for two weeks. Dose: 2.5 mg DNA/kg. Results were expressed as mean ± S.D. (n = 6).

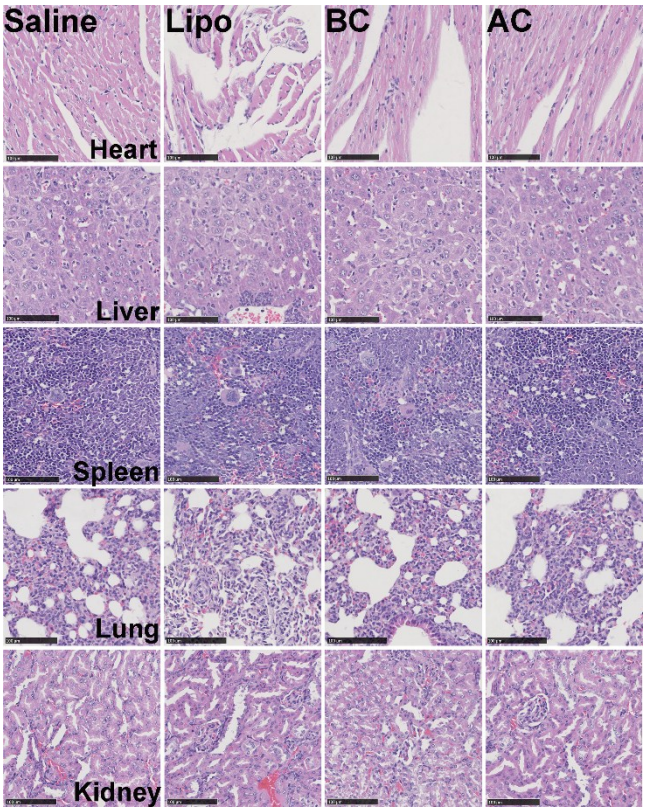


Fig. S12. Representative images of main organs (Heart, Liver, Spleen, Lung and Kidney) separated from mice after HE staining. Scale bar: 100 μm.

Reference

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