

Supplementary Information

A novel design of polynuclear co-delivery system for safe and efficient cancer therapy

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Materials and methods

Materials

Agmatine, 1,6-hexanediamine, DL-Dithiothreitol (DTT), Amiloride, Chlorpromazine and N,N'-bis(acryloyl)cystamine were provided from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS), LysoTracker® Red DND-99 and Lipofectamine 2000® (Lipo 2000) were purchased from Invitrogen™ (Thermo Fisher Scientific, USA). Trypsin with EDTA solution (0.25 %) and Trypsin without EDTA solution were obtained from Gibco (Burlington, ON, Canada). RPMI 1640, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Hoechst 33342, 4',6-diamidino-2-phenylindole (DAPI) and the Annexin V-FITC Apoptosis Detection Kit were purchased from KeyGEN Biotech (Nanjing, China). Bovine serum albumin (BSA, Mw = 6.6 kDa), 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl) and N-Hydroxysuccinimide (NHS) were purchased from HEOWNS (Tianjin, China). Phosphotungstic acid, Me-β-CD, sodium dodecyl sulfate (SDS), Urea, Tween 20, NaN₃ and Triton X-100 were purchased from Sinopharm chemical Reagent Co., Ltd. (Shanghai, China). PLGA (lactide:glycolide ratio 50:50; Mw, 5 kDa) (PLGA) was purchased from Daigang Biomaterial Co., Ltd. (Jinan, China). And 1,2-distearoyl-snglycero-3-phosphoethanolamine-N-[carboxy(polyethylene glycol)2000] (DSPE-PEG) were purchased from A.V.T, Inc. (Shanghai, China). Doxorubicin hydrochloride (DOX·HCl) was purchased from China Langchem Inc. Co. Ltd. Indotricarbocyanine (DiR) was purchased from Fanbo Biochemicals. All other chemicals and reagents were of analytical grade.

Bcl-2 siRNA (siBcl-2), scrambled siRNA (siScr), FAM-siRNA obtained from Guangzhou RiboBio Co., LTD. (Guangzhou, China). The β-tubulin (9F3) rabbit mAb, HRP-linked antirabbit IgG, and Bcl-2 specific antibody (50E3) rabbit mAb were purchased from Cell Signaling Technology. The siRNA sequences were as follows: siBcl-2: 5'-CCGGGAGAUAGUGAUGAAGdTdT-3', siScr: 5'-UUCUCCGAACGUGUCACGUDdTdT-3'. The

primers for the quantitative polymerase chain reaction (qPCR) were obtained from Genewiz, Inc. (Suzhou, China). The sequences of the primers were as follows: Bcl-2 primers (forward: 5'-AACATCGCCCTGTGGATGAC-3', reverse: 5'-AGAGTCTTCAGAGACAGCCAGGAG-3'), β -actin primers (forward: 5'-CGCGAGAAGATGACCCAGATC-3', reverse: 5'-CATGAGGTAGTCAGTCAGGTCCC-3').

Cell culture

The cancer cells (MCF-7, SMMC7721, A549 cells) were provided by the cell library of the Chinese Academy of Sciences. The cell lines were cultured in RPMI 1640 (KeyGEN, Nanjing, China). The cell culture media were supplemented with 10 % FBS, 100 $\mu\text{g}\cdot\text{mL}^{-1}$ streptomycin and 100 $\text{U}\cdot\text{mL}^{-1}$ penicillin. Cells were grown at 37 °C in a humidified atmosphere with 5 % CO_2 .

Animals

The female athymic nude mice used in *in vivo* studies weighed between 18-20 g and were 6 to 8 weeks old. They were obtained by Jiangsu Experimental Animal Center (Yangzhou, Jiangsu, China). All animals were housed under pathogen-free conditions during the studies, and received care in compliance with the institutional guidelines outlined in the Guide for the Care and Use of Laboratory Animals. All procedures were approved by the Institutional Animal Ethical Committee of China Pharmaceutical University. To set up the tumor xenograft model, MCF-7 cells (1×10^7) were suspended in 100 μL of PBS and were administered by subcutaneous injection into the right armpit of the mice.

Synthesis of polyagmatine

The poly[N,N'-bis(acryloyl)cystamine-*alt*-agmatine) (PCA) was synthesized by the Michael reaction according to a previously described method.¹ Briefly, 3.6 mmol of N,N'-cystaminebisacrylamide (CBA) and 3 mmol of agmatine hydrochloride was dissolved in 1 mL of mixed solvent (methanol: water = 3: 1, v/v). Then, 22.2 mg of calcium chloride and 6 mmol of triethylamine were added. And then the reaction was performed at 70 °C under argon for 3 days. Finally, 2.16 mmol of triethylamine was added and the reactions continued under argon for 1 day. The obtained crude product was purified through dialysis (MWCO=3.5 kDa) for another 2 days and dried by lyophilization. The structure of PCA was verified by ¹H NMR spectroscopy (Bruker AV-300, USA). The molecular weight of PCA was measured by gel permeation chromatography (GPC) (LC-20 AB, Shimadzu, Japan).

The control polymer poly[N,N'-bis(acryloyl)hexanediamine-*alt*-agmatine) (PHA) was synthesized with N,N'-bis(acryloyl)hexanediamine instead of CBA by the method described above.

Synthesis of copolymer

The poly(lactic-co-glycolic acid)-*b*-poly[N,N'-bis(acryloyl)cystamine-*alt*-agmatine]] (PLGA-PCA) was synthesized by a simple amidation reaction.² Briefly, PCA (728 mg) and PLGA (666 mg) were dissolved in DMSO (8 mL), and then 75 mg of 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl), 45 mg of N-Hydroxysuccinimide (NHS) and 54 µL of triethylamine were added. The reaction was stirred for 2 days. The final product was purified through dialysis (MWCO=14 kDa) for 2 days against deionized water and dried through lyophilization. The structure of PLGA-PCA was verified by ¹H NMR spectroscopy (Bruker AV-300, USA), and then the polymer was stored at -20 °C until further use.

The poly(lactic-co-glycolic acid)-*b*-poly[N,N'-bis(acryloyl)hexanediamine-*alt*-agmatine]] (PLGA-PHA) was synthesized with PHA instead of PCA by the method described above.

Acid-base titration

The buffering capacity of the PLGA-PCA and PLGA-PHA copolymers was determined by acid-base titration over a pH range of 10.0 to 3.0 according to a previously reported method.² Briefly, PLGA-PCA (2 mg), and PLGA-PHA (2 mg) and PEI 25K (2 mg) were each dissolved in 10 mL of 0.15 M NaCl solutions. The solutions were adjusted to an initial pH of 10.0 using 0.1 M NaOH and then titrated with a 0.1 M HCl solution until the pH dropped to 3.0, as recorded by a pH meter (Sartorius, Germany). Meanwhile, 0.15 M NaCl solutions were titrated in the same way (as controls).

Preparation and characterization of PLGA-PCA NPs/siRNA

The binding degree of siRNA by PLGA-PCA NPs was examined using agarose gel electrophoresis. PLGA-PCA NPs/siRNA polyplexes were prepared at different weight ratios of polymer/siRNA, loaded onto a 2 % agarose gel containing GelRed staining and run at 110 V in conventional TAE buffer for 20 min. The gel was then imaged under UV illuminator (Tanon 1600, China).

PLGA-PHA NPs/siRNA polyplexes were prepared by above same method.

Encapsulation of DOX and drug release

PLGA-PCA (10 mg) and DOX (2.0 mg) were dissolved in DMSO (100 μ L), and the above solution was added dropwise into deionized water (2 mL) to form nanoparticles under stirring. Then the nanoparticles was dialysed (MWCO=5 kDa) against deionized water for 1 day. The drug loading and encapsulation efficiency were measured by UV-vis spectrophotometry at 480 nm. The amount of drug release through dialysis was measured by UV-vis spectrophotometry (480 nm) at pH values of 7.4 and 5.0 at 37 °C.

DOX/PLGA-PHA were prepared by above same method.

Preparation of polynuclear nanoparticle

The DOX loading into the PLGA-PCA NPs (or PLGA-PHA NPs) was first prepared by method described above. Then, the LBC/DOX/siBcl-2 (or LBH/DOX/siBcl-2) polynuclear nanoparticles were formed by mixing the siBcl-2 with isovolumetric LBC/DOX NPs (or LBH/DOX NPs) solution through vortexing and further incubated at room temperature for 15 min. Then, the polynuclear nanoparticles were obtained by adding BSA/lipid-PEG mixture solution into above-described solution and incubating at room temperature for 15 min.

The mean hydrodynamic size of the LBC/DOX/siBcl-2 NPs was measured with a Zeta Plus particle size analyzer (Brookhaven Instruments, USA). The micrographs of the LBC/DOX/siBcl-2 polynuclear nanoparticle (w/w, PLGA-PCA:siBcl-2 = 10:1, DSPE-PEG:BSA:PLGA-PCA NPs = 0.5:0.5:1) were observed by transmission electron microscopy (TEM, Hitachi H-7650, Japan). Nanoparticles for TEM were stained with 1 % phosphotungstic acid and imaged using Hitachi an H-7650 microscope (Hitachi Company) operating at 80 kV.

Cytotoxicity of blank polynuclear nanoparticle

The cytotoxicities of blank polynuclear nanoparticles against MCF-7, SMMC7721, and A549 cells were investigated. The cells were inoculated into 96-well plate (1×10^4 cells/well). After incubating 24 h, 200 μ L of 10 % FBS-contained media were discarded, 100 μ L of FBS-free media of PEI25K, LBH NPs and LBC NPs were added at predetermined concentration (1-200 μ g/mL) and further incubated for 24, 72 h. Afterward, 20 μ L of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) solutions were added and further incubated for 4 h at 37 °C in the dark. After removing each media carefully, 150 μ L of DMSO was added to dissolve the formazan crystal. Then the absorbance of solution at 490 nm was measured by a Microplate Reader (Thermo, USA). No treated cells are as a blank.

Hemolytic activity test

The hemolytic activity was assessed as a previous reported.³ Briefly, 200 μ L of fresh diluted mouse blood was added to 800 μ L PBS (pH 7.4) containing various amounts of PEI25K, LBH NPs and LBC NPs (0.05, 0.1, 0.25, 0.5, 1, 1.5 mg) at 37 $^{\circ}$ C for 3 h. 200 μ L of blood mixed with 800 μ L of PBS was used as the negative control, while PBS containing Triton X-100 served as the positive control. After 4 h, all sample solutions were centrifuged at 2000 rpm for 5 min, 200 μ L supernatant was seeded in 96-well plates and analyzed for released hemoglobin at 540 nm using Thermo Multiskan GO (Thermo Scientific, USA). Percentage hemolysis was measured using the following formula:

$$\text{Relative rate of hemolysis (\%)} = \left[\frac{(A_s - A_n)}{(A_p - A_n)} \right] \times 100 \%$$

where A_s , A_n and A_p represent the absorbance of samples, negative and positive controls, respectively.

Cytotoxicity

The cytotoxicity was investigated by MTT assay to MCF-7, SMMC7721, A549 cells. 1.0×10^4 cells per well were seeded in 96-well plates and attached for 24 h. Then they were treated with different concentrations of DOX, LBC/siBcl-2, LBH/DOX/siBcl-2, LBC/DOX/siScr, LBC/DOX/siBcl-2, the mixture of LBC/DOX and LBC/siBcl-2. No treated cells were served as a control. After 24 and 72 h, the media were removed, each well was washed twice with cold PBS, and then 20 μ L MTT solutions (0.5 mg/mL) was added. The cells were further incubated for 4 h at 37 $^{\circ}$ C. After removing each media carefully, 150 μ L of DMSO was added to dissolve the formazan crystal. The absorbance at 490 nm was recorded by a Microplate Reader (Thermo, USA).

Cell apoptosis

MCF-7 cells were treated with free DOX, LBC/siBcl-2, LBC/DOX, LBH/DOX/siBcl-2, the mixture of Lipo2000/siBcl-2 and DOX, and LBC/DOX/siBcl-2 for 2 days. No treated cells are as

a control. The treated cells were collected and washed two times with cold PBS for quantitative determination of apoptosis. And then the cells were stained with both FITC Annexin V and propidium iodide (PI), these samples were analyzed by Fluorescence Activated Cell Sorting (FACS), and 1×10^4 events per sample were counted. The apoptosis rate was denoted as the percentage of Annexin V-FITC positive cells.

In vitro siBcl-2 transfection

The Bcl-2 mRNA transcription was evaluated by the quantitative real-time polymerase chain reaction (qRT-PCR) as a previous reported.³ MCF-7 cells (3×10^5) were seeded in 6-well plates for 24 h, and different preparations were added and incubated for 24 h. *In vitro* Bcl-2 transfection, total RNA was isolated using the RNeasy[®] mini-kits (Qiagen, USA) according to the manufacturer's protocol.

Western blotting

The protein expression in MCF-7 cells dealt with different preparations was determined using Western blotting analysis as a previous reported.³ MCF-7 cells cultured in 6-well plates were treated with different preparations for 2 days. Then cells were collected and processed according to traditional WB protocol. Finally, protein bands were visualized by enhanced chemiluminescence (ECL) detection reagents, and captured by Tanon-4200 Gel Imaging System (Tanon Science, China).

Antitumor efficacy in vivo

Antitumor effect *in vivo* was investigated on MCF-7 tumor-bearing nude mice when the tumor grew $\sim 100 \text{ mm}^3$. The mice were weighed and separated into 6 groups by random (n=4): (i) control group (PBS); (ii) free DOX; (iii) LBC/DOX; (iv) LBC/siBcl-2; (v) LBH/DOX/siBcl-2 and (vi) LBC/DOX/siBcl-2. The dosage of siBcl-2 (or siScr) and DOX were set to 0.2 mg/kg and 2.5 mg/kg, respectively. The mice were administrated once every three days over a 12-

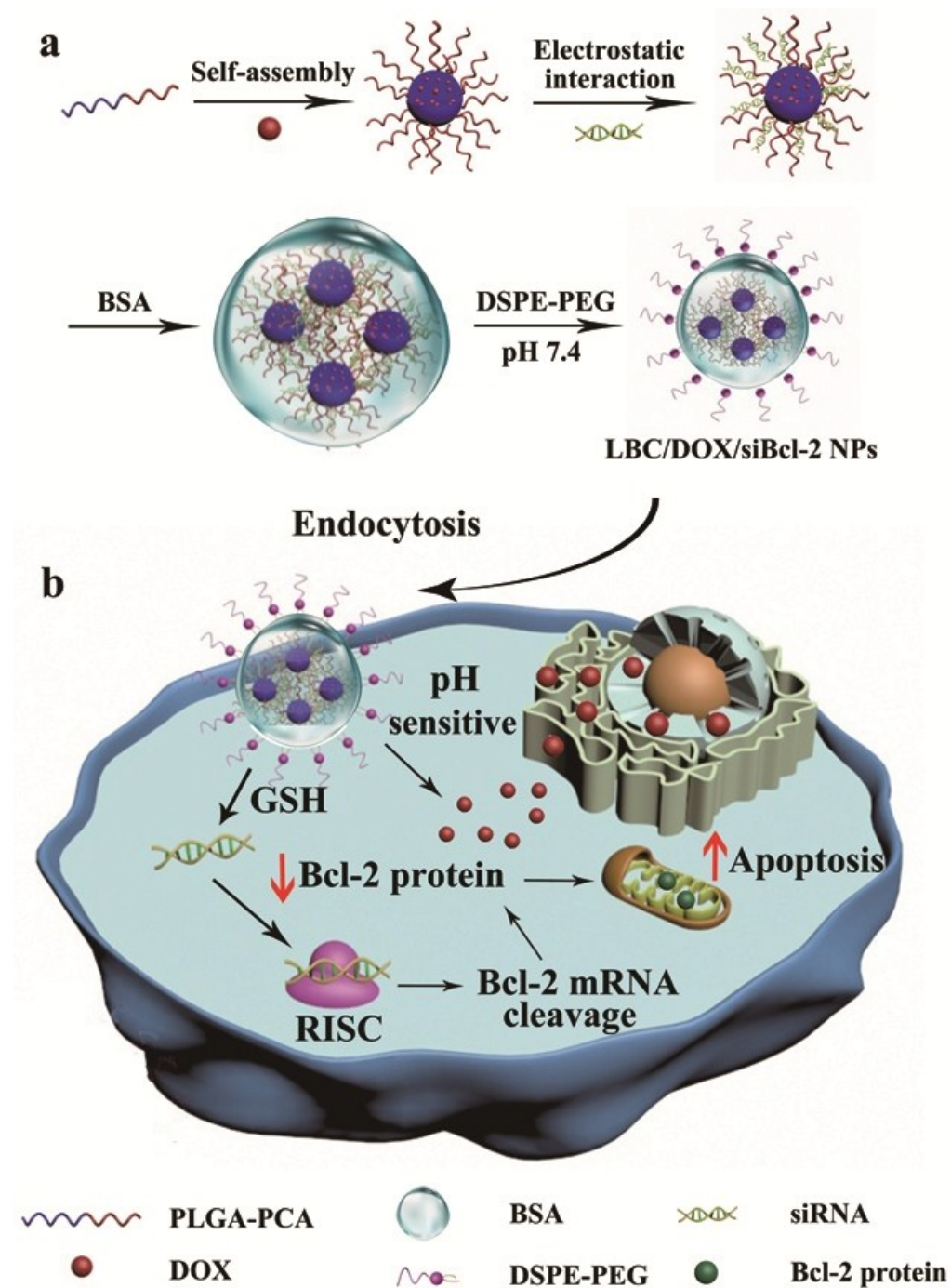
day therapeutic period. The mice weight and tumor volume were determined during duration of therapy. The tumor volume (V_T) is calculated according to the formula: $V_T = (\text{length} \times \text{width}^2)/2$. At day 24, All the mice were sacrificed and dissected. All of the organs and tumor tissues were collected for hematoxylin-eosin (H&E) staining and transferase-mediated dUTP nick end-labeling (TUNEL) staining.

Biodistribution

LBC/DiR nanoparticles were prepared by the same method described above. To determine the biodistribution, MCF-7 tumor-bearing nude mice were injected free DiR and LBC/DiR at a dose of 1.0 mg/kg of DiR via tail vein. The real-time distribution of free DiR and LBC/DiR were evaluated using an *in vivo* Imaging System (IVIS Spectrum, PerkinElmer, USA) equipped with DiR filter sets (excitation/emission, 720/790 nm).

Statistical analysis

Quantitative results were reported as the mean \pm standard deviation (SD). The statistical difference between individual groups was evaluated by oneway ANOVA.



Scheme S1. Schematic illustration of the preparation (a) and synergistically anticancer study of LBC/DOX/siBcl-2 polynuclear nanoparticle (b).

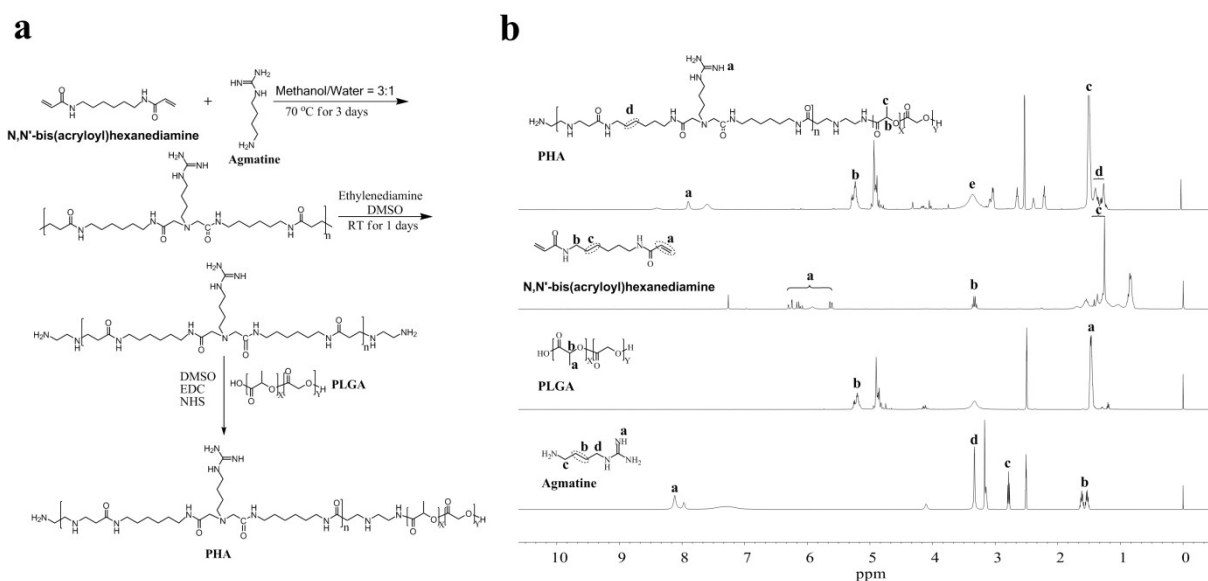


Fig. S1. Synthesis scheme (a) and ^1H -NMR (b) of PHA.

The ^1H NMR spectra of PLGA-PCA, PLGA, CBA and agmatine in d_6 -DMSO confirmed their structures. For CBA, the typical peaks representing terminated double bonds disappeared at $\delta = 5.5$ to 7.0 ppm in the PLGA-PCA copolymer. For PLGA, the peaks at $\delta = 5.0$ to 5.25 ppm demonstrated the existence of methine protons. For agmatine, the peaks at $\delta = 8.0$ to 8.25 ppm demonstrated the existence of guanidyl protons. The special signals of PLGA and agmatine were presented in PLGA-PCA at the corresponding chemical displacement, indicating that PCA was successfully conjugated with PLGA. As shown in Fig. S1b, the control PLGA-PHA was also successfully characterized by ^1H NMR spectra.

Table S1. GPC analysis of PCA and PHA copolymers.

	Mw (kDa)	Mn (kDa)	PDI
PCA	7.28	5.35	1.36
PHA	8.42	5.97	1.41

Table S2. Drug loading efficiency and drug encapsulation efficiency of DOX-loaded nanoparticles

Copolymer	*EE (%)	*LE (%)
PLGA-PCA	52.3	10.8
PLGA-PHA	46.1	9.6

*EE: Drug Encapsulation Efficiency.

*LE: Drug Loading Efficiency.

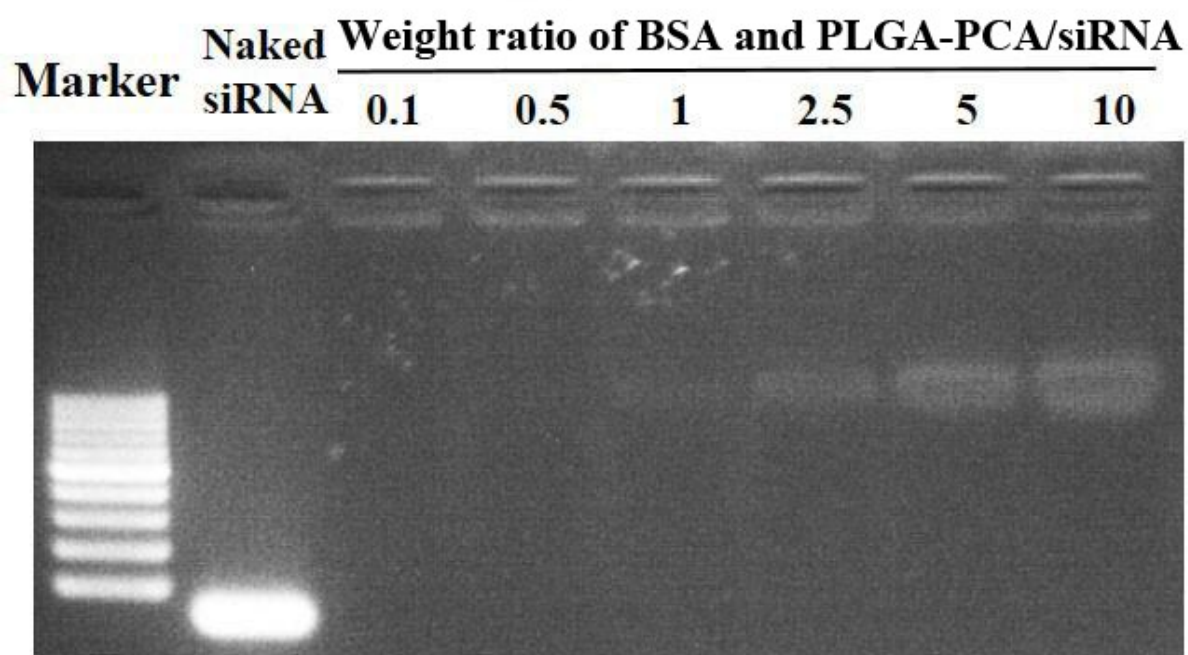


Fig. S2. Agarose gel electrophoresis assay of the competition of BSA and PLGA-PCA/siRNA nanoparticles.

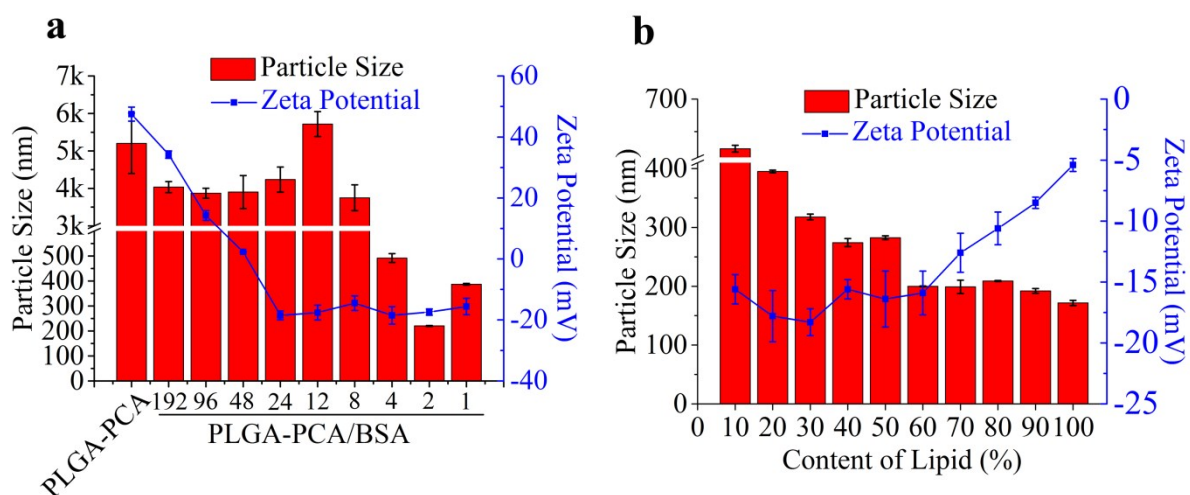


Fig. S3. *In vitro* characterization of materials/siRNA complexes. a) Hydrodynamic size and zeta potential of nanoparticles formed by PLGA-PCA/siRNA (10:1, wt:wt), variable weight ratios of BSA and lipid (lipid:PLGA-PCA=0.5:1, wt:wt) (n=3, mean \pm SD). b) Hydrodynamic size and zeta potential of nanoparticles formed by PLGA-PCA/siRNA (10:1, wt:wt), variable weight ratios of lipid and BSA (BSA:PLGA-PCA= 0.5:1, wt:wt) (n = 3, mean \pm SD).

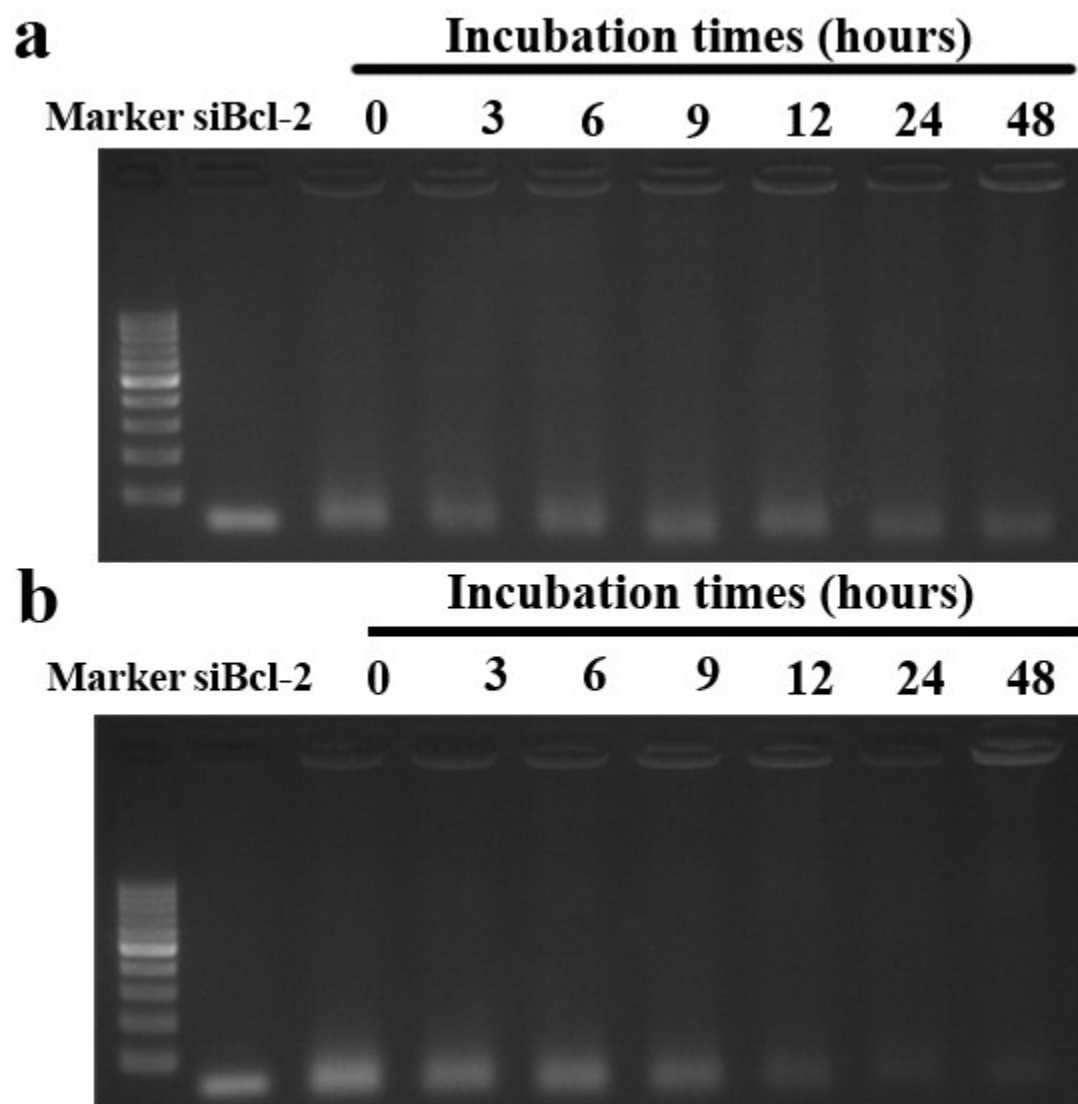


Fig. S4. Electrophoresis bands of siRNA in (a) LBC/siRNA complexes and (b) LBH/siRNA complexes after being incubated in 50 % serum for different time periods.

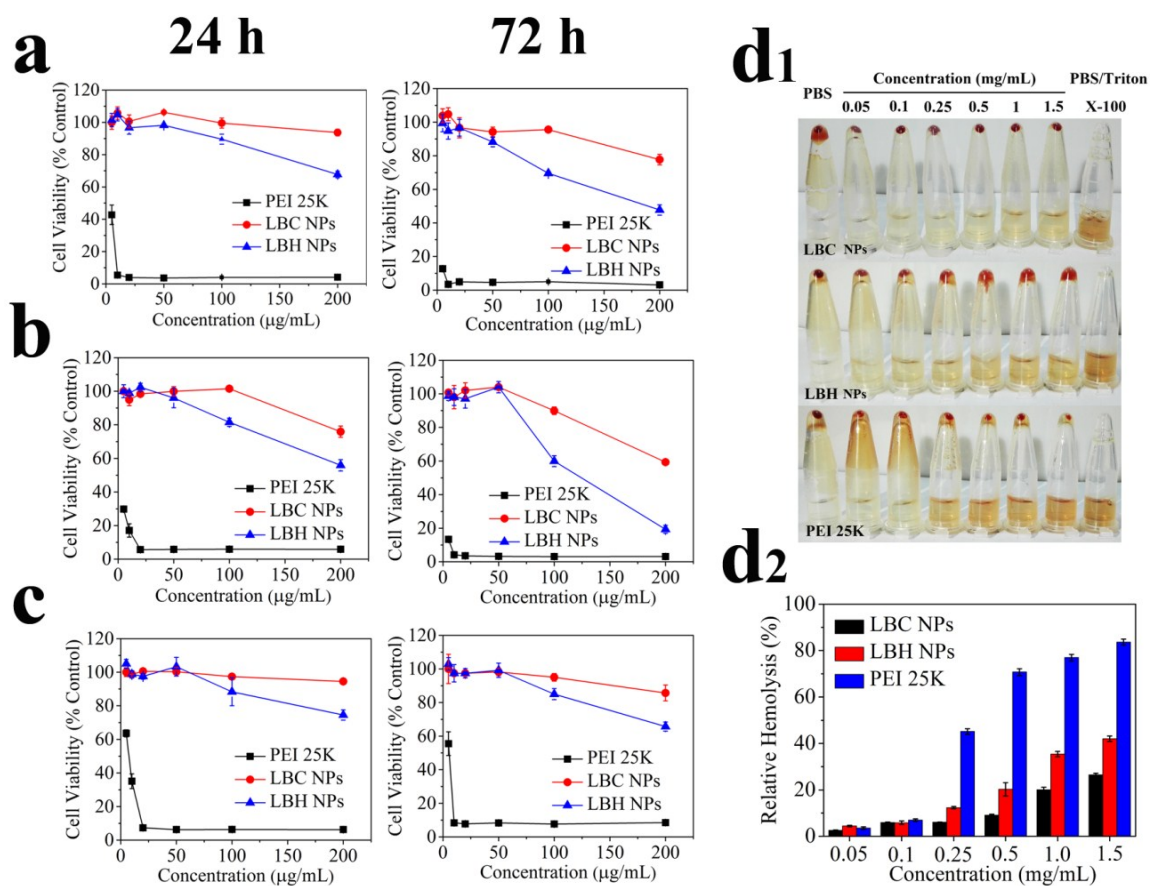


Fig. S5. Biocompatibility evaluation. Cell viability of LBC and LBH on SMMC-7721 (a), A549 (b), MCF-7 (c) cells for 24 h and 72 h. (d1) Comparison of LBC and LBH on the hemolytic activity by visual observation. (d2) Relative hemolytic activity of LBC and LBH at various concentrations.

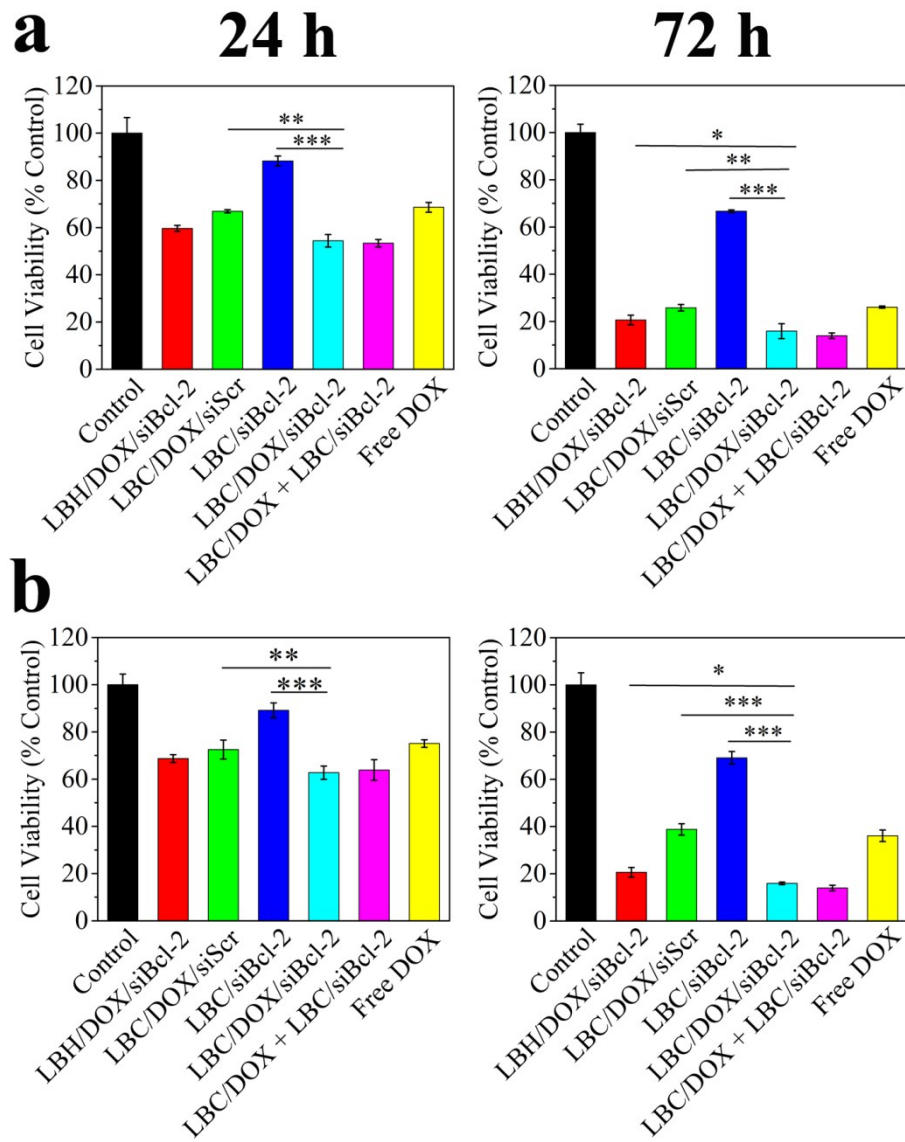


Fig. S6. Inhibiting effects of the co-delivery of siBcl-2 and DOX by LBC/DOX/siBcl-2 nanoparticles on the proliferation of SMMC7721 (a) and A549 (b) cells at two different time points (24 and 72 h). Asterisks (*) denote statistically significant differences calculated by one-way ANOVA test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

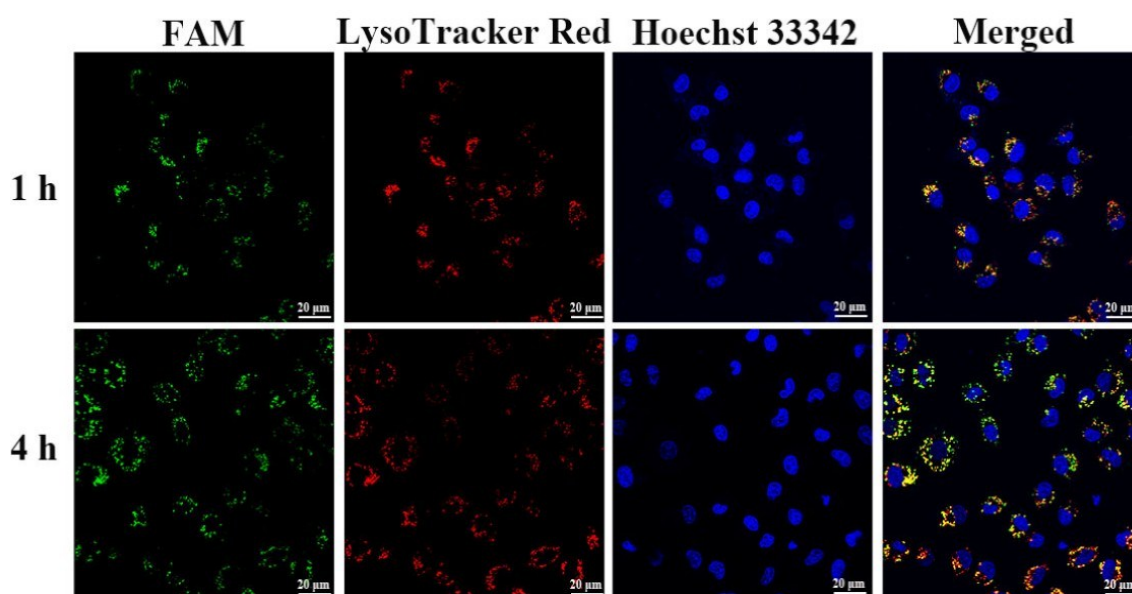


Fig. S7. CLSM determined intracellular localization of siRNA in MCF-7 cells after incubation for 1h and 4 h. Scale bar represented 20 μm. Lysosomes were labelled with LysoTracker Red DND-99 (red), the nuclei were labelled with Hoechst 33342 (blue) and FAM-siRNA (green).

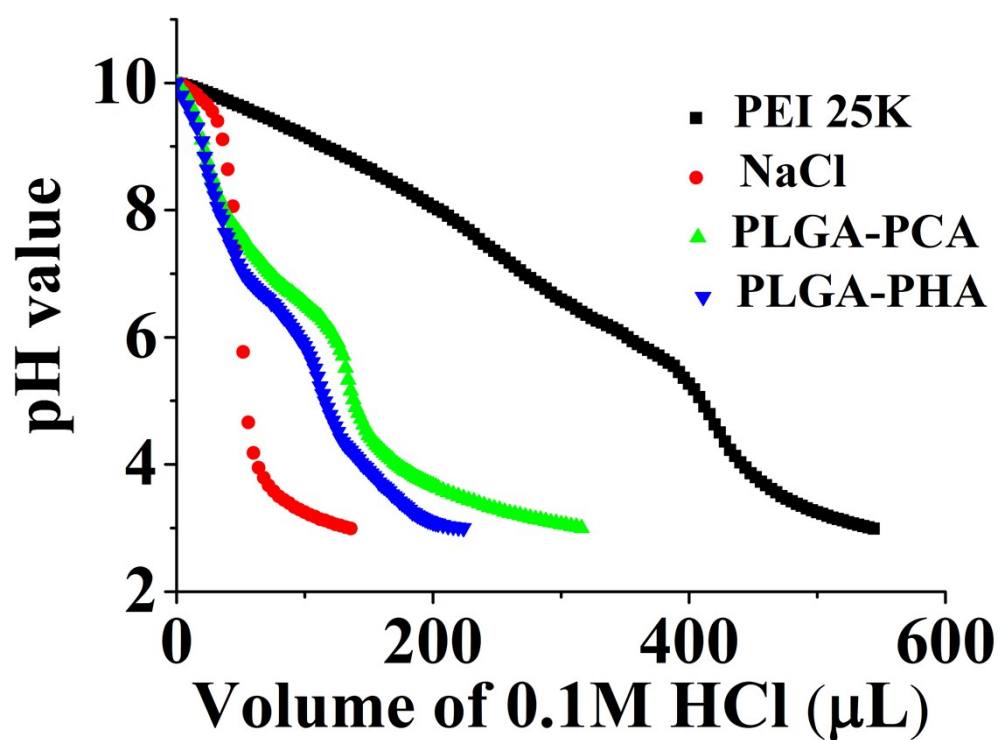


Fig. S8. The buffering capacity of various materials.

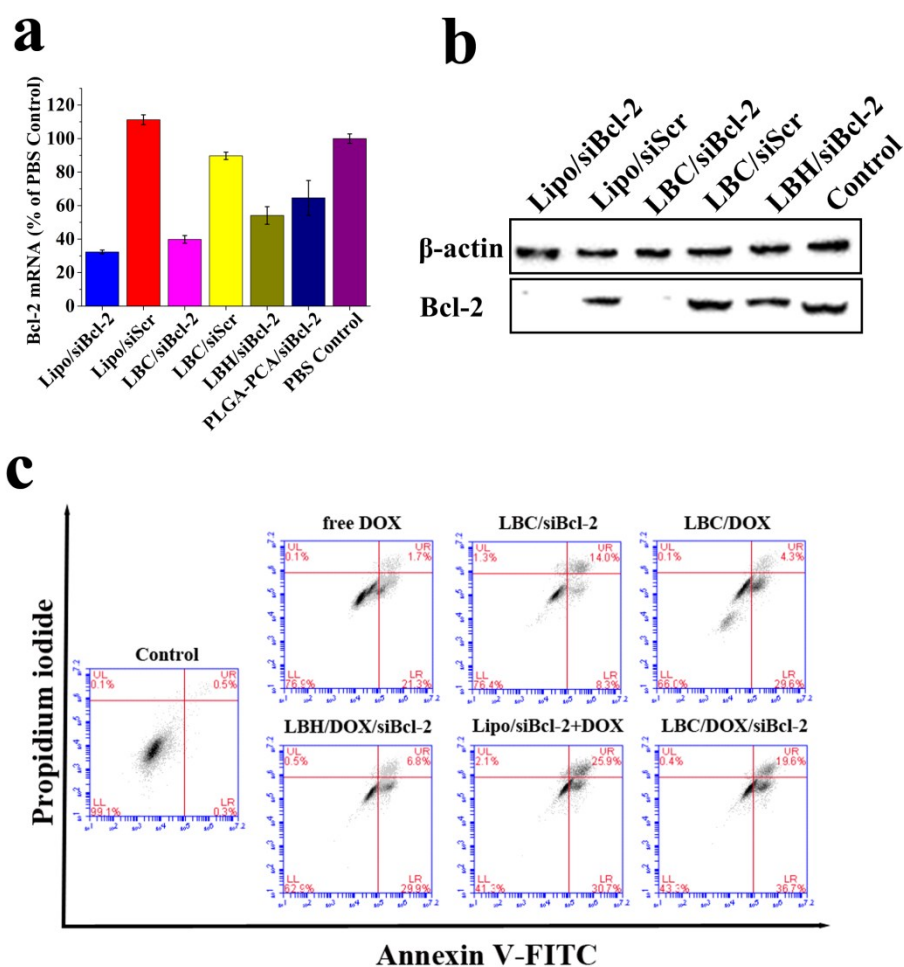


Fig. S9. (a) Bcl-2 mRNA expression determined by qRT-PCR. (b) Representative Bcl-2 protein expression determined by western blotting analysis. (c) Cell apoptosis after transfection with different formulations determined by the Annexin V-FITC/PI assay. The viable, early apoptotic, and late apoptotic cell populations (%) are shown in the lower left, lower right, and upper right quadrants, respectively.

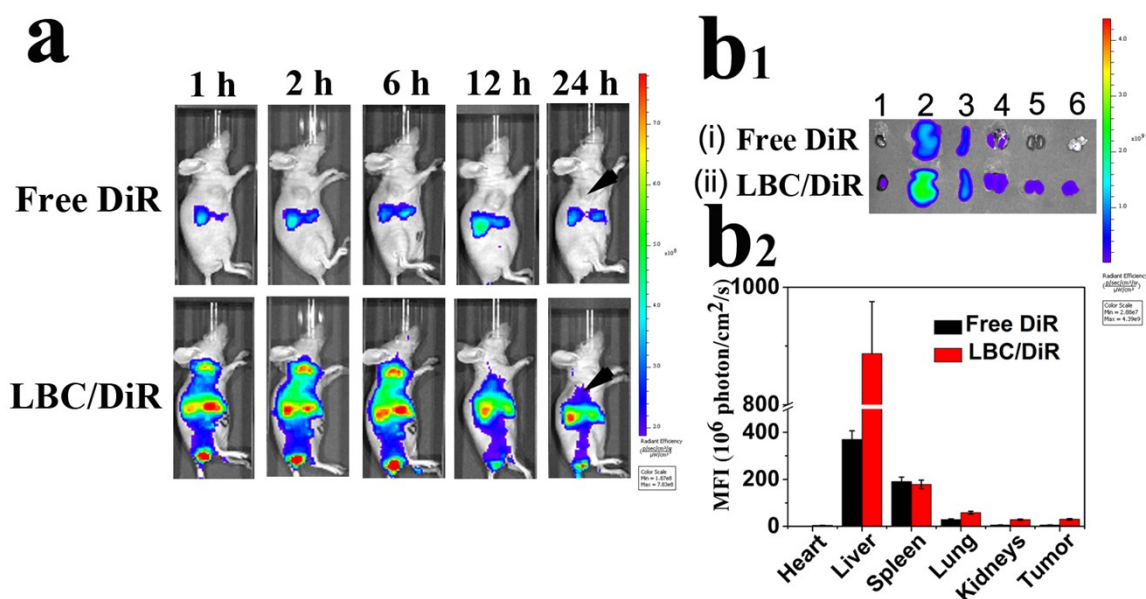


Fig. S10. (a) Biodistribution of DiR monitored using the in vivo imaging after intravenous injection of different formulations into the MCF-7 tumor-bearing mice. The black arrows indicate the region of the tumor. (b) Accumulation of DiR in the tumor and different organs detected using the ex vivo imaging at 24 h post-injection of different formulations: (i) DiR; (ii) LBC/DiR. (1) heart; (2) liver; (3) spleen; (4) lung; (5) kidney; (6) tumor. (c) The corresponding mean fluorescence intensity of tumor and major organs at 24 h postinjection.

Reference

1. J. Wu, N. Kamaly, J. Shi, L. Zhao, Z. Xiao, G. Hollett, R. John, S. Ray, X. Xu and X. Zhang, *Angew. Chem. Int. Ed.*, 2014, **53**, 8975-8979.
2. R. L. Xie, Y. J. Jang, L. Xing, B. F. Zhang, F. Z. Wang, P. F. Cui, M. H. Cho and H. L. Jiang, *Int. J. Pharm.*, 2015, **478**, 19-30.
3. B. F. Zhang, L. Xing, P. F. Cui, F. Z. Wang, R. L. Xie, J. L. Zhang, M. Zhang, Y. J. He, J. Y. Lyu, J. B. Qiao, B. A. Chen and H. L. Jiang, *Biomaterials*, 2015, **61**, 178-189.