Supporting Information

A Modular Assembly pH-Sensitive Charge Reversal siRNA Delivery System

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Synthesis of amino-acid based cationic lipid

*Synthesis of 1,5-dihexadecyl-L-glutamate (**G2C$_{14}$**):*

L-Glutamic acid (11.8 g, 80.2 mmol) and *p*-toluene sulfonic acid (TsOH) (18.3 g, 96.2 mmol) were dissolved in 350 mL of methylbenzene, and refluxed for 1 h at 110 °C. Tetradecylalcohol (47.8 g, 176.7 mmol) was added to the solution, followed by stirring for 12 h under reflux. The reaction mixture was evaporated with vacuum distillation to remove methylbenzene and then dissolved in dichloromethane (CH_2Cl_2). The CH_2Cl_2 solution was washed successively with 5% (w:v) sodium carbonate solution (100 mL × 2) and distilled water (100 mL × 1) and then evaporated. **G2C$_{14}$** was recrystallized from 100 mL of methanol (MeOH) to obtain a white powder with a yield of 55.4%.

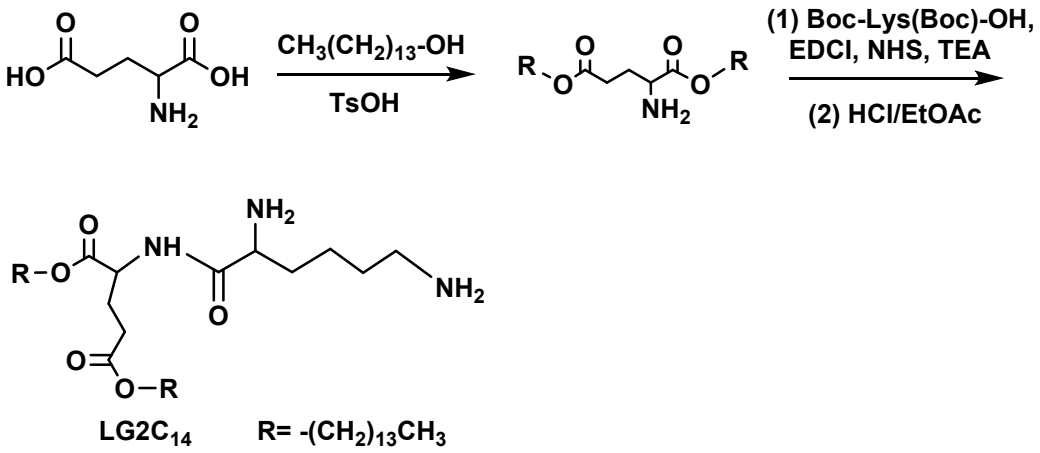
^{1}H-NMR (300 MHz, $CDCl_3$): δ 4.11 (t, J = 6.8 Hz, 2H, $COOCH_2$), 4.07 (t, J = 6.8 Hz, 1H, $COOCH_2$), 3.50 (dd, J = 5.4, 8.0 Hz, 1H, NH_2CH), 2.46 (t, J = 7.5 Hz, 2H, CH_2CO), 2.12-2.05 (m, 1H, NH_2CHCH_2), 1.90-1.79 (m, 2H, NH_2CHCH_2), 1.65-1.54 (m, 4H, $COOCH_2CH_2$), 1.31-1.26 (m, 44 H, $CH_{2\ (myristoyl)}$), 0.88 (t, J = 7.0 Hz, 6H, CH_2CH_3).

*Synthesis of ditetradecyl 2-(2,6-diaminohexanamido) pentanedioate (**LG2C$_{14}$**)*

The **LG2C$_{14}$** was synthesized as described elsewhere [1]. Briefly, Boc-Lys(Boc)-OH (3 g, 8.7 mmol), (3-dimethylaminopropyl) ethyl-carbodiimid monohydrochloride 3-propanediamine (EDC) (3.3 g, 17.3 mmol) and *N*-hydroxysuccinimide (NHS) (2.0 g, 17.3 mmol) were dissolved in 60 mL of chloroform ($CHCl_3$) with stirring for 3 h at room temperature. **G2C$_{14}$** (4.7 g, 8.7 mmol) and triethylamine (TEA) (0.88 g, 8.7 mmol) were dissolved in 40 mL of $CHCl_3$ with stirring for 1 h. Subsequently, two solutions above were mixed with stirring for 12 h. When the reaction was completed, the reaction solution was washed distilled water (20 mL × 3) and dried with anhydrous sodium sulfate before evaporated. The amino group-protected intermediate was obtained by column chromatography (CH_2Cl_2:MeOH=150:1) with a yield of 54.6%. Then, the intermediate (4.1 g, 4.7 mmol) was dissolved in 45 mL of ethyl acetate solution saturated with HCl gas for 12 h at 0 °C and the **LG2C$_{14}$** was obtained with a yield of 74.8%.

^{1}H-NMR (300 MHz, $CDCl_3$): δ 4.15-4.09 (m, 4H, $COOCH_2CH_2$), 4.02-3.97 (m, 1H, CONHCHCOO), 3.11-2.97 (m, 2H, $NH_2CH_2CH_2$), 2.52-2.5 (m, 2H, CH_2CO), 2.47-2.22 (m, 1H, $CONHCHCH_2$), 2.10-2.08 (m, 1H, $CONHCHCH_2$), 2.05-1.88 (m, 2H,

OCONHCHCH$_2$), 1.84-1.72 (m, 2H, NH$_2$CHCH$_2$), 1.65-1.54 (m, 6H, OCONHCHCH$_2$+COOCH$_2$CH$_2$), 1.31-1.26 (m, 46 H, NH$_2$CH$_2$CH$_2$CH$_2$+CH$_2$ $_{(myristoyl)}$), 0.88 (t, J = 7.0 Hz, 6H, CH$_2$CH$_3$).



Scheme S1. Synthesis of amino-acid based cationic lipid (LG2C$_{14}$)

Synthesis of amino-acid based cationic lipid with azide group

*Synthesis of ditetradecyl 2- (6-amino-2- (tert-butoxycarbonyl) hexanamido) pentanedioate (**L(Boc)G2C$_{14}$**)*

Boc-Lys(Z)-OH (2.5 g, 6.55 mmol), EDC (2.51 g, 13.1 mmol) and NHS (1.5 g, 13.1 mmol) were dissolved in 50 mL of CHCl$_3$ with stirring for 3 h at room temperature. **G2C$_{14}$** (5.25 g, 6.55 mmol) and TEA (0.66 g, 6.55 mmol) were dissolved in 50 mL of

$CHCl_3$ with stirring for 1 h. Subsequently, two solutions above were mixed with stirring for 12 h. When the reaction was completed, the reaction solution was washed distilled water (20 mL × 3) and dried with anhydrous sodium sulfate before evaporated. The amino group-protected intermediate was obtained by column chromatography (CH_2Cl_2:MeOH=150:1) with a yield of 59.3%. Then, the intermediate (2 g, 2.2 mmol) and ammonium formate (2.8 g, 44.3 mmol) were dissolved in the mixed solvent (tetrahydrofuran:MeOH=10:1). Palladium carbon (20%) was added into the reaction solution with stirring for 3 h at 45 °C [2]. **L(Boc)G2C$_{14}$** was obtained by drying with anhydrous sodium sulfate with a yield of 93.2%.

^{1}H-NMR (300 MHz, $CDCl_3$): δ 6.76 (d, *J* = 7.7 Hz, 1H, OCONHCH), 5.29 (brs, 1H, CONHCH), 4.71-4.53 (m, 3H, CONHCHCOO+CH_2NH_2), 4.19-3.93 (m, 3H, $COOCH_2$+OCONHCHCO), 3.19-3.05 (m, 2H, $NH_2CH_2CH_2$), 2.43-2.31 (m, 2H, CH_2CO), 2.21-2.18 (m, 1H, $CONHCHCH_2$), 2.02-1.95 (m, 1H, $CONHCHCH_2$), 1.88-1.79 (m, 1H, $OCONHCHCH_2$), 1.65-1.54 (m, 5H, $OCONHCHCH_2$+ $COOCH_2CH_2$), 1.48-1.34 (m,

11H, $NH_2CH_2CH_2$+$(CH_3)_3C$), 1.31-1.26 (m, 46 H, $NH_2CH_2CH_2CH_2$+$CH_{2\ (myristoyl)}$), 0.88 (t, J = 7.0 Hz, 6H, CH_2CH_3).

*Synthesis of 2- ((2-azidoethyl) carbamoyl) benzoic acid (**N_3-HHPA**)*

2-Chloroethylamine hydrochloride (5.0 g, 43.1 mmol) and sodium azide (8.4 g, 129.0 mmol) were dissolved in 100 mL of water with stirring for 15 h at 80 °C [3]. When the reaction was complete, pH of the reaction solution was adjusted to a value of 12 to 14 by adding potassium hydroxide slowly. The reaction solution was then extracted by ethyl ether (100 mL × 3), colorless oil was obtained by drying with anhydrous sodium sulfate with a yield of 56.7%. Colorless oil (2.1 g, 24.4 mmol) and hexahydrophthalic anhydride (HHPA) (3.7 g, 24.4 mmol) were dissolved in 50 mL of $CHCl_3$ with stirring for 3 h at room temperature [4]. After removal of $CHCl_3$, the crude product was purified by column chromatography (CH_2Cl_2:MeOH=50:1) with a yield of 78.3%.

^{1}H-NMR (300 MHz, $CDCl_3$): δ 11.8 (brs, 1H, COOH), 7.86 (s, 1H, $CONHCH_2$), 3.36-3.11 (m, 4H, $CONHCH_2CH_2N_3$), 2.74-2.56 (m, 1H, CHCOOH), 2.48-2.53 (m, 1H,

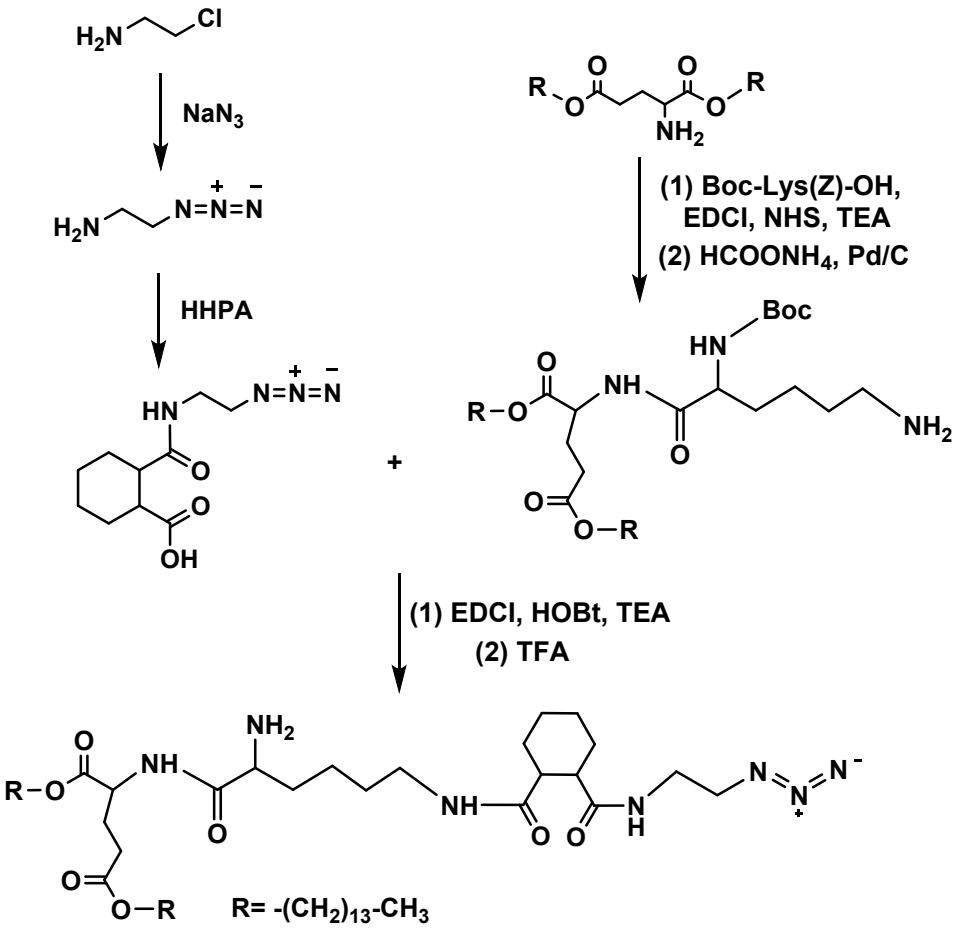
CHCONH), 2.05-1.86 (m, 2H, CH_2CH_2CH), 1.71-1.49 (m, 3H, CH_2CH_2CH+ $CH_2CH_2CH_2$), 1.45-1.22 (m, 3H, $CH_2CH_2CH_2$).

*Synthesis of ditetradecyl 2- (2-amino-6- (2- ((2-azidoethyl)carbamoyl) cyclohexane-carboxamido)hexanamido) pentanedioate (**N_3-LG2C$_{14}$**)*

N_3-HHPA (1.44 g, 5.99 mmol), EDC (1.72 g, 8.99 mmol) and N-hydroxybenzotrizole (HOBt) (1.5 g, 13.1 mmol) were dissolved in 20 mL of tetrahydrofuran with stirring for 3 h at room temperature. **L(Boc)G2C$_{14}$** (4.60 g, 5.99 mmol) and TEA (0.61 g, 5.99 mmol) were dissolved in 20 mL of tetrahydrofuran with stirring for 1 h. Subsequently, two solutions above were mixed with stirring for 12 h. When the reaction was completed, the reaction solution was washed distilled water (10 mL × 3) and dried with anhydrous sodium sulfate before evaporated. The amino group-protected intermediate was obtained by column chromatography (CH_2Cl_2:MeOH=80:1) with a yield of 69.5%. Then, the intermediate (4.1 g, 4.1 mmol) was dissolved in 16 mL of the mixed solvent (trifluoroacetic acid:CH_2Cl_2=1:1) with stirring for 3 h at 0 °C. When

the reaction was completed, pH of the reaction solution was adjusted to a value of 7 by adding 5% sodium bicarbonate solution slowly. The reaction solution was then extracted by CH_2Cl_2 (20 mL × 3) and dried with anhydrous sodium sulfate. After removal of CH_2Cl_2, the crude product was purified by column chromatography (CH_2Cl_2:MeOH=40:1) with a yield of 48.8%.

^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.91-7.80 (m, 1H, COONH), 6.69-6.50 (m, 1H, COONH), 6.45-6.01 (m, 1H, COONH), 4.54 (dd, *J* = 5.4, 8.0 Hz, 1H, CONHCHCOO), 4.13 (t, *J* = 6.8 Hz, 2H, $COOCH_2$), 4.07 (t, *J* = 6.8 Hz, 1H, $COOCH_2$), 3.51-3.32 (m, 5H, $CONHCH_2CH_2N_3$+$NH_2CHCONH$), 3.30-3.16 (m, 2H, $CONHCH_2$), 2.73-2.61 (m, 2H, NHCOCH+NHCOCH), 2.43-2.31 (m, 2H, CH_2CO), 2.21-1.95 (m, 4H, $CH_2CHCONH$+$CH_2CHCONH$+$CONHCHCH_2$), 1.88-1.26 (m, 12H, $CH_{(hexane\ ring)}$+$CONHCH_2CH_2CH_2CH_2$), 1.31-1.26 (m, 46 H, $NH_2CH_2CH_2CH_2$+ $CH_{2\ (myristoyl)}$), 0.88 (t, *J* = 7.0 Hz, 6H, CH_2CH_3).



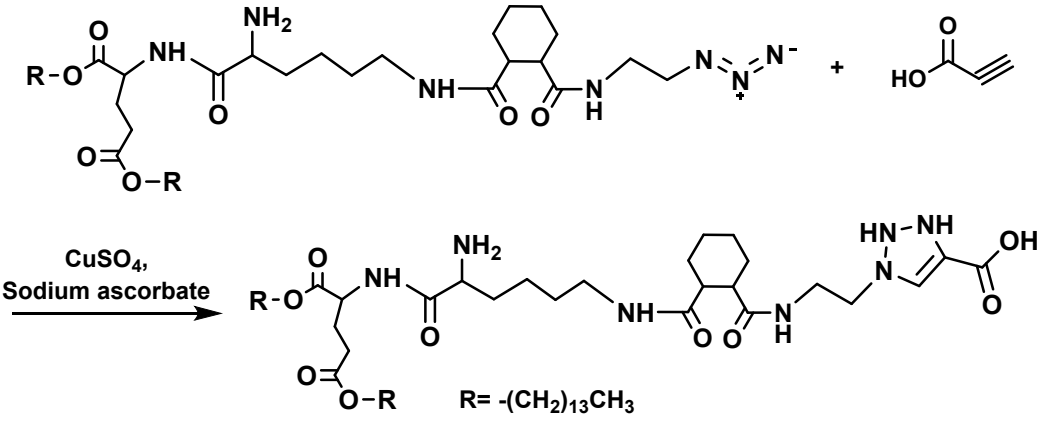
Scheme S2. Synthesis of amino-acid based cationic lipid with azide group (N_3-LG2C_{14}).

Reaction of cationic lipid with azide group and propiolic acid

Propiolic acid (0.32 g, 4.5 mmol), sodium ascorbate (1.3 g, 6.7 mmol) and copper sulfate anhydrous (0.17 g, 0.67 mmol) were dissolved in 14 mL of water, then **N_3-LG2C_{14}** (1 g, 1.12 mmol) dissolved in the 14 mL of CH_2Cl_2 was added with stirring for 12 h at room temperature [5]. When the reaction was completed, 20 mL of CH_2Cl_2 was

added into the reaction solution, washed by distilled water (10 mL × 3) and dried with anhydrous sodium sulfate before evaporated. The crude product was purified by column chromatography (CH_2Cl_2:MeOH=5:1) with a yield of 74.5%.

^{1}H-NMR (300 MHz, $CDCl_3$): δ 5.34 (s, 1H, $CH_{(Triazole)}$), 4.65-4.40 (m, 3H, CONHCHCOO+$CONHCH_2CH_2N$), 4.39-4.20 (m, 4H, $CONHCH_2CH_2N$+$CHNH_2$), 4.20-4.01 (m, 4H, $COOCH_2$+$COOCH_2$), 3.85-3.59 (m, 1H, $NH_2CHCONH$), 3.10-2.98 (m, 2H, $CONHCH_2$), 2.73-2.61 (m, 2H, NHCOCH+NHCOCH), 2.43-2.31 (m, 2H, CH_2CO), 2.21-1.95 (m, 4H, $CH_2CHCONH$+$CH_2CHCONH$+$CONHCHCH_2$), 1.88-1.26 (m, 12 H, $CH_{(hexane\ ring)}$+$CONHCH_2CH_2CH_2CH_2$), 1.31-1.26 (m, 46 H, $NH_2CH_2CH_2CH_2$+CH_2 $_{(myristoyl)}$), 0.88 (t, J = 7.0 Hz, 6H, CH_2CH_3).

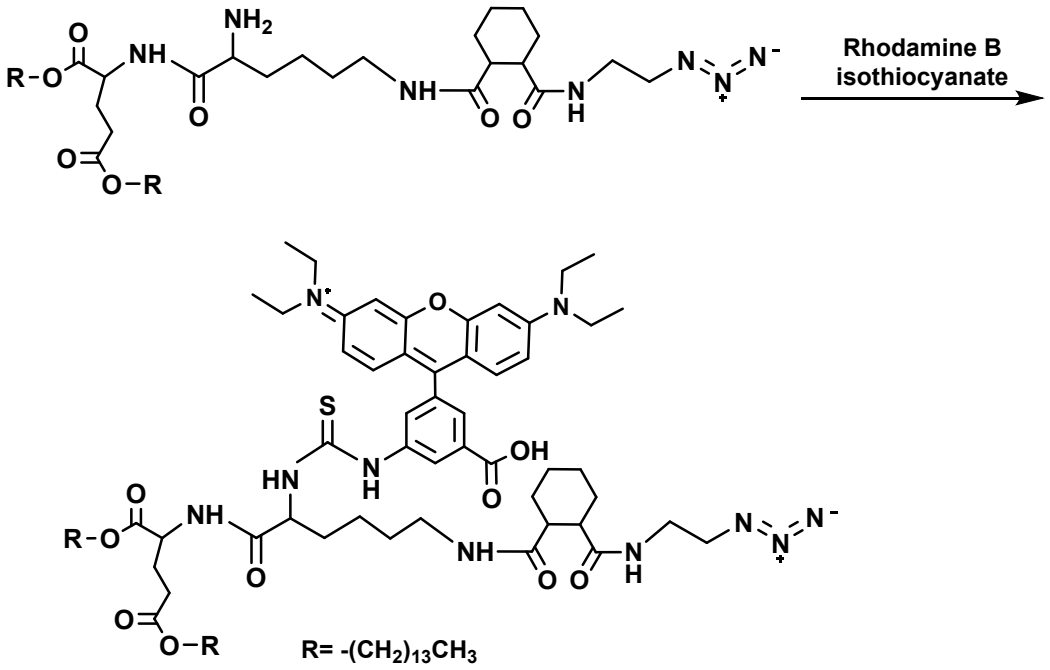


Scheme S3. Reaction between N_3-LG2C_{14} and propiolic acid

Synthesis of Rhodamine B-labeled cationic lipid (RhB-N_3-LG2C_{14})

Rhodamine B isothiocyanate (0.011 g, 0.0204 mmol) and **N_3-LG2C_{14}** (0.02 g, 0.0225 mmol) were dissolved in 10 mL of mixed solution ($CHCl_3$:MeOH=1:1) with stirring for 12 h at room temperature. When the reaction was completed, the product was obtained after evaporation of solvent.

^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.39, 8.52 (m, 2H, $CH_{(benzene)}$), 6.9-6.27 (m, 2H, $CH_{(benzene)}$-N), 6.51-6.98 (m, 3H, $CH_{(benzene)}$ =N^+), 4.41 (q, 1H, NHCH), 4.16 (q, 1H, NHCH), 4.02-4.15 (t, 4H, $COOCH_2$), 3.49 (m, 4H, CH_2NCH_2), 3.2 (m, 2H, CH_2CH_2NH), 2.88-3.25 (q, 2H, $NHCHCH_2$), 2.7 (m, 1H, CHCOOH), 2.5 (m, 1H, CHCONH), 2.19 (m, 2H, $NHCHCH_2CH_2$), 2.05 (m, 2H, $NHCHCH_2$), 1.6 (m, 2H, CH_2CH_2NH), 1.5-1.8 (m, 4H, $CHCH_2$), 1.58-1.66 (m,4H, $COOCH_2CH_2$), 1.4-1.5 (m, 4H, CH_2CH_2), 1.28 (m, 44H, $CH_{2(myristoyl)}$), 0.88 (t, 6H, CH_2CH_3).



Scheme S4. Synthesis of Rhodamine B-labeled cationic lipid (RhB-N_3-LG2C_{14})

References

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[3] Inverarity, I. A.;Viguier, R. F.;Cohen, P.; Hulme, A. N. Biotinylated anisomycin: a comparison of classical and "click" chemistry approaches. *Bioconjugate chemistry* **2007**, *18*, 1593-1603.

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[5] Hou, J. L.;Li, Z. H.;Fang, Q. H.;Feng, C. R.;Zhang, H. W.;Guo, W. K.;Wang, H. H.;Gu, G. X.;Tian, Y. P.;Liu, P.;Liu, R. H.;Lin, J. P.;Shi, Y. K.;Yin, Z.;Shen, J.; Wang, P. G. Discovery and Extensive in Vitro Evaluations of NK-HDAC-1: A Chiral Histone Deacetylase Inhibitor as a Promising Lead. *J Med Chem* **2012**, *55*, 3066-3075.

Supplementary Figures

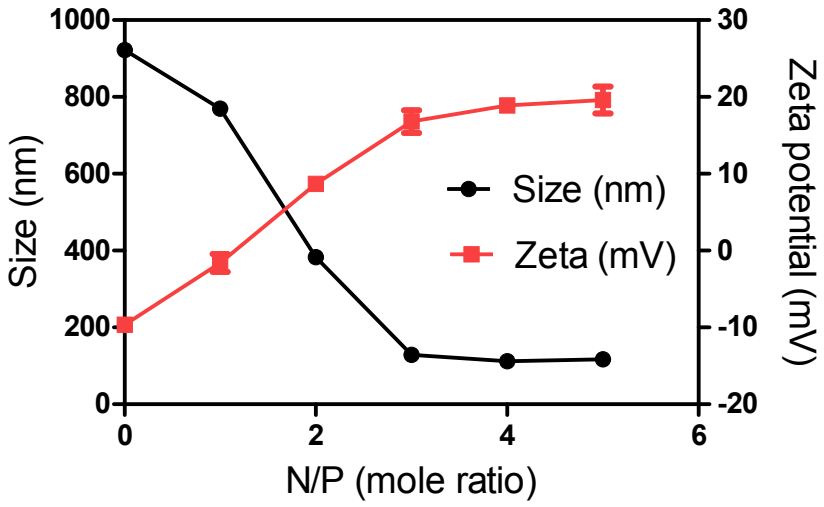


Figure S1. Particle size and zeta potential of N_3-CL/siRNA at different N:P (0, 1, 2, 3, 4, 5) measured by a dynamic light scattering (DLS) analyzer.

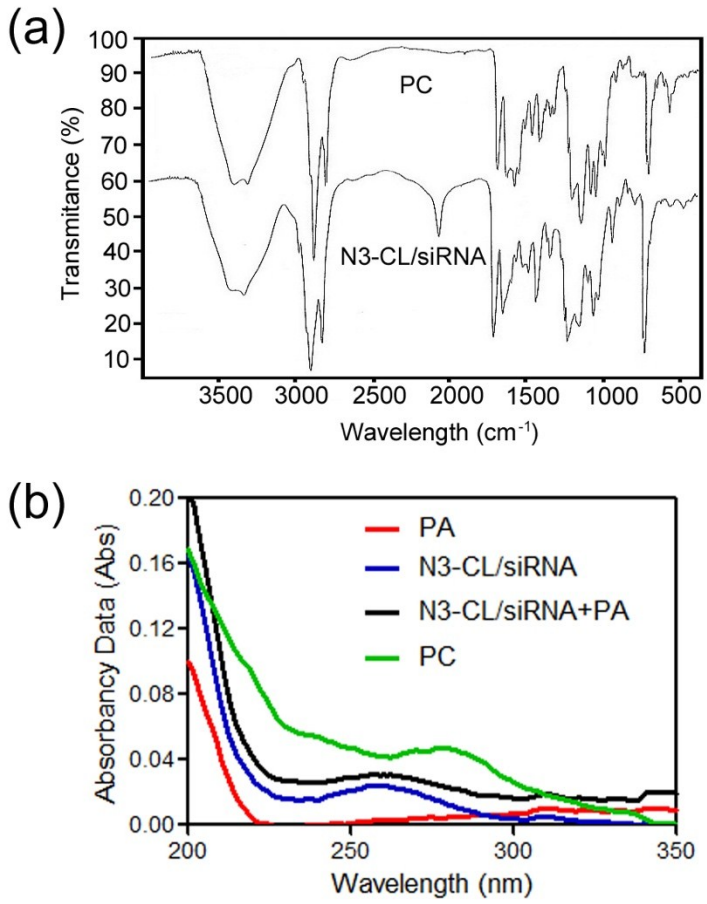


Figure S2. (a) Infrared spectra of the PC and N_3-CL/siRNA. (b) UV-Vis spectra of PA, N_3-CL/siRNA, N_3-CL/siRNA + PA and the PC.

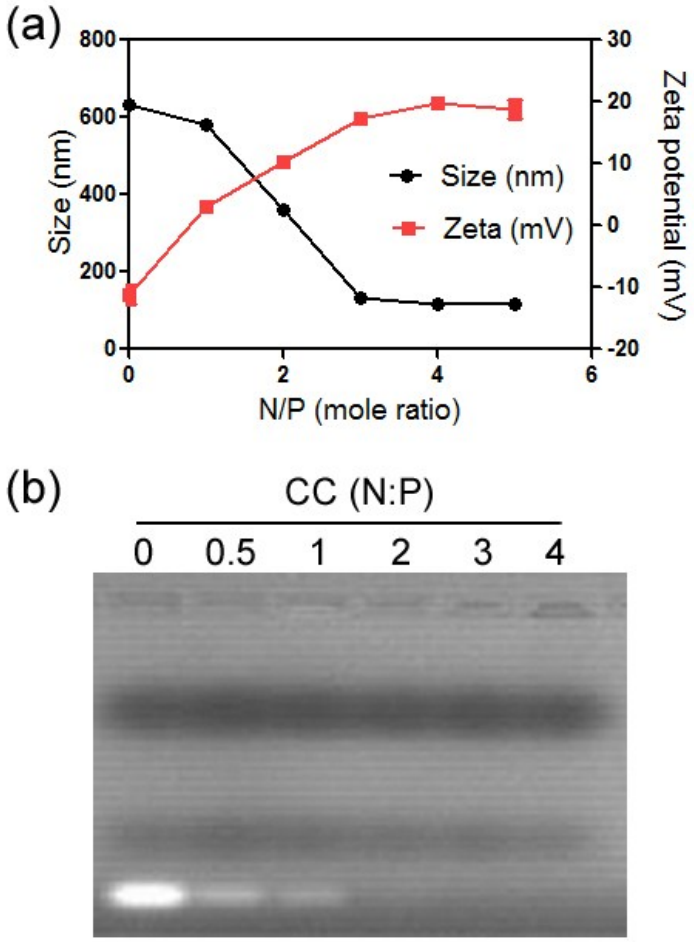


Figure S3. (a) Particle size and zeta potential of the CC at different N:P (0, 1, 2, 3, 4, 5).

(b) Agarose gel electrophoresis assay of the CC at different N:P (0, 0.5, 1, 2, 3, 4).

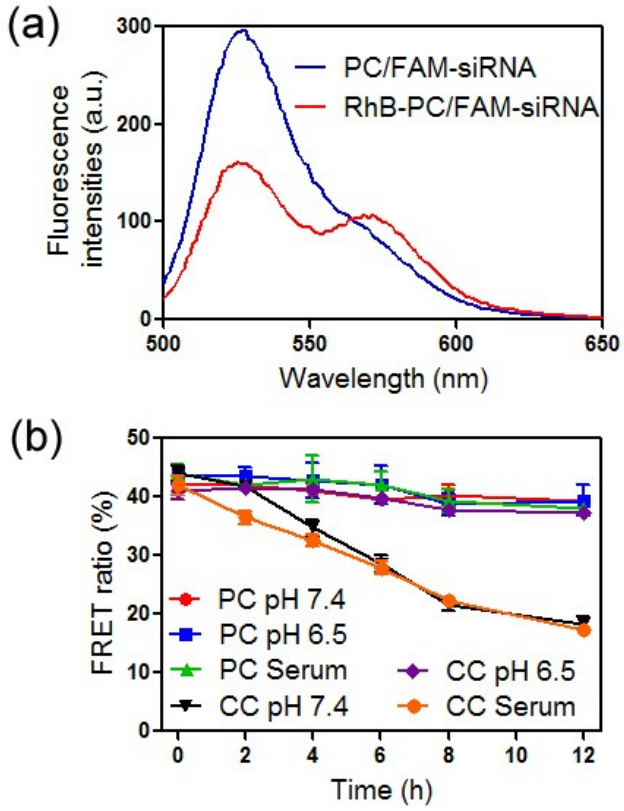


Figure S4. (a) Fluorescence intensities of the PC load with FAM-siRNA (PC/FAM-siRNA) and the double-labeled PC (RhB-PC/FAM-siRNA) containing RhB-N_3-LG2C_{14} and FAM-siRNA with an excitation wavelength of 485 nm. (b) Change in FRET efficiency of the double-fluorescence labeling PC and CC as a function of incubation time at different pH values (pH 7.4 and pH 6.5) or in the presence of serum.

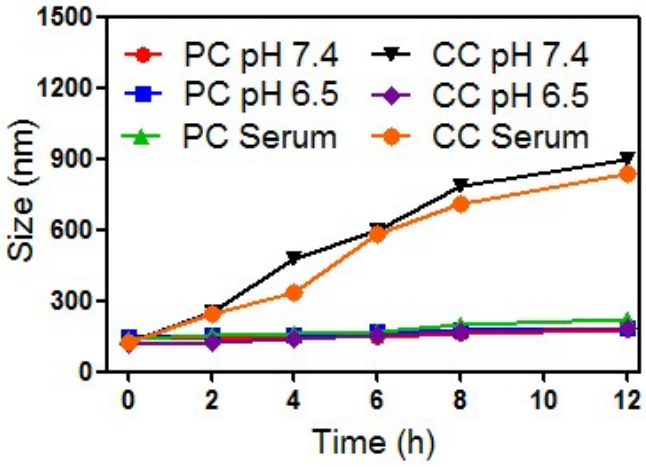


Figure S5. Change in the particle size of the PC and CC as a function of incubation time at different pH values (pH 7.4 and pH 6.5) or in the presence of serum.

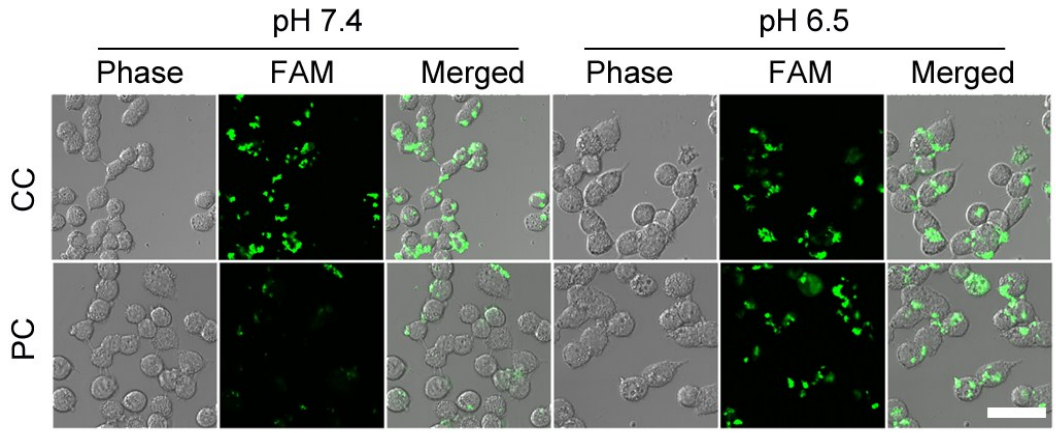


Figure S6. Cellular uptake images of Bel-7402 cells incubated with CC or PC loaded with FAM-siRNA for 3 h at different pH values (pH 7.4 and pH 6.5) observed by CLSM. The concentration of FAM-siRNA was 200 nM. Scale bar indicates 20 μm.

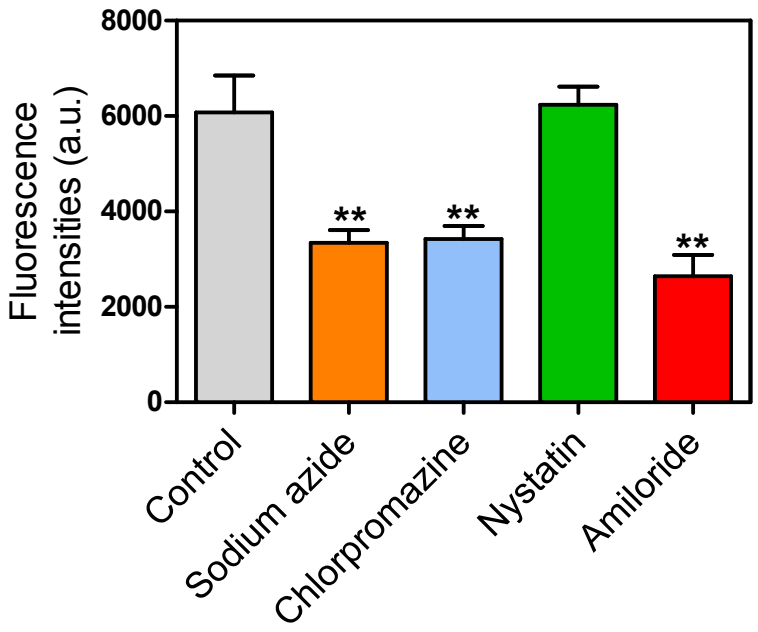


Figure S7. Endocytosis pathway on Bel-7402 cells of the PC loaded with FAM-siRNA determined by the flow cytometry. The concentration of FAM-siRNA was 200 nM. $^{**}P<0.01$, comparing with the control group.

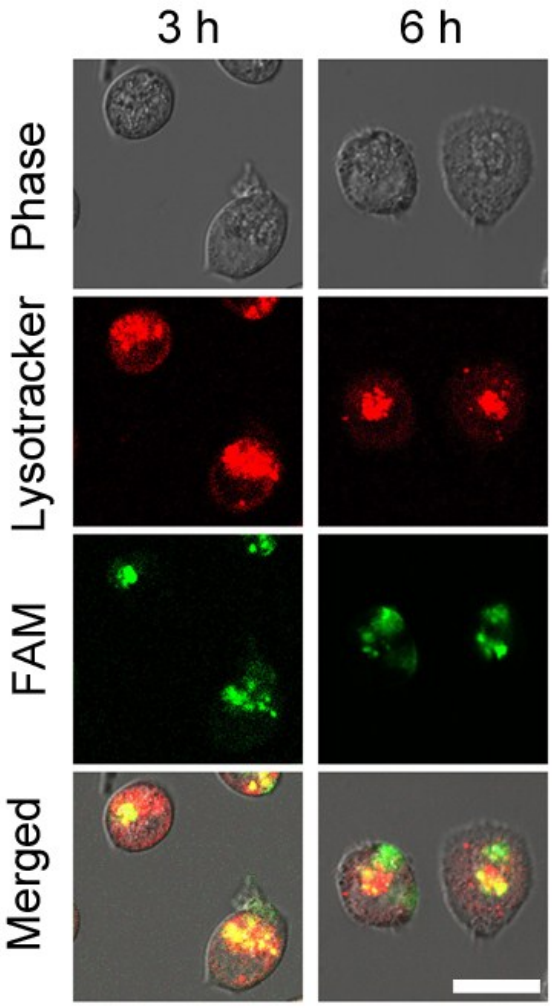


Figure S8. Intracellular location of the PC loaded with FAM-siRNA in Bel-7402 cells. The late endosomes and lysosomes were stained with LysoTracker Red. Scale bar indicates 10 μm.

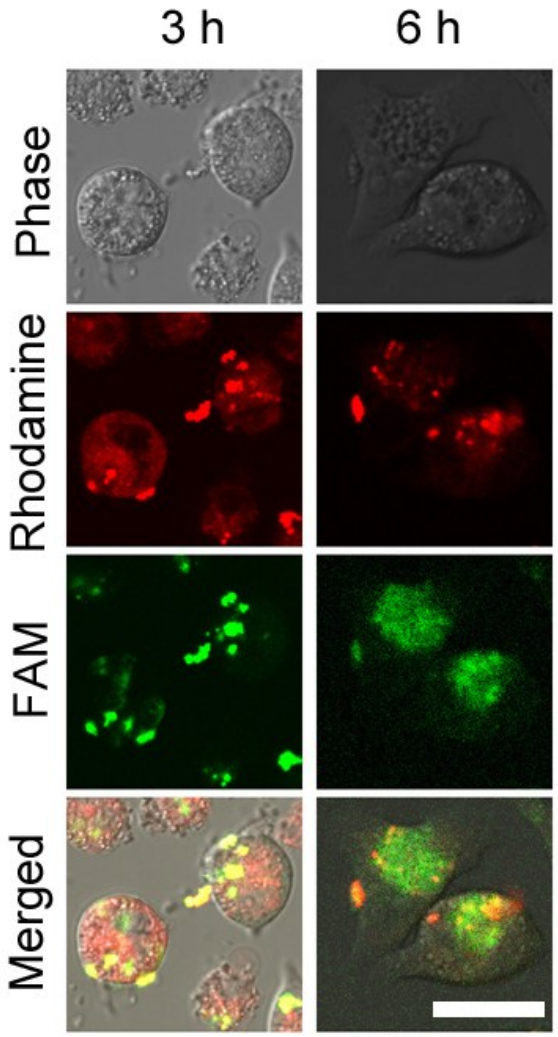


Figure S9. Intracellular disassembly of the RhB-labeled PC loaded with FAM-siRNA in Bel-7402 cells. Scale bars indicate 10 μm.

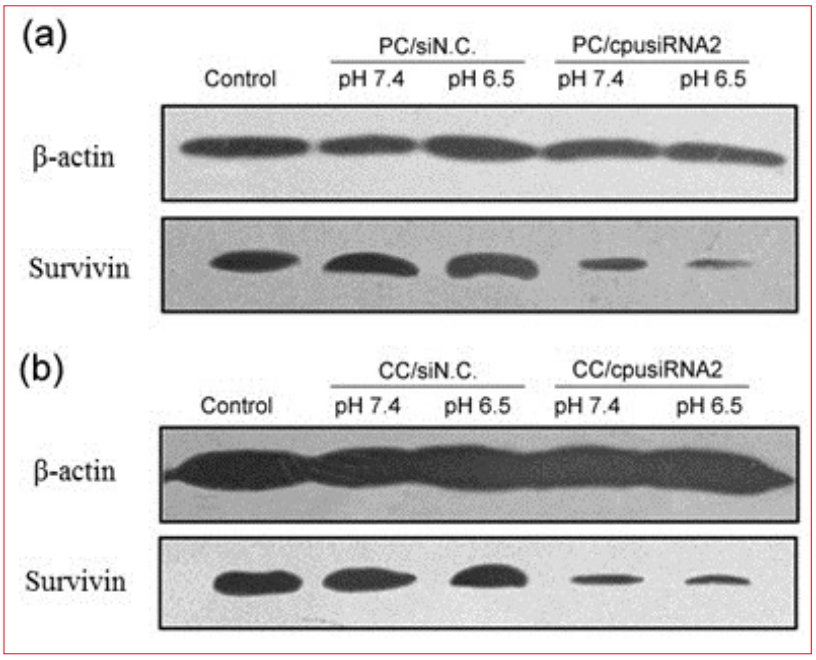


Figure S10. (a, b) Survivn protein expression in Bel-7402 cells following transfection with the PC and CC at different pH values (pH 7.4 and pH 6.5) determined by the Western blot analysis.

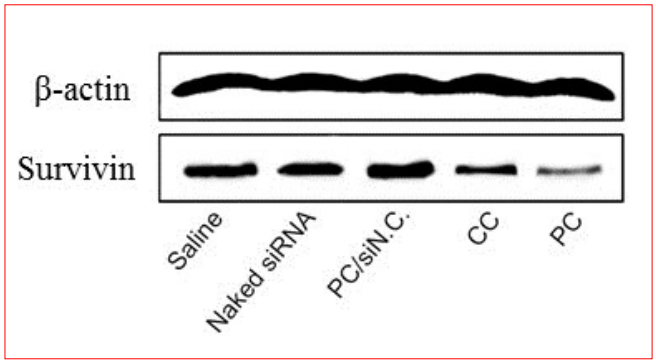


Figure S11. Survivn protein expression *in vivo* determined by the western blot analysis.

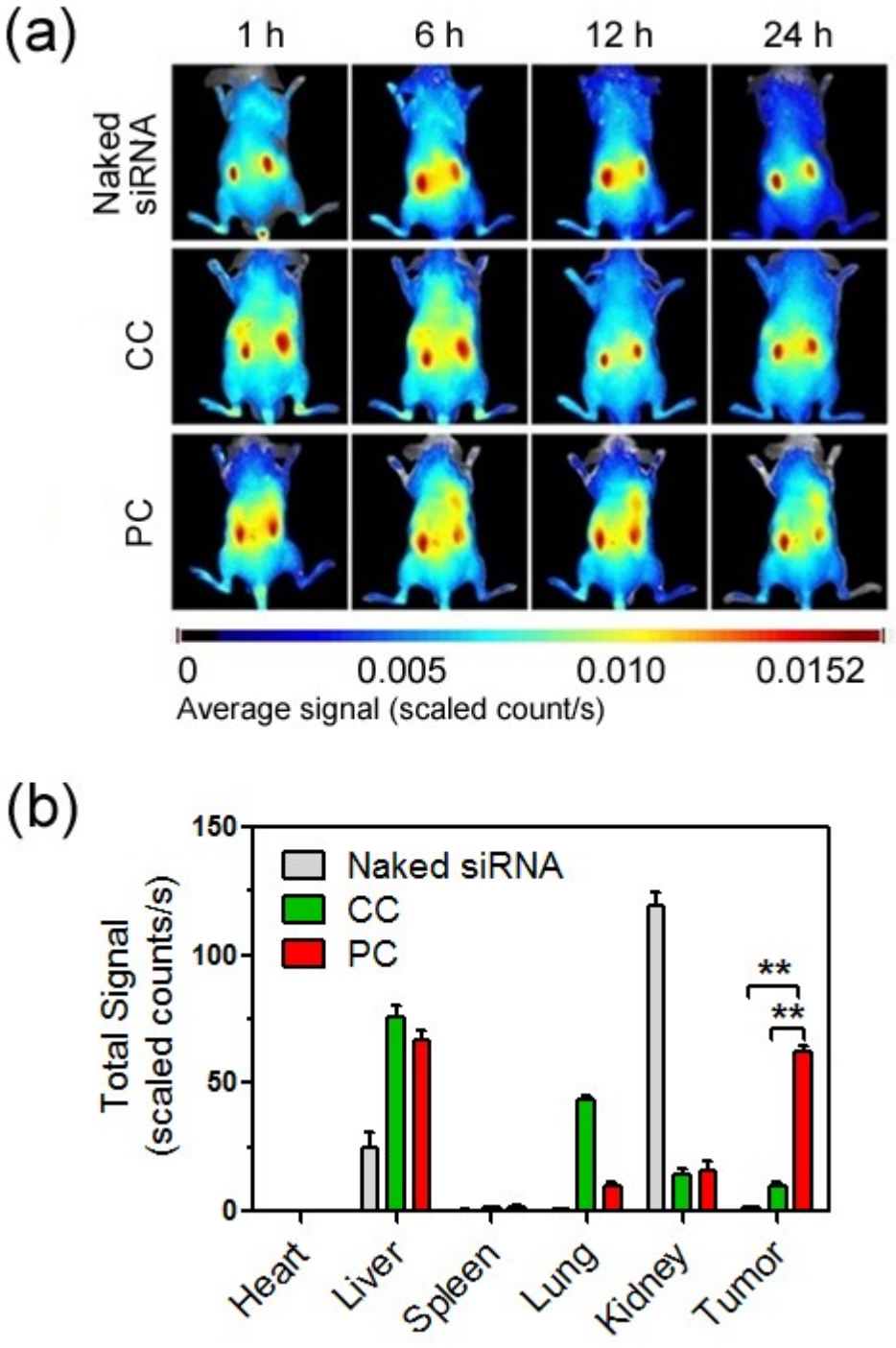


Figure S12. (a) *In vivo* biodistribution of Cy5-siRNA monitored using the *in vivo* imaging after intraveous injection of naked Cy5-siRNA, CC and PC loaded with Cy5-

siRNA into the Heps tumor-bearing ICR mice, at a dose of 1 mg/kg. (b) Total fluorescence signal of Cy5-siRNA in the tumor and different normal tissues quantified with the ROI analysis.

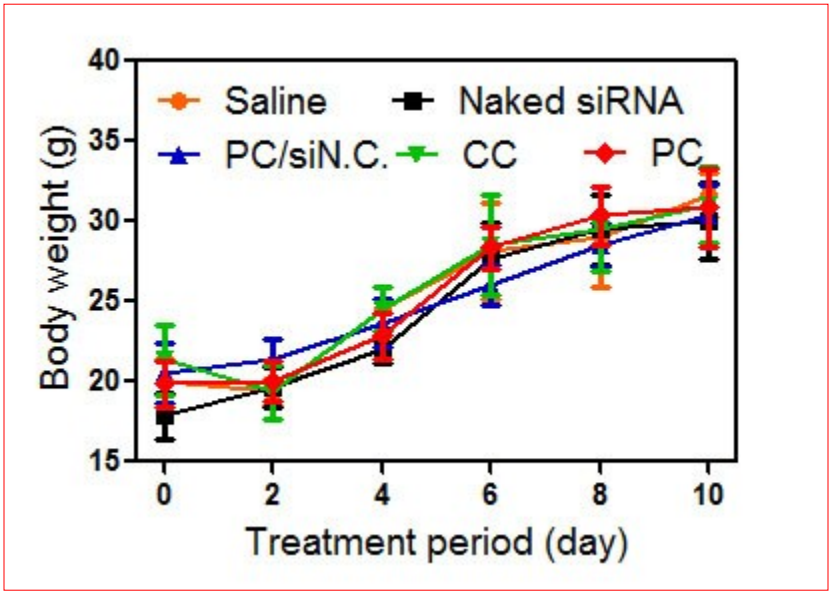
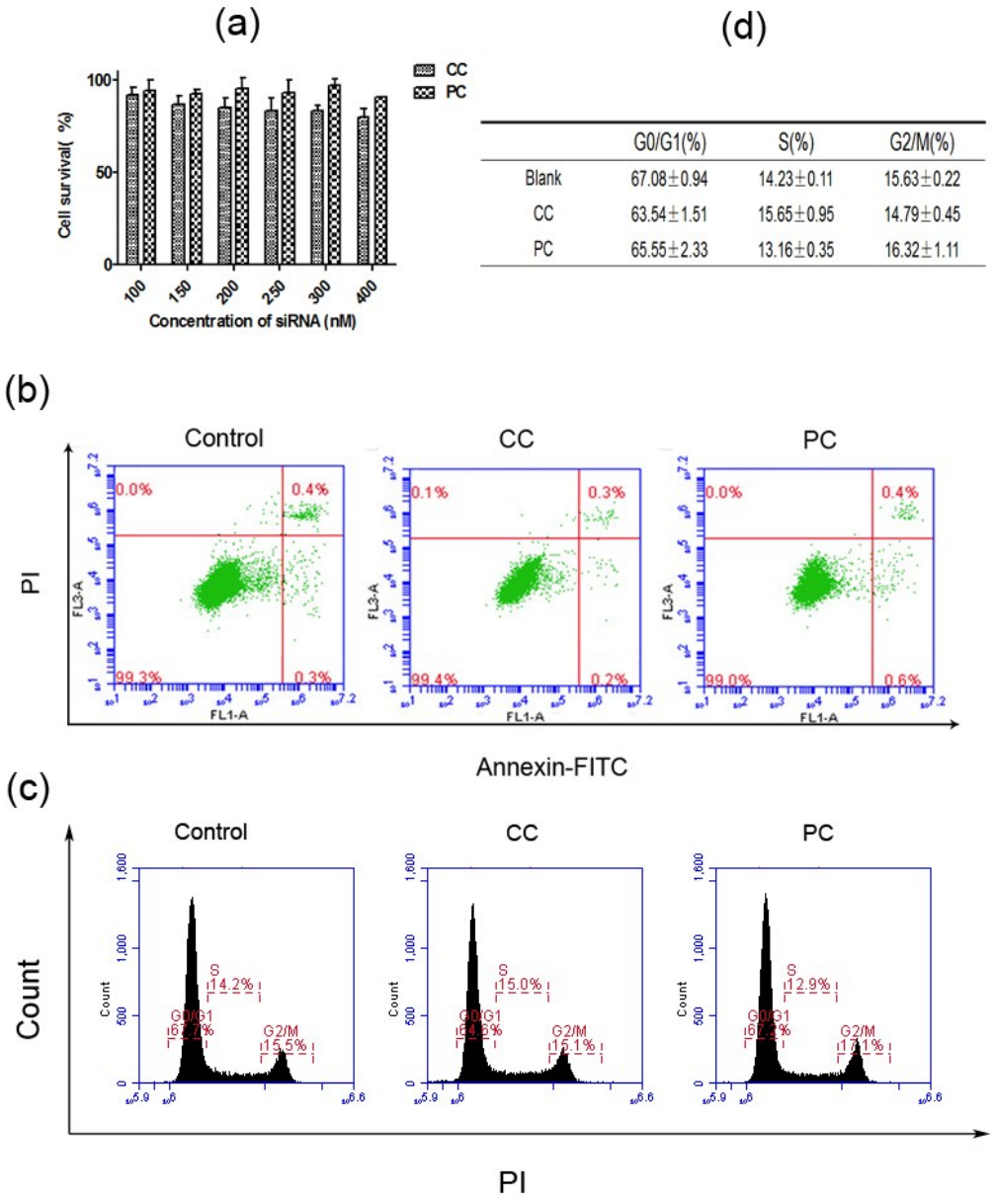


Figure S13. The body weight variation of the Heps tumor-bearing ICR mice during the treatment with different formulations.



	G0/G1(%)	S(%)	G2/M(%)
Blank	67.08±0.94	14.23±0.11	15.63±0.22
CC	63.54±1.51	15.65±0.95	14.79±0.45
PC	65.55±2.33	13.16±0.35	16.32±1.11

Figure S14. (a) Toxicity of L02 cells treated with CC and PC. Cytotoxicity was estimated with MTT assay. (b) Apoptosis after treated with different formulations in L02 cells determined by the Annexin V-FITC/PI assay. The viable (Q3), early apoptotic (Q2),

and late apoptotic (Q4) cell populations (%) are shown in the lower left, lower right and upper right quadrants, respectively. (c,d) Flow cytometric analysis of cell cycle in L02 cells with CC and PC. (c) FACS analysis. (d) Percentage of L02 cells in every phase of cell cycle upon different treatment.

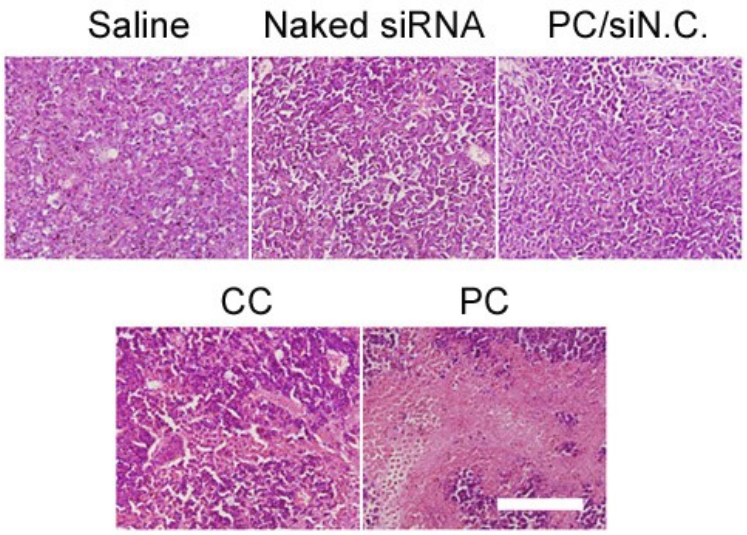


Figure S15. Images of tumor sections with HE staining from Heps tumor-bearing ICR mice detected by a fluorescence microscope. Scale bar indicates 100 μm.

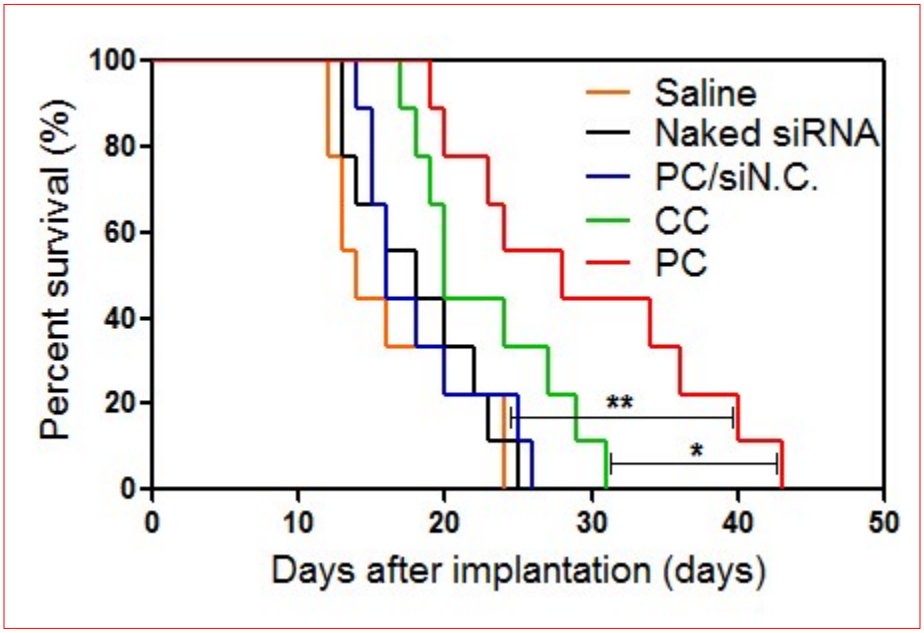


Figure S16. Percent survival of the Heps tumor-bearing ICR mice after the treatment with saline, naked siRNA, PC/siN.C., CC and PC loaded with cpusiRNA2 at a dose of 1.2 mg/kg. **P<0.01, *P<0.05 (analyzed with the Log-rank test).