摘要

阳离子脂质体能够有效提高小干扰 RNA（siRNA）的基因治疗效果。然而，阳离子脂质体在体内循环时易被网状内皮系统清除。尽管 PEG 修饰能够延长阳离子脂质体的血液循环时间，PEG 修饰严重影响 siRNA 的包埋率，细胞内吞和内涵体逃逸，从而导致 siRNA 的基因沉默效果低。基于此，我们设计合成了 PCB 修饰的脂质分子 DSPE-PCBn，用于构建新型长循环阳离子脂质体。其中，DSPE-PCB20 在生理条件下可以有效抵抗负电荷蛋白质对阳离子脂质体/siRNA 复合物的非特异性吸附，增强阳离子脂质体/siRNA 复合物的血清稳定性，效果与 DSPE-PEG 2000 相当。同时，由于 PCB 独特的结构组成，一方面 PCB 分子中含有大量的正电荷季胺基团，其对阳离子脂质体复合 siRNA 药物的能力影响小。在 N/P 比为 20/1 时，其对 siRNA 的包埋率为 92%，而 PEG 聚合物仅为 73%。另一方面 PCB 分子中含有大量的负电荷羧酸根基团，其在内涵体的酸性条件下可以质子化，增加了阳离子脂质体表面的正电荷，促进阳离子脂质体对负电荷内涵体膜的扰动，从而有利于 siRNA 药物逃离内涵体进入细胞质，发挥基因沉默作用。DSPE-PCB20 阳离子脂质体因具有显著的血清稳定性、siRNA 药物复合能力和内涵体逃逸能力，其负载的 ApoB siRNA 药物可以有效下调肝部载脂蛋白 B（Apolipoprotein B，ApoB）的表达，从而降低了血液中胆固醇的含量，在治疗高胆固醇血症方面具有很好的应用前景。

Journal of Controlled Release 176 (2014) 104–114

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Enhanced endosomal/lysosomal escape by distearoyl phosphoethanolamine-polycarboxybetaine lipid for systemic delivery of siRNA

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ARTICLE INFO

Article history:
Received 17 May 2013
Accepted 8 December 2013
Available online 21 December 2013

Keywords:
siRNA delivery
Cationic liposomes
Polycarboxybetaine
pH-sensitive
Endosomal/lysosomal escape

ABSTRACT

Cationic liposome based siRNA delivery system has improved the efficiencies of siRNA. However, cationic liposomes are prone to be rapidly cleared by the reticuloendothelial system (RES). Although modification of cationic liposomes with polyethylene glycol (PEG) could prolong circulation lifetime, PEG significantly inhibits siRNA entrapment efficiency, cellular uptake and endosomal/lysosomal escape process, resulting in low gene silencing efficiency of siRNA. In this study, we report the synthesis of zwitterionic polycarboxybetaine (PCB) based distearoyl phosphoethanolamine-polycarboxybetaine (DSPE-PCB) lipid for cationic liposome modification. The DSPE-PCB_{20} cationic liposome/siRNA complexes (lipoplexes) show an excellent stability in serum medium. The siRNA encapsulation efficiency of DSPE-PCB_{20} lipoplexes could reach 92% at N/P ratio of 20/1, but only 73% for DSPE-PEG lipoplexes. The zeta potential of DSPE-PCB_{20} lipoplexes is 8.19 ± 0.53 mV at pH 7.4, and increases to 24.6 ± 0.87 mV when the pH value is decreased to 4.5, which promotes the endosomal/lysosomal escape of siRNA. The DSPE-PCB_{20} modification could enhance the silencing efficiency of siRNA by approximately 20% over the DSPE-PEG 2000 lipoplexes at the same N/P ratio *in vitro*. Furthermore, DSPE-PCB_{20} lipoplexes could efficiently mediate the down-regulation of Apolipoprotein B (ApoB) mRNA in the liver and consequently decrease the total cholesterol in the serum *in vivo*, suggesting therapeutic potentials for siRNA delivery in hypercholesterolemia-related diseases.

1. Introduction

The specific and robust effect of RNA interference (RNAi) on gene expression renders small interfering RNA (siRNA) a valuable therapeutic agent for the treatment of various diseases [1,2]. However, the intrinsic deficiencies of siRNA, such as short plasma half-life, poor membrane penetrability and lack of target ability, have limited the development of its therapeutic application [3,4]. To realize the therapeutic potential of siRNA, it is significant to develop efficient and safe delivery vehicles that can overcome the potential bottleneck for siRNA delivery [5].

Both viral and nonviral vectors have been used to deliver siRNA, and mediate targeted gene silencing *in vitro* and *in vivo* [6]. However, inherent shortcomings such as immunogenic and inflammatory responses restrict the widespread use of viral vectors [7–9]. In nonviral vectors, cationic liposomes are desirable because they are of low immunogenity, and easy to scale up and chemically modify [10–12]. However, cationic liposomes have nonspecific interaction with plasma protein *in vivo* and are prone to be rapidly cleared by the reticuloendothelial system (RES) before reaching the diseased site, resulting in low gene silencing efficiency of siRNA *in vivo* [13–15].

A strategy to address this issue is the introduction of polyethylene glycol (PEG)-lipids such as 1, 2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000] (DSPE-PEG 2000) to cationic liposomes for increasing the serum stability and blood circulation lifetime [16,17]. The product is referred to as "stealth liposome" [18]. Unfortunately, the steric barrier due to PEGylation interferes with the siRNA encapsulation efficiency, cellular uptake and subsequent endosomal/lysosomal escape of cationic liposome/siRNA complexes (lipoplexes) [19–21]. Because of the poor endosomal/lysosomal escape, and subsequent degradation of the lipoplexes in the

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http://dx.doi.org/10.1016/j.jconrel.2013.12.007

late lysosomes by a variety of enzymes, siRNA could not be released into the cytoplasm efficiently and hence the gene silencing efficiency of the PEGylation lipoplexes is very low [22,23].

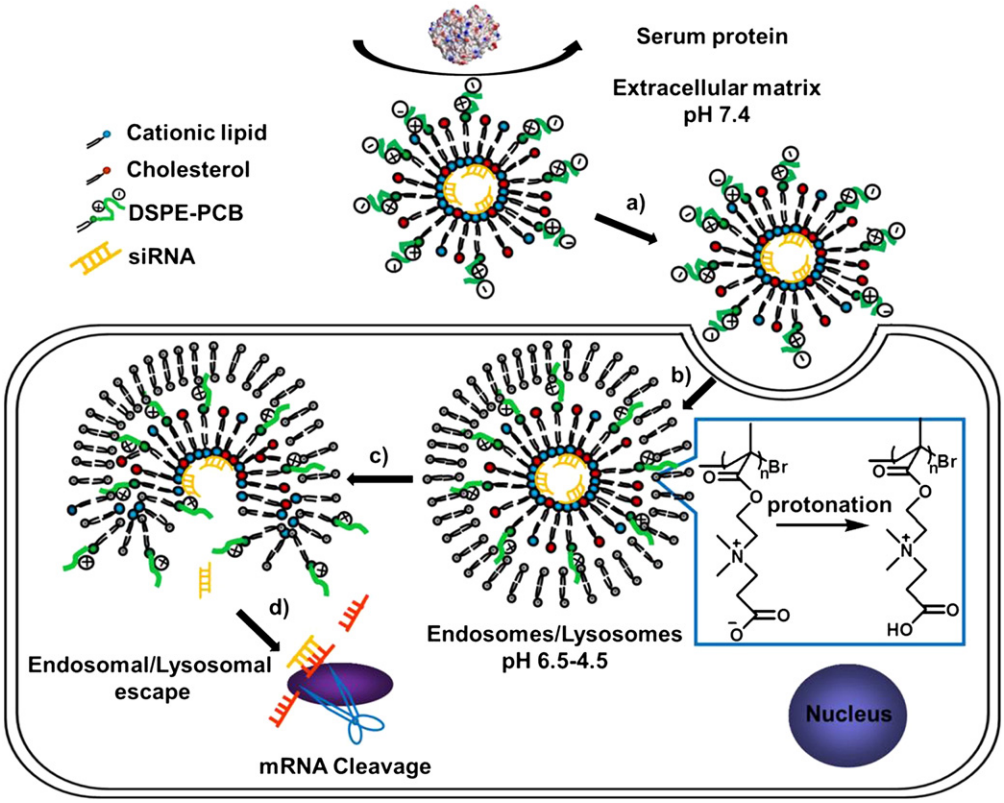
Zwitterionic polymers such as poly (sulfobetaine) (PSB) and poly (carboxybetaine) (PCB), have both cationic and anionic charge groups alone, and can highly resist nonspecific protein adsorption due to their strong hydration layer via electrostatic interactions [24–26]. As potentially excellent candidates for resisting protein adsorption, zwitterionic polymers have been used in the application of gene delivery. Interestingly, when zwitterionic polymers are introduced into cationic gene vectors, the mechanism of action may be very different from PEGylation. Zwitterionic polymers exhibit high resistance to nonspecific protein adsorption without sacrificing transfection efficiency. In our previous work, we demonstrated that zwitterionic PSB improved cellular uptake and gene transfection efficiency of cationic polymers [27]. Jiang and co-workers also proved that zwitterionic PCB-ethyl ester could package DNA and exhibit relatively high transfection efficiency [28]. Among the zwitterionic polymers, PCB is highly resistant to nonspecific protein adsorption at the pH of 7.4, and the negative carboxyl acid groups of the PCB could be protonated at low pH, which makes them sensitive to acidic environment [29,30]. After protonation, the PCB has only cationic quaternary ammonium groups. This unique behavior led us to hypothesize that we can construct a novel cationic liposome system by conjugating PCB with lipid molecules for cationic liposome hydrophilic modification. The cationic liposomes containing PCB-lipids could resist protein adsorption in the circulatory system as PEG-lipids do. Furthermore, neutral PCB-lipids could be protonated in endosomes/lysosomes, which might promote the fusion of cationic liposomes with endosomal/lysosomal membrane and thus enhance the endosomal/lysosomal escape of siRNA (Scheme 1). The pH-sensitive PCB-lipid modification might overcome the barriers associated with the use of PEG and enhance the siRNA silencing efficiency. To the best of our knowledge, this is the first example of PCB-lipid modified cationic liposomes without loss of their endosomal/lysosomal penetrability capacity.

Herein, we modified DSPE lipid with zwitterionic polymer PCB and evaluated the efficiency of DSPE-PCB cationic liposome/siRNA complexes in inhibiting the expression of Apolipoprotein B (ApoB) mRNA in liver. First, we synthesized the DSPE-PCB_n with atom transfer radical polymerization (ATRP) and applied them in cationic liposomes for delivering the siRNA. In order to contrast, DSPE-PEG 2000 based cationic liposome/siRNA complexes delivery system under the same condition was also prepared. The physicochemical properties of the DSPE-PEG and DSPE-PCB lipoplexes, including serum stability, pH sensibility, cytotoxicity, intracellular uptake, endosomal/lysosomal escape and specific gene silencing efficiency *in vitro* were evaluated. Furthermore, we examined the *in vivo* distribution of the lipoplexes in C57BL/6 mice and performed the *in vivo* functional study to assess their potential application *in vivo*.

2. Materials and methods

2.1. Materials

2-(*N*,*N*′-dimethylamino)ethyl methacrylate (DMAEMA, 98%), 2-bromoisobutyryl bromide (97%) were obtained from Alfa Aesar. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) (Rho-PE) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (ammonium salt) (NBD-PE) were from Avanti Polar Lipids. β-Propiolactone (98%), cholesterol (95%), dimethyldioctadecylammonium bromide (DDAB) and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) were purchased from J&K Scientific Ltd. 1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE) and 1, 2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000] (DSPE-PEG 2000) were purchased from Shanghai Advanced Vehicle



Scheme 1. Schematic diagram of DSPE-PCB lipoplexes for siRNA delivery with enhanced siRNA endosomal/lysosomal escape ability. a) Resistant nonspecific protein adsorption. b) Cellular endocytosis. c) Protonation of DSPE-PCB in endosomes/lysosomes. d) mRNA Cleavage.

Technology Ltd. Co. Copper (I) bromide (CuBr, 99%), *N,N,N′,N″,N″*-pentamethyldiethylenetriamine (PMDETA, 99%) and (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich. LysoTracker Red was obtained from Invitrogen. Lipofectamine™ 2000 was also from Invitrogen and used as per manufacturer's instructions. Cy5-labeled siRNA (Cy5-NC), FAM-labeled siRNA (FAM-NC), Luc-siRNA (targeting firefly luciferase) and ApoB siRNA (targeting ApoB mRNA) were custom synthesized by Suzhou Ribo Life Science Co. Ltd. (Jiangsu, China). Their sequences were as follows: Cy5-labeled siRNA: sense: 5′-Cy5-CCUUGAGGCAUA CUUCAAAdTdT-3′, antisense: 5′-UUUGAAGUAUGCCUCAAGGdTdT-3′; FAM-labeled siRNA: sense: 5′-CCUUGAGGCAUACUUCAAAdTdT-3′, antisense: 5′-UUUGAAGUAUGCCUCAAGGdTdT-3′; Luc-siRNA: sense: 5′-CCCUAUUCUCCUUCUUCGCdTdT-3′, antisense: 5′-GCGAAGAAGGAG AAUAGGGdTdT-3′; ApoB siRNA: sense: 5′-GUCAUCACACUGAAUACC AAUdTdT-3′, antisense: 5′-AUUGGUAUUCAGUGUGAUGACACdTdT-3′. All other reagents used were obtained commercially at analytical grade.

2.2. Sample synthesis

2.2.1. Synthesis of CB monomer

CB monomer was synthesized according to the method reported by Jiang and co-workers [31]. Briefly, β-propiolactone (0.43 g, 6 mmol) in 5 mL of dried dichloromethane was added dropwise to 25 mL of dried dichloromethane containing DMAEMA (0.79 g, 5 mmol). The reaction mixture was stirred under nitrogen protection at 10 °C for 12 h. The white precipitate was washed with 50 mL of dried dichloromethane and 50 mL of anhydrous acetone. The product was then dried under reduced pressure to obtain the final CB monomer product.

2.2.2. Synthesis of DSPE-Br initiator

Triethylamine (0.2 mL, 1.6 mmol) was added to 20 mL of dried dichloromethane containing DSPE (0.60 g, 0.8 mmol), and the mixture was stirred at 25 °C for 1 h. 2-Bromoisobutyryl bromide (0.18 g, 0.8 mmol) was then injected by syringe into the solution. After the injection was completed, the system was stirred for 12 h at 60 °C. The product was washed with deionized water for three times, and the dichloromethane was removed using the rotary evaporator.

2.2.3. Synthesis of DSPE-PCB_n polymer

DSPE-PCB_n was synthesized by the ATRP using DSPE-Br as the initiator. A typical synthesis procedure with the theoretical degree of polymerization (DP) of 50 is described as follows. DSPE-Br (17.94 mg, 0.02 mmol) was dissolved in 10 mL of dichloromethane, and CB (229.27 mg, 1.00 mmol) monomer was dissolved in 10 mL of ethanol. DSPE-Br, CB and CuBr (5.74 mg, 0.04 mmol) were added to a clean and dry Schlenk flask. The Schlenk flask was degassed by three freeze–pump–thaw cycles and recharged with nitrogen. PMDETA (6.93 mg, 0.04 mmol) dissolved in 1 mL of ethanol was injected into the frozen system. The Schlenk flask was stirred for 24 h at 60 °C. The impurities and unreacted monomers were removed by dialyzing in a Cellu SepH1-membrane (MWCO3500) against dichloromethane and deionized water for 48 h, respectively, and freeze-dried to obtain the final product. DSPE-PCB_n with theoretical DP of 20 was also prepared in a similar way.

The ^{1}H NMR spectra was recorded on a Bruker 300 MHz spectrometer. The degree of polymerization of DSPE-PCB_n and the structure of all the products were confirmed by ^{1}H NMR spectra.

2.3. Preparation and characterization of cationic liposomes and lipoplexes

Cationic liposomes were prepared by thin lipid film method [32,33]. DDAB (9.46 mg, 15 μmol), cholesterol (5.8 mg, 15 μmol) and DSPE-PCB_n (6 μmol) at a molar ratio of 1:1:0.4 were dissolved in chloroform. The organic phase was removed at 55 °C on a rotary evaporator to obtain a thin lipid film. Then, the lipid film was hydrated with 15 mL of phosphate buffer saline (PBS). After sonication at 37 °C for 30 min, the solution was extruded 5 times using EmulsiFlex-C5 high-pressure homogenizer (Avestin, Canada). The final total lipid concentration was 2.4 mM. The DDAB/cholesterol with same concentration, or DDAB/cholesterol with same concentration and DSPE-PEG 2000 (16.8 mg, 6 μmol) were dissolved in chloroform for the preparation of bare cationic liposomes or DSPE-PEG cationic liposomes.

Lipoplexes were prepared by diluting siRNA and cationic liposomes separately in equal volumes at designed N/P ratio (N: nitrogen moiety of DDAB, P: phosphorus one of siRNA). The cationic liposomes and siRNA were mixed together for 30 min at room temperature. The mean particle diameter of the cationic liposomes and lipoplexes was determined by dynamic light scattering (DLS), and the surface charge of the cationic liposomes and lipoplexes was analyzed by the zeta potential using a Zetasizer Nano ZS instrument (Malvern Instruments). Further morphological analysis was carried out by cryogenic transmission electron microscopy (Cryo-TEM, FEI Tecnai 20, The Netherlands).

2.4. Gel retardation assay

Lipoplexes with different N/P ratios were formed as described above. 20 μL of lipoplexes were mixed with 4 μL of 6× loading buffer, and then the mixture was loaded onto 2% agarose gel containing GelGreen™ nucleic acid stain. Electrophoresis was carried out at a voltage of 90 V for 10 min in 1× TAE running buffer. Finally, the results were recorded at UV with light wavelength of 254 nm.

2.5. siRNA encapsulation efficiency (E.E.)

To evaluate the efficiency of siRNA encapsulated in cationic liposomes, the fluorescence of un-encapsulated Cy5-labeled siRNA was quantified using fluorescence spectrophotometer (excitation at 633 nm, emission at 670 nm). The cationic liposomes and Cy5-labeled siRNA were mixed together for 30 min at room temperature to prepare cationic liposome/Cy5-labeled siRNA complexes at different N/P ratios. After centrifugation at 5000 ×*g* for 5 min, the fluorescence of the Cy5-labeled siRNA in the supernatant was detected using a fluorescence spectrophotometer (Hitachi, Japan). The encapsulation efficiency was calculated using the formula:

$$\text{Encapsulation efficiency } (\%) = (F_0 - F_1)/F_0 \times 100\%$$

where F_0 was the fluorescence of Cy5-labeled siRNA used for encapsulation, F_1 was the fluorescence of Cy5-labeled siRNA in the supernatant.

2.6. Serum stability of lipoplexes

The lipoplexes (N/P = 10/1) at a concentration of 1 mg/mL were incubated in DMEM containing 10% FBS at 37 °C under gentle stirring. At each time point, the mean diameter of the lipoplexes was calculated in triplicate using DLS.

2.7. Cytotoxicity measurement by MTT assay

To measure the cytotoxicity of the cationic liposome/siRNA complexes, MTT cell viability assay was performed against Hela-Luc cells. Briefly, Hela-Luc cells were seeded in 96-well plates at a concentration of 4×10^3 cells/well in 100 μL of DMEM containing 10% FBS, 1% penicillin (100 units/mL) and streptomycin (100 μg/mL) at 37 °C under a 5% CO_2 atmosphere for 24 h. To determine the cytotoxicity of the lipoplexes, lipoplexes with 0.25 μg siRNA at various N/P ratios, were added to 100 μL of DMEM with 10% FBS medium. After 24 h incubation, 20 μL of the MTT solution (5 mg/mL in PBS) was added to each well and incubated for additional 4 h at 37 °C. The medium and MTT were then replaced with 100 μL of DMSO, and the samples

were incubated at 37 °C for 5 min to dissolve the MTT formazan. The plates were gently shaken for 10 min to ensure the dissolution of formazan. The absorbance was measured at 490 nm using a Tecan microplate reader (Tecan, Switzerland). Lipofectamine™ 2000 was used as a positive control. Untreated Hela-Luc cells were used as a negative control and its cell viability was defined as 100%.

2.8. Flow cytometry measurements

FAM-labeled siRNA was used to determine the cellular uptake of the lipoplexes using flow cytometry. Briefly, Hela-Luc cells were seeded in 12-well plates at 1×10^5 cells per well in 500 μL of culture medium for 24 h. The cells were then incubated with lipoplexes at N/P ratio of 10/1, which contains 4 μg of siRNA in DMEM with 10% FBS medium. After 4 h incubation, the cells were rinsed three times with cold PBS, trypsinized and harvested in PBS. Then the samples were assessed with BD Calibur flow cytometry (BD Co., USA) to determine the fluorescence intensity of FAM (green).

2.9. Confocal microscopy

The intracellular trafficking of cationic liposomes with FAM-labeled siRNA was assessed using confocal microscopy. Briefly, 1×10^4 Hela-Luc cells were seeded in Petri dishes for 24 h, and the medium was replaced with DMEM containing 10% FBS, which involved the lipoplexes at N/P ratio of 10/1 including 0.17 μg FAM-labeled siRNA, and the cells were then incubated for 4 h at 37 °C. Subsequently, the cells were washed three times with PBS followed by staining with LysoTracker Red for 20 min at 37 °C. The cells were then washed three times with PBS and fixed with 4% paraformaldehyde for 10 min. Nuclei was stained with DAPI for 10 min. Red fluorescence of LysoTracker Red, blue fluorescence of DAPI and green fluorescence of FAM-labeled siRNA were observed using a Zeiss LSM780 confocal microscopy (Zeiss Co., Germany). To follow the endosomal/lysosomal escape of the lipoplexes, DSPE-PCB_{20} lipoplexes and DSPE-PEG lipoplexes with 1 μg FAM-labeled siRNA at N/P ratio of 10/1 were incubated with Hela-Luc cells for 4 h and 8 h, respectively. Following the operation mentioned above, cells were observed using confocal microscopy.

2.10. IN Cell Analyzer 2000

The endosomal/lysosomal escape of cationic liposomes with FAM-labeled siRNA was taken and analyzed with the IN Cell Analyzer 2000 system (GE Healthcare). Briefly, 4×10^3 Hela-Luc cells were seeded in 96-well plates in 100 μL medium at 37 °C for 24 h. The medium was replaced with fresh medium, which involved the lipoplexes at N/P ratio of 10/1 including 1/6 μg FAM-labeled siRNA, and the cells were then incubated for 4 h and 8 h at 37 °C, respectively. Subsequently, the cells were stained as described in confocal microscopy. The intensity of green fluorescence in red (I_1) and total green fluorescence (I_0) was calculated for each well by the IN Cell Analyzer 2000 system and the co-localization ratio was taken as a measurement of the endosomal/lysosomal escape of FAM-labeled siRNA. The co-localization ratio was calculated using the formula:

$$\text{Co-localization ratio } (\%) = I_1/I_0 \times 100\%.$$

2.11. Zeta potential measurements of lipoplexes at different pH

The cationic liposome/siRNA complexes at N/P ratio of 10/1 were incubated in phosphate buffer (PB) solution of different pH at 37 °C for 30 min. The zeta potential of the lipoplexes was measured with a Zetasizer Nano ZS instrument.

2.12. Lipid mixing assay of lipoplexes at different pH

Cationic liposomes containing DDAB: Cholesterol:DSPE-PCB_{20}: Rho-PE: NBD-PE = 1:1:0.4:0.1:0.1 (molar ratio) or DDAB: Cholesterol:DSPE-PEG 2000: Rho-PE: NBD-PE = 1:1:0.4:0.1:0.1 (molar ratio) were prepared by the method mentioned above. Cationic liposomes were mixed with siRNA at N/P ratio of 10/1. Anionic liposomes containing DSPG: Cholesterol: DOPE = 0.2:1:0.4 (molar ratio) were prepared the same way. For mixing experiments, 8 μL of DSPE-PCB_{20} lipoplexes were added to 1920 μL of PBS (pH = 7.4) or PB solution (pH = 5.0) and the fluorescence was measured as F_{min}. 72 μL of anionic liposomes were added, and the fluorescence (F) was measured after 5 min incubation at 37 °C. 50 μL of 15% C12E10 were then injected, and the fluorescence (F_{max}) was measured after 1 min. The lipid mixing was calculated using the formula:

$$\text{Lipid mixing } (\%) = (F - F_{min})/(F_{max} - F_{min}) \times 100\%.$$

All fluorescence measurements were made on a Spex Fluorolog fluorimeter at Ex/Em = 465/520 nm.

2.13. In vitro gene silencing efficiency

To assess the silencing capability of the lipoplexes *in vitro*, Hela-Luc cells were seeded in 24-well plates at 5×10^4 cells per well and grown to 60%–70% confluent prior to transfection. Lipoplexes at N/P ratio of 10/1 containing 1 μg Luc-siRNA, were added to 250 μL of culture medium containing 10% FBS, followed by incubation with cells at 37 °C under 5% CO_2 atmosphere for 24 h. The lipoplexes were then replaced with fresh culture medium and incubated for another 24 h. The cells were washed with 0.5 mL of PBS and lysed with reporter lysis buffer at 37 °C for 30 min. 50 μL of the lysate were mixed with 50 μL of substrate (Luciferase Assay System, Promega Co.) and the luciferase activity for each well was determined using a LB 960 microplate luminometer (Berthold Technologies, Germany). Luciferase down-regulation relative to untreated cells was determined for each condition.

2.14. In vivo distribution study

Male C57BL/6 mice 5–7 weeks old, with weight of 20–25 g, were purchased from the Academy of Military Medical Sciences of China. All procedures involving experimental animals were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of Peking University. Lipoplexes with Cy5-labeled siRNA at N/P ratio of 10/1 were administered to each mouse via tail vein injection at 2.5 mg siRNA/kg in a total volume of 200 μL. At each given time point, the fluorescence signal of the Cy5 from whole body was detected using a Kodak *in vivo* imaging system (Kodak In-Vivo Imaging System FX Pro. Carestream Health, USA). At designated times, mice were sacrificed, and the liver samples were harvested and examined.

2.15. In vivo ApoB siRNA down-regulation study

To assess the capability of cationic liposomes mediating gene silencing in the liver, ApoB siRNA (targeting ApoB mRNA expressed in the liver) was used. Male C57BL/6 mice were treated with cationic liposome/ApoB siRNA complexes at N/P ratio of 10/1 via tail vein injection at 2.5 mg siRNA/kg (n = 4). Three days later, the mice were sacrificed and the liver tissues and serum specimens were collected, followed by the evaluation of several parameters.

2.16. Statistical analysis

The data is summarized as the mean value with standard deviation of triplicate measurements. Unpaired Student's *t*-test was used to assess

statistical differences ($p < 0.05$) between the group means. All experiments were done in triplicate with a minimum of three independent experiments.

3. Results and discussion

3.1. Synthesis and characterization of DSPE-PCB$_n$ polymer

Herein, we synthesized DSPE-PCB$_n$ lipid for cationic liposomes modification. CB monomer was prepared by the conjugation of DMAEMA and β-propiolactone through the ring open reaction. ^{1}H NMR spectra recorded for CB is shown in Fig. S1a. ^{1}H NMR (300 MHz, D_2O): δ = 6.06: $-CH{=}CCH_3-$; δ = 5.85: $-CH{=}CCH_3-$; δ = 4.58: $-OCH_2CH_2N-$; δ = 3.70: $-OCH_2CH_2N-$; δ = 3.59: $-NCH_2CH_2C-$; δ = 3.10: $-NCH_3CH_3-$; δ = 2.64: $-NCH_2CH_2COO-$; δ = 1.84: $CH_2{=}CCH_3-$ [31]. The ^{1}H NMR spectra suggested the formation of the CB monomer (yield 95.29%).

The subsequent esterification reaction of the terminal amino group of the DSPE with 2-bromoisobutyryl bromide afforded DSPE-based ATRP initiator (DSPE-Br). The final structure of DSPE-Br was confirmed by ^{1}H NMR spectra (Fig. S1b). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.20: $-OCHCH_2O-P-$; δ = 4.40: $-CH_2COOCH_2-$; δ = 4.18: $-P-OCH_2CH_2-$; δ = 3.98: $-P-OCH_2CH-$; δ = 3.44: $-OCH_2CH_2N-$; δ = 2.25: $-COCH_2CH_2-$; δ = 1.86: $-BrCCH_3CH_3$; δ = 1.58: $-COCH_2CH_2-$; δ = 1.23: $-(CH_2)_{14}-$; δ = 0.88: $-CH_2CH_3$ (yield 74.53%) [34].

In the last step, DSPE-PCB$_n$ polymers were synthesized by the ATRP of DSPE-Br and CB with the CuBr/PMDETA as the catalyst system. The ^{1}H NMR spectra of DSPE-PCB$_n$ polymers are shown in Fig. S1c and Fig. S1d. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 4.10: $-OCH_2CH_2N-$; δ = 3.0–4.8: $-OCH_2CH_2NCH_2CH_2-$; δ = 2.60: $-NCH_2CH_2COO-$; δ = 2.25: $-NCH_3CH_3-$; δ = 1.80: $-BrCCH_2CH_3$; δ = 1.20–.28: $-(CH_2)_{14}-CH_3$; δ = 1.00: $-BrCCH_3-$. The ^{1}H NMR spectra suggested the formation of the DSPE-PCB$_n$ polymers. The peaks of DSPE-Br initiator ($-(CH_2)_{14}-CH_3$) and CB monomer ($-OCH_2CH_2N-$) concluded that the DPs of DSPE-PCB$_n$ polymer were 10 and 20 for the theoretical DPs of 20 and 50 products, respectively. Thus, DSPE-PCB$_{10}$ and DSPE-PCB$_{20}$ which had approximately equivalent molecular weights to commercially available product DSPE-PEG 2000 were used to investigate the influence of PCB on the property of cationic liposomes.

3.2. Size, zeta potential and morphology of the cationic liposomes and lipoplexes

The size of cationic liposomes and lipoplexes was characterized by DLS. As shown in Fig. 1a, the particle diameter of bare cationic liposomes, DSPE-PCB$_{10}$ cationic liposomes, DSPE-PCB$_{20}$ cationic liposomes and DSPE-PEG cationic liposomes were 126.9 ± 9.1 nm, 115.7 ± 9.6 nm, 119.0 ± 3.4 nm and 110.0 ± 4.5 nm, respectively. The spherical shape of cationic liposomes was confirmed by Cryo-TEM images (Fig. 1b). The average zeta potential of bare cationic liposomes, DSPE-PCB$_{10}$ cationic liposomes, DSPE-PCB$_{20}$ cationic liposomes and DSPE-PEG cationic liposomes were 44.5 ± 2.4 mV, 32.7 ± 3.2 mV, 23.4 ± 1.7 mV and 19.1 ± 0.4 mV, respectively. The zeta potential of DSPE-PCB$_{10}$ and DSPE-PCB$_{20}$ cationic liposomes were lower than that of bare cationic liposomes, indicating that PCB had a shielding effect on the surface charge of cationic liposomes as PEG did. After complexing with siRNA, the size of the lipoplexes increased and the zeta potential decreased due to the electrostatic interactions between cationic liposomes and siRNA. The fluorescence intensity of Cy5-labeled siRNA in supernatant after complexing was lower than that before complexing with cationic liposomes (Fig. 1c), indicating that siRNA had complexed with cationic liposomes.

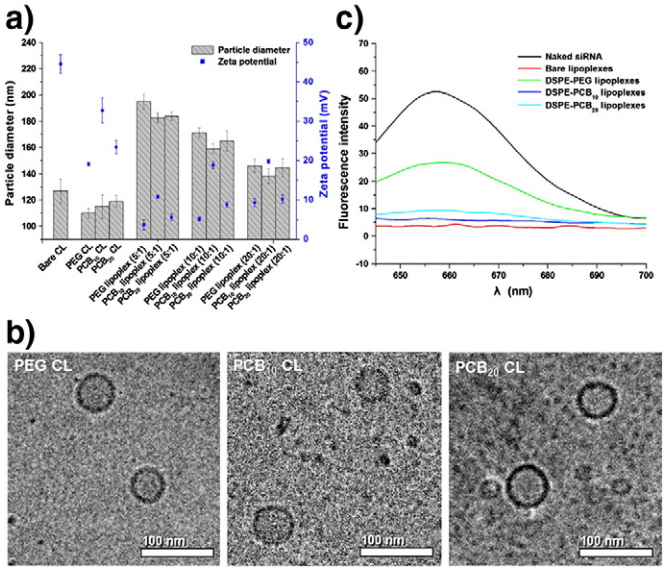


Fig. 1. a) Mean particle diameter and zeta potential of cationic liposomes and lipoplexes at various N/P ratios. b) Cryo-TEM images of cationic liposomes. c) Fluorescence intensity of Cy5-labeled siRNA in the supernatant after complexing with cationic liposomes (N/P = 10/1).

3.3. siRNA condensation results of the lipoplexes

Modification of cationic liposomes with PEG extends the circulation lifetime of the cationic liposomes. However, the PEGylation results in low gene entrapment efficiency due to the decline of positive charge [35,36]. It has been reported that zwitterionic poly (sulfobetaine) (PSB) with a quaternary ammonium group did not interfere with the DNA condensation of polymers [27]. This indicated that PCB, which had a positive quaternary ammonium group, might also have less effect on the entrapment efficiency compared to that of neutral PEG. Herein, agarose gel electrophoresis retardation assay was performed to investigate the influence of PCB coating on encapsulation ability of cationic liposomes. As shown in Fig. 2a, the complete retardation of siRNA was achieved at N/P ratios of 1/1, 5/1 and 10/1 for bare lipoplexes, DSPE-PCB$_{10}$ lipoplexes and DSPE-PCB$_{20}$ lipoplexes, respectively. DSPE-PCB$_{10}$ and DSPE-PCB$_{20}$ showed a slight down-regulation compared with bare lipoplexes. However, DSPE-PEG lipoplexes could retardate the siRNA completely at higher N/P ratio of 20/1. It was revealed that siRNA condensation capacity of DSPE-PCB$_{10}$ and DSPE-PCB$_{20}$ lipoplexes was higher than that of DSPE-PEG lipoplexes. The siRNA encapsulation efficiency was further measured using fluorescence spectrophotometer. It was demonstrated that the encapsulation efficiency of siRNA for DSPE-PCB$_{20}$ lipoplexes could reach 92% at N/P ratio of 20/1, but only 73% for DSPE-PEG lipoplexes. Even at N/P ratio of 5/1, the encapsulation efficiency of DSPE-PCB$_{20}$ lipoplexes was 67%, with only 39% for DSPE-PEG lipoplexes obtained (Fig. 2b). The rational reason might be that the cationic quaternary amine group of zwitterionic polymer PCB could facilitate the condensation of siRNA, which was favorable for the following gene transport.

3.4. Stability of the lipoplexes

In order to achieve significantly gene silencing efficiency *in vivo*, it is required that the delivery system for siRNA should be sufficiently stable in the circulatory system. The serum stability of bare lipoplexes, DSPE-PEG lipoplexes, DSPE-PCB$_{10}$ lipoplexes and DSPE-PCB$_{20}$ lipoplexes was evaluated in DMEM culture medium containing fetal bovine serum (FBS). The average particle diameter was measured to evaluate the stability of lipoplexes. As shown in Fig. 3,

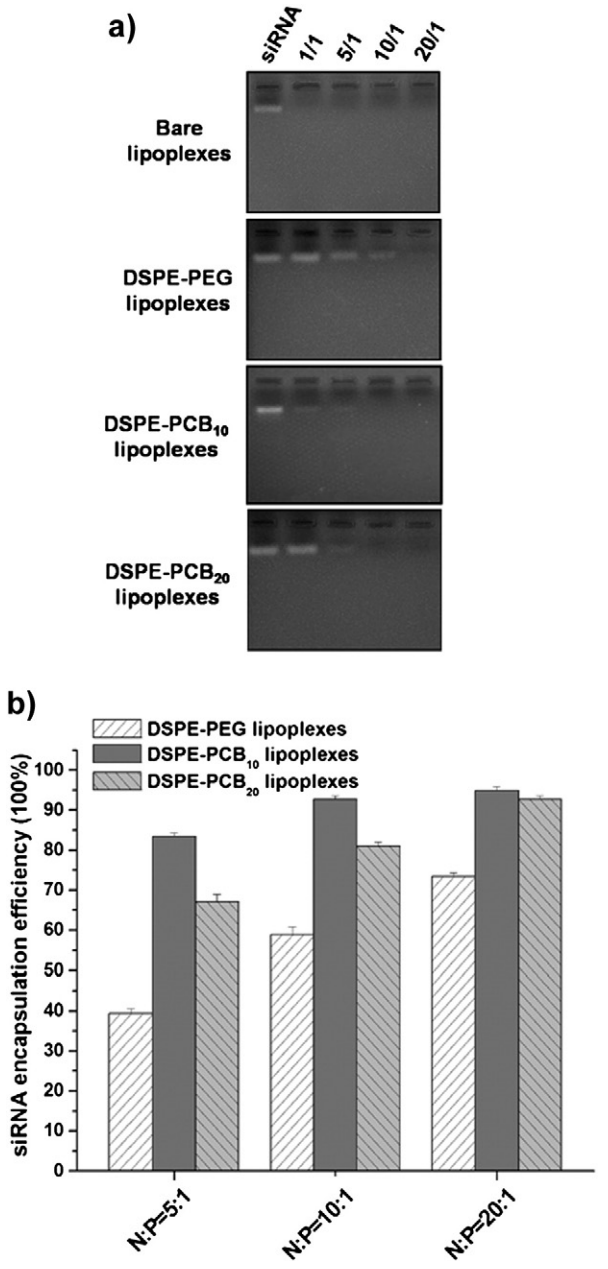


Fig. 2. a) Agarose gel electrophoresis retardation assay of bare cationic lipoplexes, DSPE-PEG lipoplexes, DSPE-PCB_{10} lipoplexes and DSPE-PCB_{20} lipoplexes. b) The siRNA encapsulation efficiency of DSPE-PEG lipoplexes, DSPE-PCB_{10} lipoplexes and DSPE-PCB_{20} lipoplexes at N/P ratios of 5/1, 10/1 and 20/1, detected by fluorescence spectrophotometer.

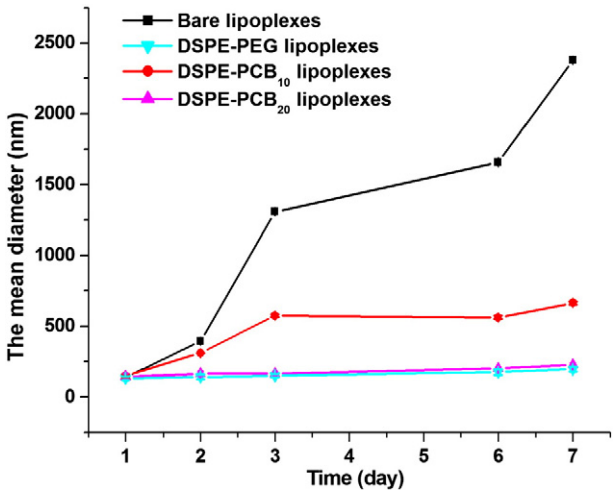


Fig. 3. Changes in particle diameters of the lipoplexes at N/P ratio of 10/1 following incubation with DMEM containing 10% FBS.

the diameter of bare cationic lipoplexes reached approximately 2400 nm after 7 days of incubation, indicating significant protein adsorption of bare cationic lipoplexes. In the case of DSPE-PCB_{20} lipoplexes, only a slight increase in size from 150 nm to approximately 200 nm after 7 days of incubation was observed. The result suggested that DSPE-PCB_{20} molecules were distributed on the outer surface of the lipoplexes, which can prevent nonspecific protein adsorption and resist aggregation of the lipoplexes. It is worth noting that the stability of DSPE-PCB_{20} was equivalent to commercial DSPE-PEG 2000 in the experimental range. The diameter of the DSPE-PCB_{10} lipoplexes increased from 140 nm to approximately 700 nm after 7 days of incubation, indicating that PCB_{10} was not as effective as PCB_{20} for protecting the lipoplexes from protein adsorption. In view of the above discussion, DSPE-PCB_{20} was selected for a detailed comparative study with DSPE-PEG 2000 in the further work, since its protein resistant adsorption ability was comparable with that of DSPE-PEG 2000.

3.5. In vitro cytotoxicity of the lipoplexes

It has been shown that the free positive charge outside lipoplexes facilitates the cellular uptake through a nonspecific electrostatic interaction between lipoplexes and negatively charged cell membranes. On the other hand, more free positive charge was also a major underlying cause for cytotoxicity. For this reason, the MTT assay was employed in Hela-Luc cells to evaluate the cytotoxicity of the DSPE-PCB_{20} lipoplexes, DSPE-PEG lipoplexes and Lipofectamine 2000. The cell viability of DSPE-PEG lipoplexes and DSPE-PCB_{20} lipoplexes reduced with the increase of N/P ratios, since higher concentration of cationic liposomes led to relatively higher cytotoxicity. Cell viability of DSPE-PCB_{20} complexes at N/P ratio of 5/1 was 97% after 24 h of incubation, and nearly 88% at N/P ratio of 20/1 (Fig. S2). The cytotoxicity of the DSPE-PCB_{20} was comparable to that of DSPE-PEG lipoplexes at the same N/P ratio, and much lower than that of Lipofectamine 2000. The results suggested that the DSPE-PCB_{20} polymer had the same good biocompatibility as DSPE-PEG 2000.

3.6. Cellular uptake and endosomal/lysosomal escape of the lipoplexes

The surface shielding of gene delivery systems with PEG has become a preferential strategy to prolong blood circulation [37]. However, the gene transport efficiency may be compromised by PEGylation, for the steric and electrostatic hindrance to entry target cells caused by PEG. Moreover, the PEG layer can still act as a barrier for the efficient escape of endosomes/lysosomes even after the lipoplexes are endocytosed by the target cell [38,39]. As a potential long circulation candidate for gene delivery, PCB has been proven to reduce protein adsorption and aggregation. A major concern is whether DSPE-PCB_{20} lipoplexes could be internalized by the cells and escape from the endosomes/lysosomes efficiently.

The cellular uptake and intracellular distribution of the lipoplexes were studied by flow cytometry and confocal microscopy. As shown in Fig. 4a, naked siRNA showed low level cellular internalization, only a tiny amount of Hela-Luc cells displayed green fluorescence. Cationic liposomes led to an increase of intracellular siRNA fluorescence, and more fluorescence was observed for Lipofectamine 2000, DSPE-PEG lipoplexes and DSPE-PCB_{20} lipoplexes. The DSPE-PCB_{20} based siRNA

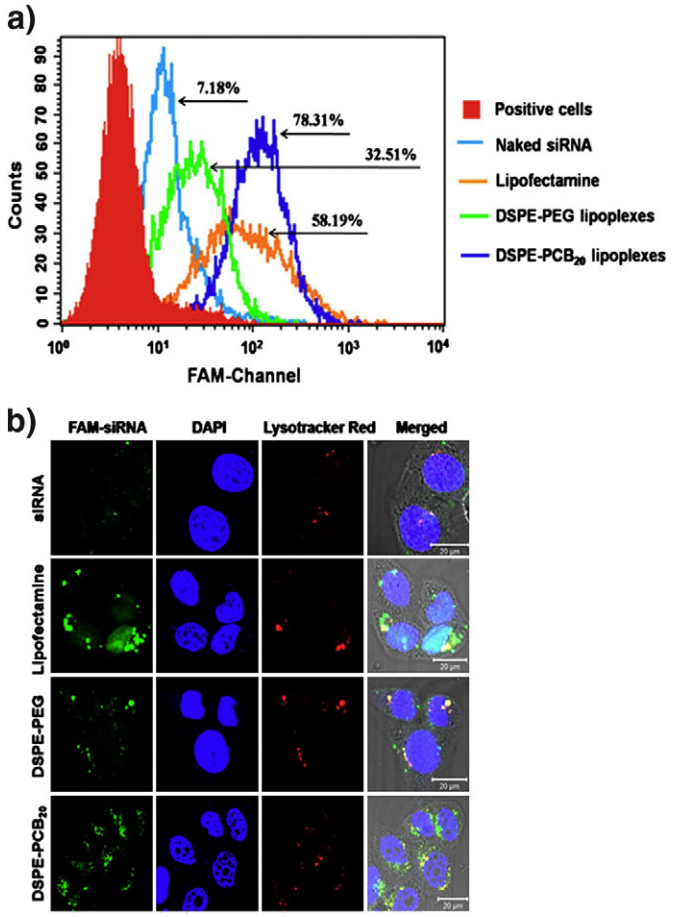


Fig. 4. a) Flow cytometry analyses of Hela-Luc cells after incubation with naked FAM-labeled siRNA and lipoplexes at N/P ratio of 10/1. b) Confocal microscopy images of intracellular trafficking of lipoplexes (N/P = 10/1) in Hela-Luc cells. Cell nuclei and endosomes/lysosomes were counterstained with DAPI (blue) and LysoTracker Red (red), respectively.

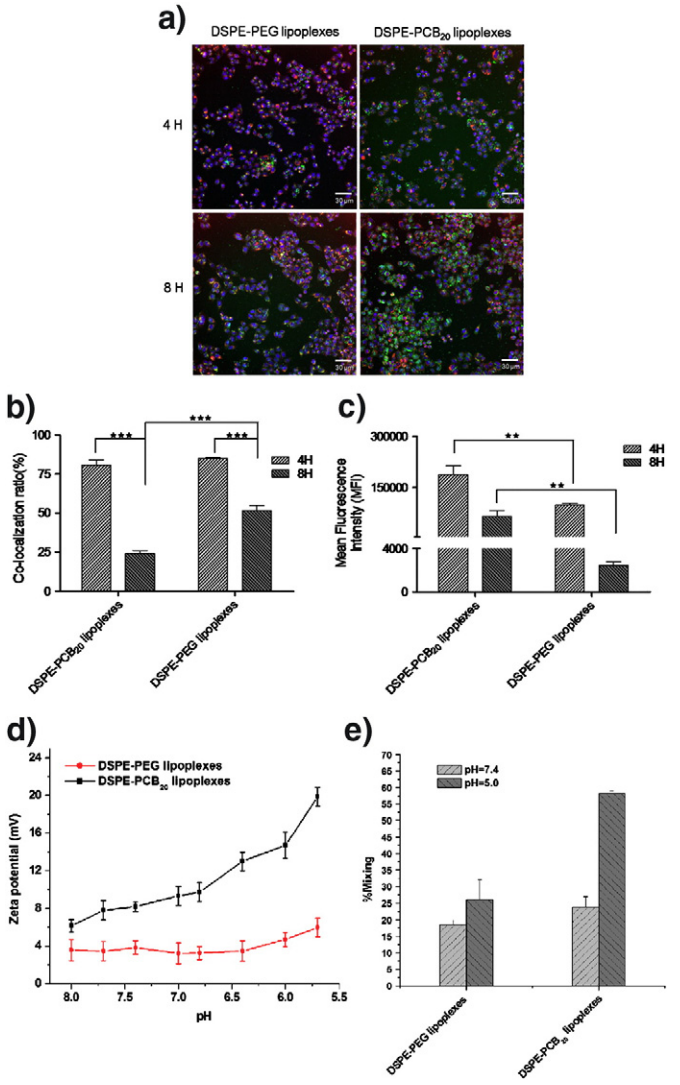


Fig. 5. a) Assessment by IN Cell Analyzer 2000 of endosomal/lysosomal escape of lipoplexes at N/P ratio of 10/1 in Hela-Luc cells after incubation for 4 h and 8 h. The endosomes/lysosomes and cell nucleic were counterstained with LysoTracker Red and DAPI, respectively. b) The co-localization ratio of intensity of green fluorescence in red and total green fluorescence was qualified with IN Cell Analyzer 2000. c) Mean green fluorescence intensity of total FAM-labeled siRNA was qualified with IN Cell Analyzer 2000. d) Changes in zeta potential of lipoplexes at N/P ratio of 10/1 following incubation with different pH of PB solution. e) Lipid mixing between lipoplexes (N/P = 10/1) and anionic liposomes at pH 7.4 and pH 5.0 detected by FRET.

lipoplexes showed 78% cellular internalization, much higher than that of DSPE-PEG lipoplexes (32%) and Lipofectamine 2000 (58%). The results were further confirmed by confocal microscopy. Stronger green fluorescence was found in cells incubated with DSPE-PCB_{20} lipoplexes than with DSPE-PEG lipoplexes and Lipofectamine 2000 (Fig. 4b). The excellent cellular uptake of DSPE-PCB_{20} lipoplexes might be related to the synergistic effect of higher siRNA encapsulation efficiency and excellent protein resistant adsorption ability of DSPE-PCB_{20} lipoplexes.

Endosomal/lysosomal escape of siRNA after entering the cell is important for subsequent post-transcriptional gene silencing in the cytoplasm [40]. Herein, intracellular trafficking of the cationic liposome/FAM-labeled siRNA complexes against Hela-Luc cells was evaluated by IN Cell Analyzer 2000 system. The endosomes/lysosomes and cell nuclei were counterstained with LysoTracker Red and DAPI, respectively. As shown in Fig. 5a, DSPE-PEG lipoplexes were almost completely entrapped in endosomes/lysosomes after 4 h, as shown by the co-location of red and green fluorescence, indicating that the lipoplexes resided in endosomes/lysosomes. After 8 h of incubation, DSPE-PEG lipoplexes carrying FAM-labeled siRNA were still mainly colocalized with LysoTracker Red stained organelles. Comparatively, the separation of green and red fluorescence was significant when cells were incubated with DSPE-PCB_{20} lipoplexes, suggesting that DSPE-PCB_{20} lipoplexes could escape from endosomes/lysosomes efficiently and release siRNA into the cytoplasm. The co-localization ratio between endosomes/lysosomes and FAM-labeled siRNA of DSPE-PCB_{20} lipoplexes and DSPE-PEG lipoplexes was also quantitatively studied by IN Cell Analyzer 2000 system, and fluorescence signals of LysoTracker Red and FAM-labeled siRNA in each well were normalized over the DAPI signal. As shown in Table 1, the number of wells for each sample was 5, and I_1 was the intensity of FAM-labeled green fluorescence colocalized with endosomes/lysosomes (red fluorescence) and I_0 was the total green fluorescence of FAM-labeled siRNA. The ratio between I_1 and I_0 was taken as a measurement of co-localization between endosomes/lysosomes and FAM-labeled siRNA. As summarized in Fig. 5b, the co-localization ratio of DSPE-PCB_{20} lipoplexes and DSPE-PEG lipoplexes for 4 h was almost the same, about 80.5 ± 3.4% and 85.1 ± 1.4%, respectively. The ratio of DSPE-PEG lipoplexes for 8 h was 51.4 ± 7.8%, while it was only 24.0 ± 4.1% for DSPE-PCB_{20} lipoplexes. It meant that the FAM-labeled siRNA of DSPE-PCB_{20} lipoplexes was less co-localized with ensosomes/lysosomes and could more efficiently escape from endosomes/lysosomes, which was consistent with the results from confocal microscopy (Fig. S3). The intensity of

Table 1
Fluorescence intensity of FAM-labeled siRNA qualified with IN Cell Analyzer 2000 system.

Wells	DSPE-PEG lipoplexes—4 h				DSPE-PCB_{20} lipoplexes—4 h				DSPE-PEG lipoplexes—8 h				DSPE-PCB_{20} lipoplexes—8 h			
	Cells	I_1 (E + 08)	I_0 (E + 08)	I_1/I_0%	Cells	I_1 (E + 08)	I_0 (E + 08)	I_1/I_0%	Cells	I_1 (E + 08)	I_0 (E + 08)	I_1/I_0%	Cells	I_1 (E + 08)	I_0 (E + 08)	I_1/I_0%
1	2915	2.01	2.41	83.40	2354	4.18	5.54	75.54	4183	0.0438	0.0756	57.94	3492	0.887	3.45	25.71
2	2617	2.65	3.04	87.17	3003	3.82	4.90	77.96	3913	0.0384	0.0645	59.33	3865	0.160	0.540	29.63
3	2568	2.03	2.41	84.23	2315	4.08	4.85	84.12	4286	0.0495	0.0937	52.83	3639	0.259	1.07	24.21
4	3780	3.28	3.86	84.97	2318	4.63	5.52	83.87	4319	0.0484	0.114	42.46	3552	0.886	3.94	22.49
5	2991	3.26	3.79	86.04	3129	5.27	6.50	81.07	4112	0.0677	0.152	44.54	3445	0.635	3.49	18.19

total green fluorescence of DSPE-PCB_{20} lipoplexes calculated by the IN Cell Analyzer 2000 was stronger than that of DSPE-PEG lipoplexes, which corresponded with the results from flow cytometry (Fig. 5c). It has been proved that non-pH responsive zwitterionic lipids lacked the ability to promote endsomal/lysosomal escape because they could not be protonated at endocytic pH [41]. Therefore, it is reasonable to assume that the improvement of endosomal/lysosomal escape of siRNA might result from the pH-sensitive PCB coating outside the DSPE-PCB_{20} lipoplexes.

To confirm this hypothesis, zeta potential of lipoplexes measurement experiment was performed at different pH values, mimicking the microenvironments that the lipoplexes might encounter upon cellular internalization through an endocytotic process. The zeta potential of DSPE-PCB_{20} cationic liposome/siRNA complexes at N/P ratio of 10/1 was evaluated by culturing the lipoplexes in PB with different pH from 7.4 to 4.5, which was consistent with the pH from the extracellular matrix to the lysosomes [42]. The zeta potential of DSPE-PEG lipoplexes had no significant change over the pH range studied, whereas DSPE-PCB_{20} lipoplexes showed a pH-dependent zeta potential from 8.19 ± 0.53 mV at pH 7.4 to 24.6 ± 0.87 mV at pH 4.5 (Fig. 5d). The increase in positive charge of DSPE-PCB_{20} lipoplexes was due to the protonation of carboxyl acid groups of PCB at the surface of lipoplexes under acidic condition. The observation was in agreement with the pH dependent kinetics of PCB as published previously [31].

To further confirm that the pH-sensitive DSPE-PCB_{20} promoted the interaction between lipoplexes and endosomal/lysosomal membrane, a fluorescence resonance energy transfer (FRET) based lipid mixing assay was conducted to quantitatively measure the lipid mixing triggered by membrane fusion. The PE-conjugated FRET probes lissamine rhodamine B (Rho-PE) and 7-nitrobenzo-2-oxa-1,3-diazole (NBD-PE) were combined into a single DSPE-PCB_{20} lipoplex or DSPE-PEG lipoplex. The distance between Rho-PE and NBD-PE is so close that the fluorescence of NBD is attenuated due to FRET with Rho. Upon lipid mixing with anionic liposome membrane, NBD signal increased due to an increase in average distance between Rho-PE probe and NBD probe as they might redistribute across the two membranes [41]. As shown in Fig. 5e, the lipid mixing between DSPE-PEG lipoplexes and anionic liposomes was 20% and there was a slight increase of 6% when the pH value decreased to 5.0. The lipid mixing between DSPE-PCB_{20} lipoplexes and anionic liposomes was 23% at pH 7.4, which is roughly equivalent to that of DSPE-PEG lipoplexes with the same pH values. While there was approximately 60% lipid mixing between DSPE-PCB_{20} lipoplexes and anionic liposomes when the pH value decreased to 5.0, two times higher than that at pH 7.4. It proved that the lipid fusion between DSPE-PCB_{20} lipoplexes and anionic membrane was correlated with the pH values, and the protonation of DSPE-PCB_{20} in acidic environment promoted the fusion of lipoplexes with anionic membrane. On the basis of these results, we speculated that PCB polymer brush was expected to be in a protonated state after the DSPE-PCB_{20} lipoplexes were internalized into cells, which resulted in a more positively charged surface of DSPE-PCB_{20} lipoplexes. The formation of positively charged PCB-lipids facilitated the destabilization of endosomal/lysosomal membrane by forming ion-pairs with anionic endosomal/lysosomal membrane lipids, and releasing siRNA into the cytoplasm. Moreover, the positively charged DSPE-PCB_{20} reduced the electrostatic hindrance to cationic DDAB lipids and promoted the fusion between cationic DDAB lipids and anionic endosomal/lysosomal membrane [43,44]. Thus, PCB polymer brush is particularly desirable for endosomal/lysosomal escape of siRNA and is an excellent alternative to PEG.

3.7. Silencing efficacy mediated by the lipoplexes in vitro

Hela-Luc cells, which could stably express luciferase, were used to evaluate the gene silencing efficiency of DSPE-PCB_{20} cationic liposome/Luc-siRNA complexes *in vitro*. As shown in Fig. 6, the silencing efficiency of DSPE-PCB_{20} lipoplexes could reach 76% at N/P ratio of 10/1, while only 41% for DSPE-PEG lipoplexes. Higher silencing efficiency of DSPE-PCB_{20} lipoplexes was attributed to the superior cellular uptake and endosomal/lysosomal escape performance of the DSPE-PCB_{20} lipoplexes.

3.8. In vivo distribution of the lipoplexes

In vivo distribution of cationic liposome/Cy5-labeled siRNA complexes was performed *via* tail vein injection. Comparison images of the biodistribution *in vivo* are shown in Fig. S4, naked siRNA exhibited weak fluorescence signal in the animal body. For the DSPE-PCB_{20} lipoplexes and DSPE-PEG lipoplexes, much siRNA uptake was observed

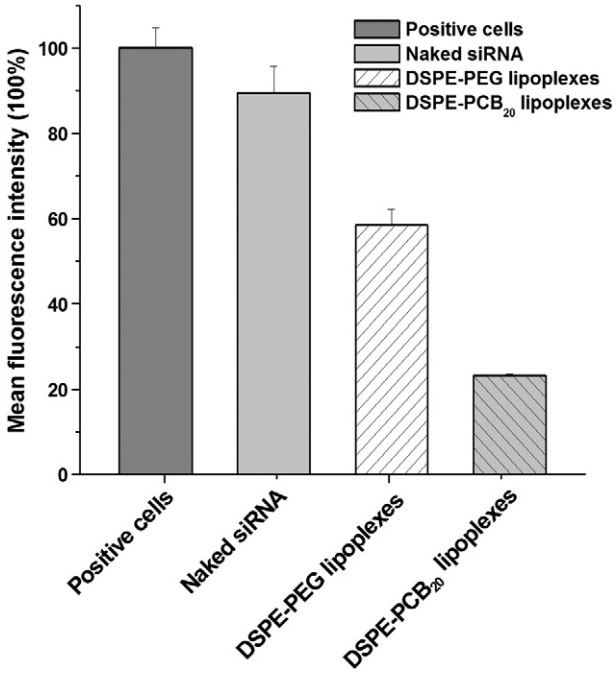


Fig. 6. *In vitro* gene silencing efficiency of lipoplexes at various N/P ratios verified by luciferase assay.

for many organs. In addition, siRNA in DSPE-PEG lipoplexes accumulated more than that of DSPE-PCB_{20} lipoplexes in bladder, which is the major excretory organ [45]. Those results indicated that siRNA in DSPE-PEG lipoplexes excreted from circulatory system was more than that in DSPE-PCB_{20} lipoplexes. Therefore, it could be concluded that DSPE-PCB_{20} lipoplexes had long duration in the circulatory system.

Apolipoprotein B (ApoB), which serves as the scaffold to solubilize cholesterol and fatty acids for secretion into the blood, was expressed in the liver [46]. Herein, the accumulation and distribution of siRNA in the liver isolated from the treated mice was observed by additional histological observations (Fig. 7). It was revealed that the magnitude of intracellular signals of DSPE-PCB_{20} lipoplexes was superior compared to that of DSPE-PEG lipoplexes in the liver tissue.

3.9. In vivo functional study of the lipoplexes

ApoB siRNA was used to evaluate the ability of DSPE-PCB_{20} based lipoplexes to mediate gene silencing in the liver. DSPE-PCB_{20} lipoplexes and DSPE-PEG lipoplexes containing ApoB siRNA at N/P ratio of 10/1 were applied to mice *via* tail vein injection. Three days later, the mice were sacrificed and specimens were collected and evaluated. Reduction in ApoB mRNA was significantly enhanced by DSPE-PCB_{20} lipoplexes compared to that of DSPE-PEG as reflected by the ApoB knockdown values. In Fig. 8a, DSPE-PCB_{20} lipoplexes treated livers showed an inhibition of 62% of ApoB mRNA expression, while only 7% for DSPE-PEG lipoplexes. A combined effect of the excellent liver accumulation and efficient cellular transport of siRNA by DSPE-PCB_{20} lipoplexes could be the main reason for the significant suppression of AopB mRNA expression. In addition, detection of cholesterol in serum experiment showed the same trend, and cholesterol in the serum of mice treated with DSPE-PCB_{20} lipoplexes was 25% lower than that of mice treated with DSPE-PEG lipoplexes at the end of 3 days of treatment (Fig. 8b). The level of

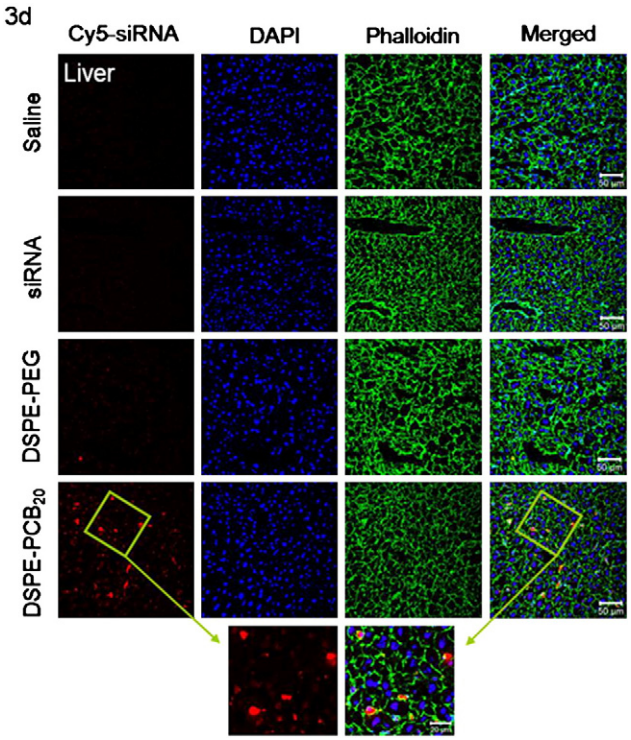


Fig. 7. Liver section observed under confocal microscopy. Cell nuclei and F actin were counterstained with DAPI (blue) and Phalloidin (green), respectively.

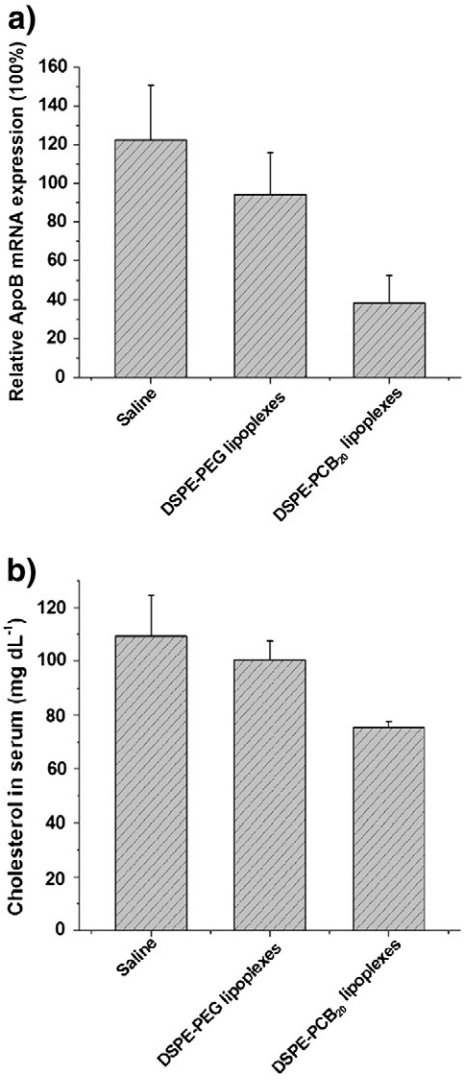


Fig. 8. *In vivo* functional study of lipoplexes in C57BL/6 mice. a) Relative ApoB mRNA expression in the liver has been analyzed after 3 days of the injection. C57BL/6 mice received intravenous injection of DSPE-PEG lipoplexes or DSPE-PCB_{20} lipoplexes at a dose of 2.5 mg ApoB siRNA/kg. b) The measurement of total CHO in serum. Each bar represents the mean ± SEM (n = 4). $^{*}P < 0.05$, and $^{***}P < 0.001$ *vs* the saline treated group.

ApoB mRNA was reduced due to the silencing of effective ApoB siRNA, and fewer lipid molecules such as cholesterol were secreted into the blood from the liver. This phenomenon resulted in consequently decreasing total cholesterol in the serum. The effect of ApoB knockdown *in vivo* with the DSPE-PCB_{20} lipoplexes may have good application in hypercholesterolemia-related diseases.

4. Conclusions

In summary, the findings reported herein show that the persistent circulation of DSPE-PCB_{20} lipoplexes can be used as a successful siRNA delivery system. At the same N/P ratio, the DSPE-PCB_{20} lipoplexes showed superior serum stability, siRNA encapsulation efficiency and cellular uptake compared to the DSPE-PEG lipoplexes *in vitro*. Quite different from DSPE-PEG, the zwitterionic DSPE-PCB_{20} could offer stability without sacrificing the endosomal/lysosomal escape capacity of lipoplexes, which was particularly favorable for siRNA delivery system. *In vivo* functional studies indicated that ApoB siRNA could be successfully delivered to the liver by the DSPE-PCB_{20} lipoplexes. The level of ApoB

mRNA was repressed and resulted in a decrease of total cholesterol in the serum, indicating that DSPE-PCB_{20} lipoplexes might have promising application in hypercholesterolemia-related disease.

Acknowledgments

This work was financially supported by the National Natural Science Foundation of China (Grants 51103159, 51203162, 81273422, 21304099, 51373177), the National Key Basic Research Program of China (2009CB930200, 2011CBA01100), the National High Technology Research and Development Program (Grants 2012AA020804, 2012AA022703, 2014AA020708), the Foundation for Innovative Research Groups of the National Natural Science Foundation of China (31221002), the National High-tech R&D Program of China (2012AA022501), the National Drug Program (2011ZX09102-011-12, 2012ZX09102301-006), the Youth Foundation of National Key Laboratory of Biochemical Engineering, Institute of Process Engineering, Chinese Academy of Sciences (2012), the Instrument Developing Project of the Chinese Academy of Sciences (YZ201253), and the Strategic Priority Research Program of the Chinese Academy of Sciences (A)-Revolution of Nano-industry Manufacturing Technology Focus).

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jconrel.2013.12.007.

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