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Doping Hydroxylated Cationic Lipid into PEGylated Cerasome Boosts *in Vivo* siRNA Transfection Efficacy

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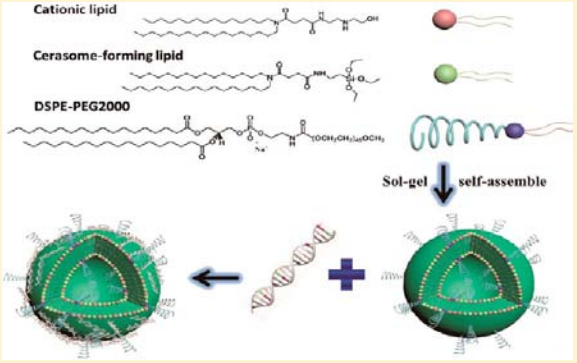
Supporting Information

ABSTRACT: The therapeutic application of small interfering RNA (siRNA) requires safe nanocarriers for specific and efficient delivery *in vivo*. Herein, PEGylated cationic cerasomes (PCCs) were fabricated by doping a cationic lipid with a hydroxyl group into nanohybrid cerasomes. Multiple properties of PCCs provide a solution to many of the limitations associated with current platforms for the delivery of siRNA. The polyorganosiloxane surface imparts PCCs with higher morphological stability than conventional liposomes. The PEGylation of the cationic cerasome could protect the cerasome nanoparticles from agglomeration and macrophage capture, reduce protein absorption, and consequently prolong the blood circulating time and enhance the siRNA delivery efficiency. In addition, incorporation of the lipid containing a hydroxyl group further facilitates endosome release. Moreover, PCCs were further used to transport siRNA into the cytosol primarily via endocytosis. When applied to systemic administration, PCCs have demonstrated effective delivery into the liver and preferential uptake by hepatocytes in mice, thereby leading to high siRNA gene-silencing activity. All these results show potential therapeutic applications of PCCs-mediated delivery of siRNA for liver diseases.



INTRODUCTION

It has been increasingly attractive to target specific genes associated with various incurable human diseases.[1,2] RNA interference (RNAi) is currently one of the leading platform technologies being developed in clinics to prevent the expression of unwanted genes, and it may represent the next generation of new therapeutics.[3] By delivering the desired small interfering RNAs (siRNAs) to the specific cells,[4] it is possible to block the production of disease-causing proteins. However, siRNA is anionic, hydrophilic, and nucleases-degradable,[5] causing cellular uptake limitations, renal elimination, and enzymatic digestion in plasma.[6] The therapeutic potential of siRNAs is severely limited by the availability of delivery systems which protect siRNA from degradation, deliver it to the target cell with high specificity and efficiency, and promote its endosomal escape and cytosolic dispersion. Therefore, it is extremely crucial to develop an ideal delivery vehicle to improve siRNA intracellular transportation, pharmacokinetics, and biodistribution properties.[7−9]

Viral-based vectors are effective at delivering exogenous constructs to facilitate gene overexpression, but their intrinsic serious drawbacks (i.e., adverse effects on host genome integrity, immunological consequences, etc.) hinder progression in the clinic.[10,11] Non-viral-based nanoparticles such as polymers,[12] inorganic nanoparticles,[13] micelles,[14] and lipid materials[15] are widely investigated. Lipid-based gene transfer was one of the earliest strategies used to introduce genetic materials into host cells.[16−18] Precise formulation of genetic materials into liposomal nanoparticles with lipids and other molecular components (i.e., polyethylene glycol (PEG)−lipid,[19,20] cholesterol,[21] peptide,[22,23] etc.) has resulted in their improved bioavailability. As one of the most promising gene delivery systems, cationic liposomes offer advantages such as biomimetic characteristic, tolerance for variations, and easy formation of lipoplexes.[24] In addition, cationic liposomes can provide reduced siRNA renal clearance and protect siRNA from enzymatic degradation. However, liposomes still have not attained their full potential as gene delivery vehicles because of

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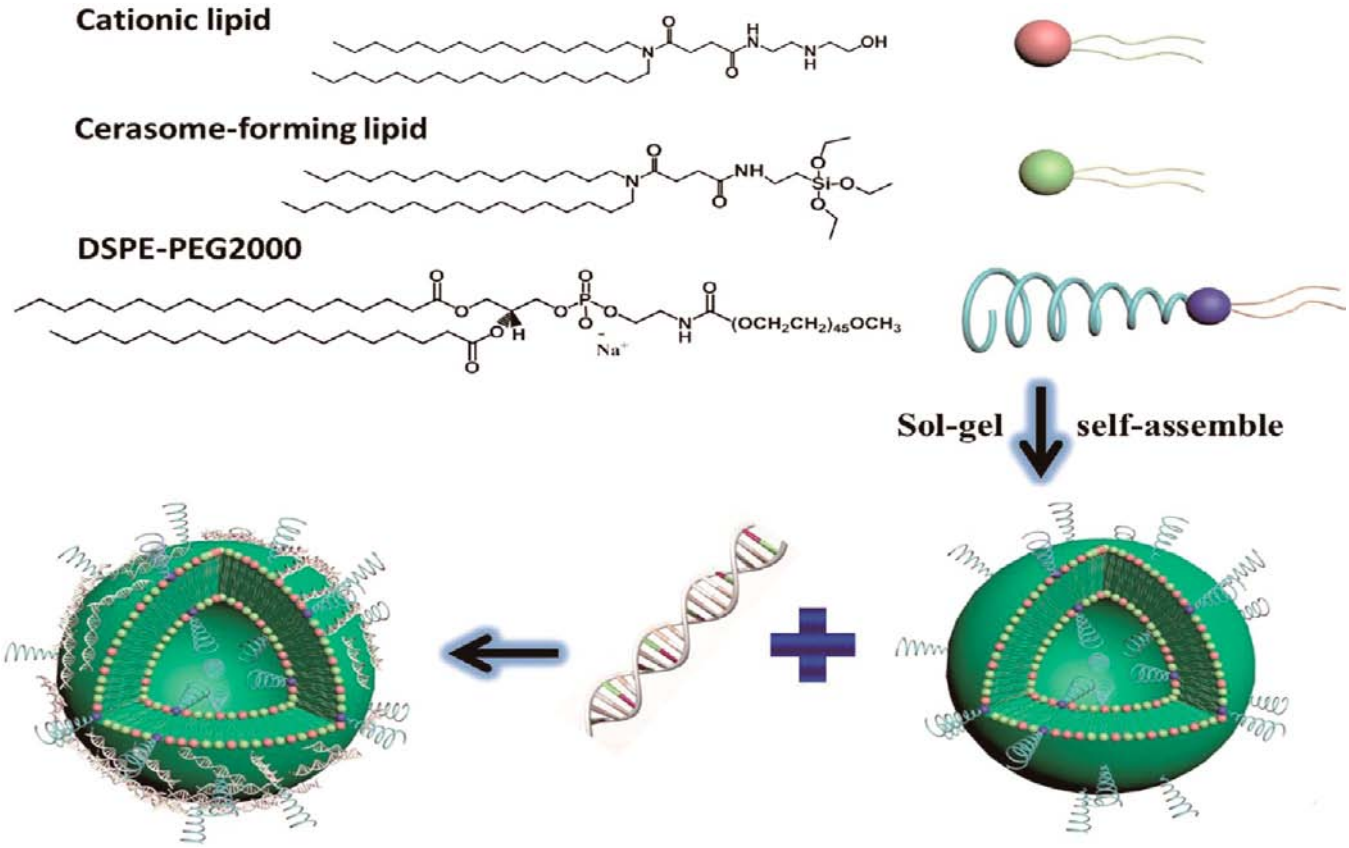
Scheme 1. Schematic Illustration of the Formation of the PEGylated Cationic Cerasome (PCC) for Efficient siRNA Delivery



their insufficient morphological stability. The systemic delivery of siRNA may be hindered by the instability of lipoplexes under physiological conditions. The main hurdles to affect the transfection efficiency of siRNA include the rapid plasma clearance of the carrier and siRNA endosome release after cell uptake. Therefore, safe strategies to enhance the transfection efficiency of siRNA remain a challenge in gene therapy. With the persistent exploration of cationic lipids with diverse structures, some research groups found that cationic lipid head-group manipulation is a strategy to further improve the transfection efficiency.[11,16,25] Nakanishi et al. found that the hydroxyl group of the cholesterol derivative affects the stability of the liposome−DNA complexes and enhances the transfection efficiency by facilitating the endosome-releasing process.[26] Because of differences between DNA and siRNA delivery, we were wondering whether the lipids with a hydroxyl group can improve siRNA transfection efficiency.

Recently, liposomal nanohybrid cerasome has attracted intensive research interests as an ideal type of drug delivery system because its surface atomic layer of polyorganosiloxane imparts higher morphological stability than conventional liposomes and its liposomal bilayer structure reduces the overall rigidity and density greatly compared to silica nanoparticles.[27−29] It has been demonstrated that cerasomes exhibit remarkably high stability toward surfactant solubilization, long-term storage, acidic media, and all factors known to destabilize conventional liposomes. Cationic cerasomes, prepared from a kind of lipid containing cationic moieties and organoalkoxysilane head-groups, were reported to be an infusible and cell-friendly carrier for siRNA delivery.[30,31] However, the synthesis procedures are very complicated and time-consuming.[30,32]

Since both the hydroxyl group and PEG chain of the lipid appear to facilitate siRNA delivery, in this study we fabricated PEGylated cationic cerasomes (PCCs) from a cerasome-forming lipid (CFL) of *N*-[*N*-(3-triethoxysilyl)propylsuccinamoyl]dihexadecylamine by doping a PEGylated lipid of DSPE-PEG2000 and a hydroxylated cationic lipid (HCL) of N^1,N^1-dihexadecyl-N^4-(2-(2-hydroxyethylamino)ethyl)-succinamide employing the sol−gel process and self-assembly technique as demonstrated in Scheme 1. The PEGylation of the cationic cerasome by incorporating DSPE-PEG2000 could protect the cerasome nanoparticles from agglomeration and macrophage capture, reduce protein absorption, and consequently prolong the blood circulating time.[7,11] The physicochemical properties and intracellular trafficking behavior of these PCCs were investigated. The delivery efficacy of siRNA was examined *in vitro* and *in vivo* as well. The unique characteristics of PCCs address many of the deficiencies associated with current liposome technology.

RESULTS AND DISCUSSION

Preparation and Characterization of PCCs. Both CFL and HCL have the same hydrophobic double-chain segment derived from dihexadecylamine. The HCL was synthesized by coupling dihexadecylamine with succinic anhydride, followed by coupling with 2-(2-aminoethylamino)ethanol. Detailed synthesis procedures and characterization data are shown in the Supporting Information. PCCs were fabricated from the mixture of CFL, HCL, and DSPE-PEG2000 according to the Bangham method[33] combined with a sol−gel reaction and self-

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assembly process. The content of DSPE-PEG2000 was fixed at 5 mol % in the mixture of CFL, HCL, and DSPE-PEG2000. Since the ratio and combination of cationic/helper lipid contribute greatly to the transfection efficiency of liposomes,[24] six molar ratios of CFL to HCL (2:1, 1:1, 1:3, 1:6, 1:9, and 0:10) were used to prepare PCCs in order to optimize the composition of PCCs. For comparative study, cationic cerasomes with no PEGylation (CCs) were also prepared from the mixtures of CFL and HCL. Upon ultrasonication, the vesicular membrane formed and self-rigidified via *in situ* sol−gel processes ($Si{-}OCH_2CH_3 + H_2O \rightarrow Si{-}OH + CH_3CH_2OH$, followed by $2Si{-}OH \rightarrow Si{-}O{-}Si + H_2O$) on the surface. Detailed results of the size and zeta-potential of PCCs prepared at various molar ratios are displayed in Table 1. The mean size

Table 1. Particle Size, Zeta-Potential, and Polydispersity of Non-PEGylated and PEGylated Cationic Cerasomes with Various Ratios of CFL to HCL

CFL:HCL	particle size (nm)	zeta-potential (mV)	polydispersity
no DSPE-PEG2000			
2:1	84.33 ± 10.88	28.59 ± 0.97	0.202
1:1	73.10 ± 14.55	26.55 ± 0.59	0.227
1:3	70.50 ± 3.12	27.34 ± 0.97	0.214
1:6	49.50 ± 6.80	26.69 ± 1.01	0.279
1:9	54.37 ± 17.60	26.59 ± 1.35	0.192
0:10	42.47 ± 11.35	30.04 ± 1.02	0.181
with 5% DSPE-PEG2000			
2:1	71.43 ± 4.72	18.33 ± 0.50	0.282
1:1	64.70 ± 14.11	26.50 ± 1.34	0.245
1:3	53.13 ± 6.00	27.18 ± 1.38	0.260
1:6	54.17 ± 8.12	28.04 ± 1.17	0.248
1:9	56.53 ± 9.94	26.41 ± 1.40	0.272
0:10	41.63 ± 3.07	33.48 ± 0.72	0.251

of PCCs was measured by dynamic light scattering (DLS). The average diameter of PCCs before siRNA binding ranged from 40 to 90 nm, depending on the molar ratio of CFL to HCL. Higher molar ratios of HCL resulted in smaller particle sizes. However, the zeta-potential showed no obvious increase with increasing HCL molar ratios. The morphology of the PCCs was analyzed by transmission electron microscopy (TEM). TEM images of the typical PCC (CFL:HCL 1:3, 5% DSPE-PEG2000), which was selected for further investigations, are shown in Figure 1. The PCC morphologies appeared to be identically spherical, and no obvious aggregation was seen after siRNA binding. The size distribution obtained by TEM was a little smaller than that from DLS, owing to the hydrate ionic radius of the carrier when conducting the DLS measurements.

To determine the optimal molar ratio of nitrogen atoms in the HCL to phosphates in the RNA (referred to as the N/P ratio) for siRNA delivery, 4% agarose gel electrophoresis was used to evaluate the binding of various amounts of siRNA to CCs or PCCs. As shown in Figure 2A, efficient siRNA binding of CCs or PCCs occurred at the N/P ratio of 5:1. As expected, PEGylation resulted in a decrease in siRNA binding efficiency due to the steric shielding effect of the PEG coating on the cerasome surface.[34] The extended particle size of some CCs/siRNA and PCCs/siRNA complexes might be attributed to a few cerasome particles being cross-linked by the siRNA (Figure 2B), but no obvious aggregates of the PCCs/siRNA complexes were observed in the TEM images (Figure 1B). The zeta-potential turned out to be negative (CCs/siRNA, −19.5 ± 0.32 mV; PCCs/siRNA, −15.34 ± 0.43 mV) at the N/P ratio of 2.5:1; when the N/P ratio was increased to 5:1, the zeta-potential of the complexes turned to be positive (CCs/siRNA, 25.31 ± 0.84; PCCs/siRNA, 20.04 ± 0.31) (Figure 2C), indicating that the binding of siRNA on the outer surface could shield the positive charge of the nanocarrier to some extent. This phenomenon might be ascribed to the effect of ionic strength.[35−37]

Silencing Efficacy of Cerasomes/siRNA Complexes *in Vitro*. The ideal nanocarrier for siRNA delivery should be nontoxic or low-toxic. The cytotoxicity of naked CCs and PCCs was evaluated in HepG2-Luc, which is a hepatocellular carcinoma-derived luciferase-expressing cell line, using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Supporting Information, Figure S2A,B). It was found that the cell viability depended on both the molar ratio of HCL and the concentration of nanoparticles. The viability decreased with increasing molar ratio of HCL and the nanoparticle concentration. The nanocarriers with CFL:HCL molar ratios of 1:3 or higher showed good biocompatibility, and the viable cell count for HepG2-Luc cells was 96.65 ± 4.65% for PCCs after 24 h exposure to nanoparticles with the final concentration of 25 μg/mL.

The cytotoxicity of both CCs/siRNA and PCCs/siRNA was evaluated in HepG2-Luc cell line using the MTT assay. As show in (Figure 3A), the viability of CCs/siRNA and PCCs/siRNA with a CFL:HCL molar ratio of 1:3 at N/P ratio of 5:1 is more than 80%. To assess the gene-silencing efficiency of carriers, an anti-luciferase siRNA was performed in HepG2-Luc cell line. A commercially available transfection reagent, Lipofectamine2000 was chosen as positive control. Each formulation showed different degrees of firefly luciferase-

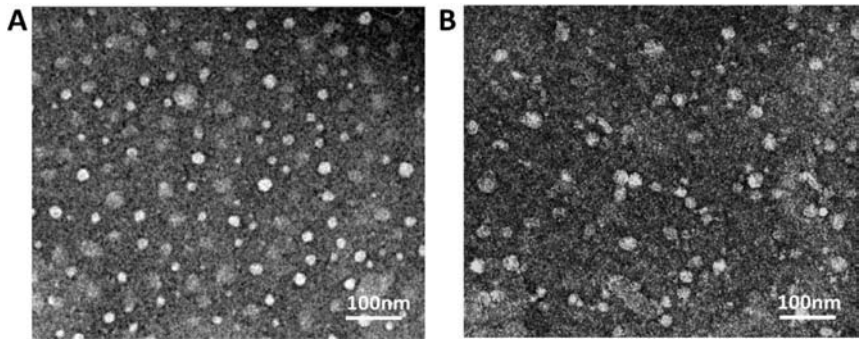

Figure 1. TEM images of PCCs (CFL:HCL = 1:3, 5% DSPE-PEG2000) before (A) and after (B) siRNA binding (N/P = 5); scale bar = 100 nm.

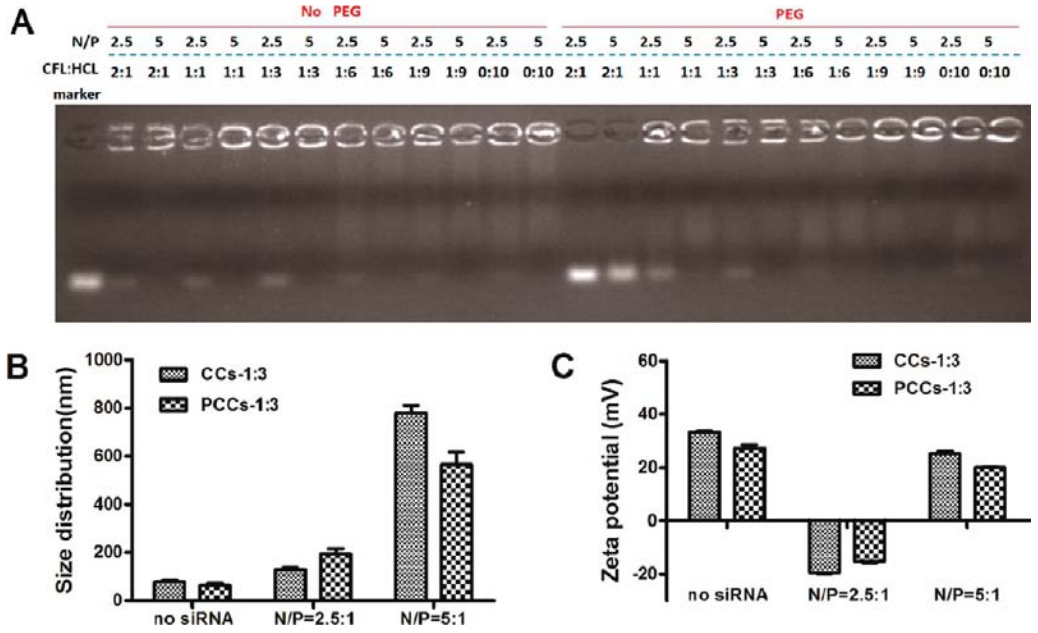

Figure 2. (A) siRNA binding ability of CCs and PCCs. Gel electrophoresis was performed through a 4% agarose gel at 90 V for 20 min. (B) Size (nm) distribution and (C) zeta-potential (mV) of CCs/siRNA and PCCs/siRNA complexes with a CFL:HCL molar ratio of 1:3.

expression inhibition (Figure 3B). To verify that the carrier is not cell specific, the transfection efficiency was also evaluated on Hela-Luc (a cervical cancer-derived luciferase-expressing cell line), and the luciferase-inhibition efficiency was in accordance with that on HepG2-Luc (Supporting Information, Figure S3A). The CCs/siRNA complexes with CFL:HCL molar ratio of 1:3 at the N/P ratio of 5:1 exhibited gene-silencing efficiency comparable to that of Lipofectamine2000, while other carriers could achieve the same efficiency by employing more siRNA.[36,30] Moreover, the gene-silencing efficiency of all nanocarriers was N/P ratio-dependent, with increased inhibition of gene expression efficiency as the N/P ratio increased from 2.5:1 to 5:1. Therefore, both CCs and PCCs with a CFL:HCL molar ratio of 1:3 (designated as CCs-1/3 and PCCs-1/3) loaded with siRNA at the N/P ratio of 5:1 were chosen for further *in vitro* and *in vivo* investigation in order to minimize toxicity without sacrificing efficiency.

Cellular Internalization of Cerasomes/siRNA Complexes and Gene-Silencing Efficiency Evaluation. HepG2-Luc cells were incubated with CCs-1/3/siRNA and PCCs-1/3/siRNA complexes respectively at the N/P ratio of 5:1 to evaluate their cellular uptake. Hoechst 33342 was used to stain nuclei, and siRNA labeled with Cy5 was employed to identify the localization of siRNA-loaded cerasomes. As show in Figure 4A (as well as Supporting Information, Figure S4), visible red fluorescence signals of Cy5-siRNA in the cytoplasm were observed after 4 h of incubation with CCs-1/3/siRNA and PCCs-1/3/siRNA complexes, indicating the internalization of CCs and PCCs.

In order to exert the function of post-transcriptional gene silencing, it is crucial for siRNA to escape from the lysosome and be released into the cytoplasm.[38] To investigate whether siRNA could escape from the lysosome, lysosomes were stained by LysoTracker Green. As show in Figures 4A and S4, obvious yellow regions were observed in CCs-1/3/siRNA-treated cells, indicating that the complexes mainly co-localized in the lysosome. In the PCCs-1/3/siRNA-treated cells, yellow regions are not that obvious, which might be attributed to the PEGlation shielding effect. Clearer results that can prove that PCCs-1/3/siRNA were co-localized in the lysosome are evident in the images with lower magnification in Figure S4. It was reported that internalization of cerasomes occurred through a clathrin-dependent endocytosis pathway due to their strong affinity for the head-groups of a variety of phospholipids and clathrin-coated vesicles because of their siliceous composition.[39] Red fluorescence signal was observed after emergence, indicating that the siRNA was released into the cytoplasm instead of remaining inside the lysosome only. In addition, the cells incubated with CCs-1/3/siRNA complexes showed stronger intracellular fluorescence than those incubated with PCCs-1/3/siRNA complexes. This could be attributed to the PEGylation of cerasomes, which could prevent the formation of essential non-bilayer intermediates and inhibit fusion with the cell and/or the endosome membrane, thus reducing the potential for cellular uptake and decreasing transfection efficiency.[11,40] Although the PEGylation might hinder the cellular uptake of the carriers and the nucleic acid escaping from endosome,[40] doping PEG into carriers still stands reasonable for any nanocarrier aimed at successful systemic delivery of siRNAs to distant organs.[3]

To exclude the effect of cell proliferation, quantitative real-time polymerase chain reaction (qRT-PCR) was applied to the HepG2-Luc cell line on Plk1 gene to investigate the silencing efficiency. Herein, expression of Plk1 gene was chosen to analyze, for it shows elevated activity in various cancers and is a key regulator of mitotic progression in mammalian cells.[39,41] Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as internal control, for it is highly conserved. CCs-1/3/siPlk1 and PCCs-1/3/siPlk1 complexes at the N/P ratio of 5:1 were incubated in HepG2-Luc cells for 24 h, and then gene expression was analyzed by qRT-PCR. As show in Figure 4B, the naked siRNA did not have an effect on Plk1 expression. In contrast, both CCs-1/3/siPlk1 and PCCs-1/3/siPlk1 groups down-regulated Plk1 gene expression in cells; the silencing efficiency reached 84.3% in the CCs-1/3 group and 70.8% in the PCCs-1/3 group when compared with the negative control.

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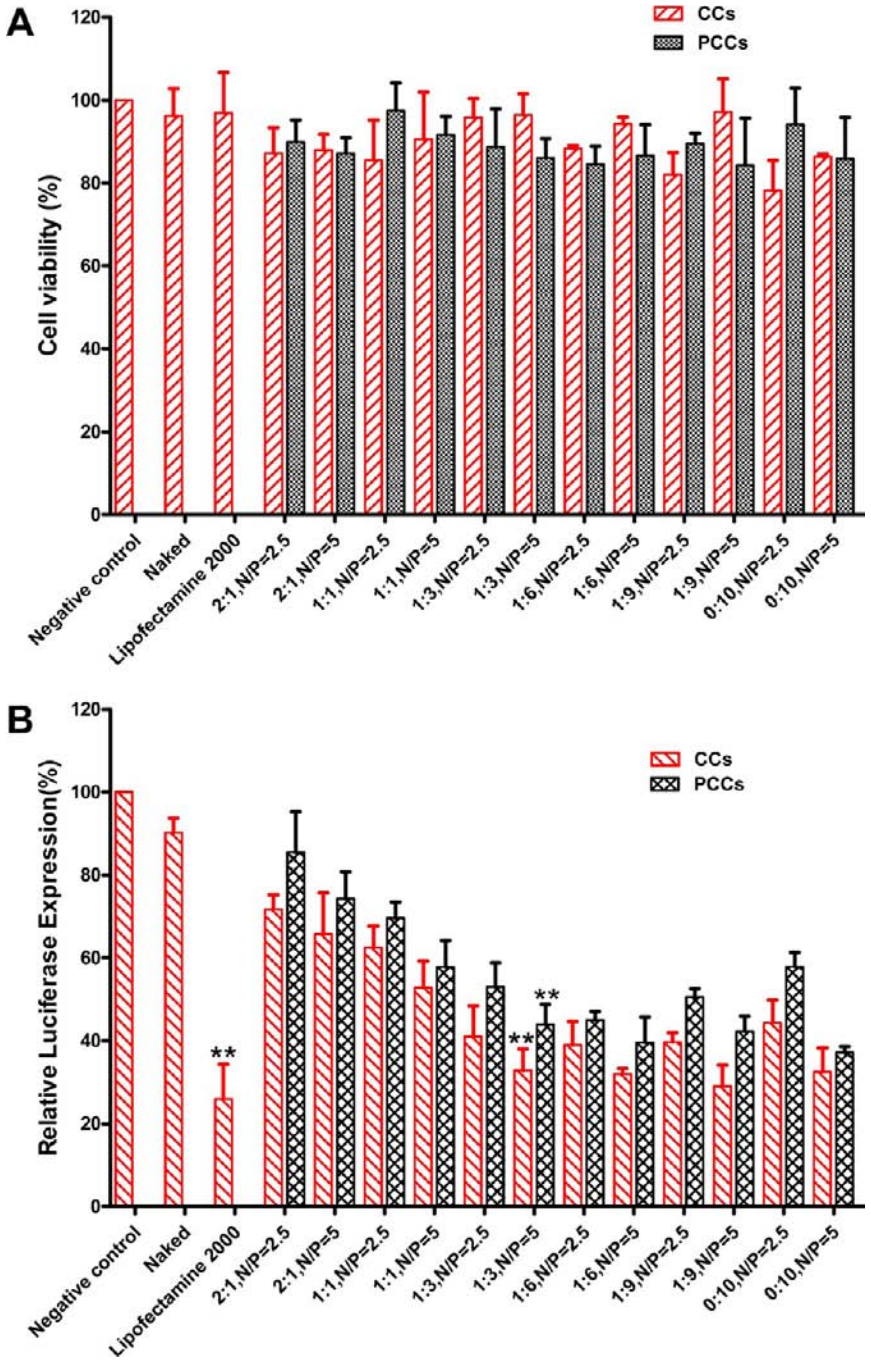

Figure 3. siRNA-loaded nanoparticles (CCs and PCCs) with various CFL:HCL molar ratios (2:1, 1:1, 1:3, 1:6, 1:9, 0:10) at N/P ratios of 2.5 and 5. (A) HepG2-Luc cell viability by MTT assay after 24 h exposure. (B) Relative firefly luciferase expression level after various treatments. The concentration of nanocarriers used is 3 mg/mL. All complexes were normalized to negative control. Each bar represents the mean ± SD of three independent experiments. $^{**}P < 0.01$.

Notably, CCs-1/3/siPlk1 was more effective than PCCs-1/3/siPlk1 and equivalent to Lipofectamine2000 in the case of gene silencing, due to efficient escape from the endosome, as demonstrated in Figure 4A. Additionally, to prove whether this carrier is cell specific or siRNA specific, qRT-PCR was applied to the Hela-Luc cell line on RHoA gene to investigate silencing efficiency mediated by CCs and PCCs. The mRNA down-regulation tendency was observed to be similar to that of Plk1 on the HepG2-Luc cell line (Figure S3B).

***In Vivo* Distribution of Cerasomes/siRNA Complexes.** To demonstrate the *in vivo* distribution of the nanoparticles, we administered CCs-1/3/siRNA and PCCs-1/3/siRNA complexes (50 μg of Cy5-siRNA) at the N/P ratio of 5:1 by tail vein injection to C57BL/6 mice and monitored the distributions by whole-body fluorescence imaging. As shown in Figure 5A, the fluorescence gradually faded over time, and it is especially noticeable on the mouse injected with naked siRNA. Both CCs-1/3/siRNA and PCCs-1/3/siRNA complexes revealed strong liver accumulation and good release behavior, as visible Cy5-siRNA signal remained detectable in the liver even 3 days after administration. In addition, both isolated-organ and whole-body fluorescence imaging proved

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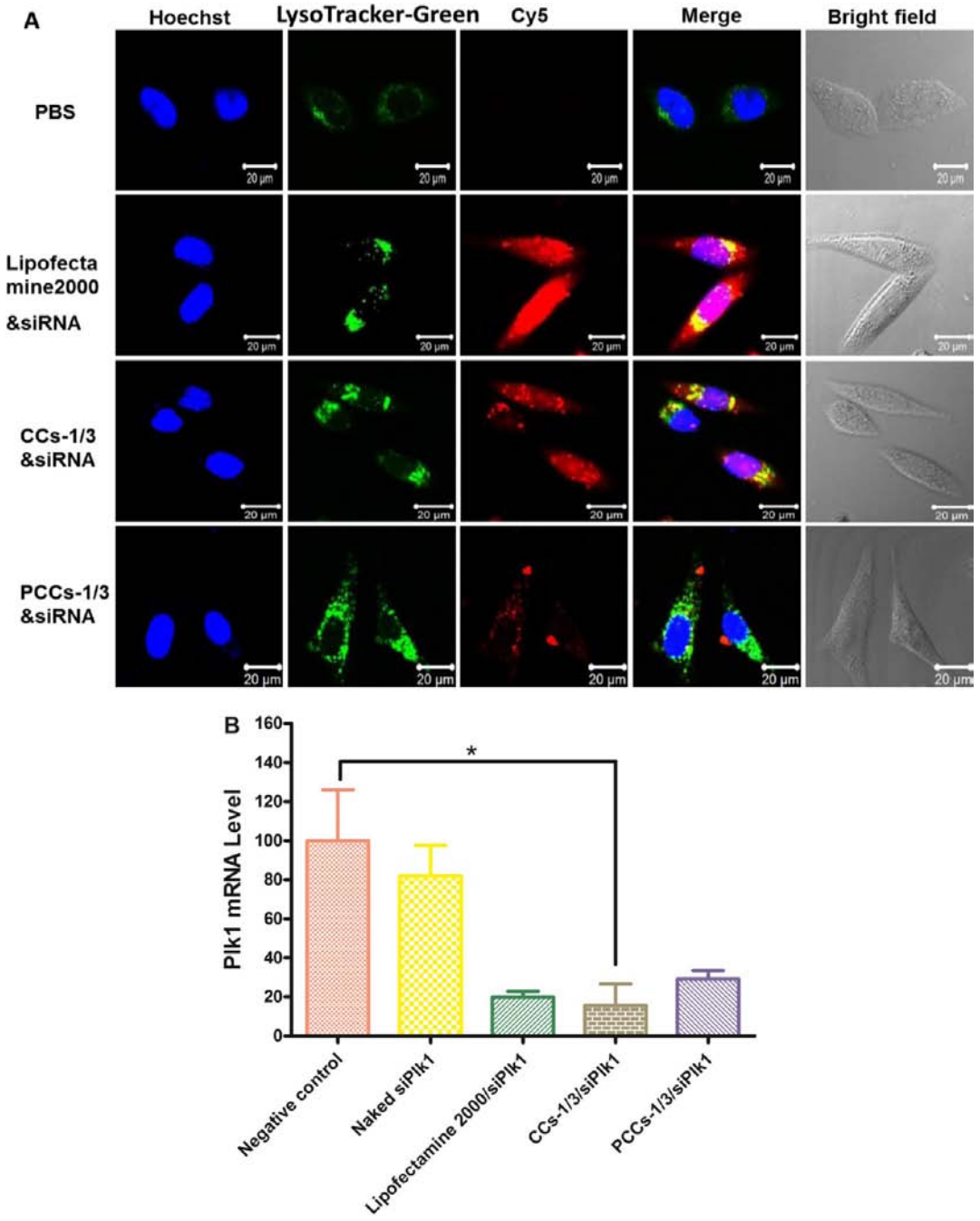

Figure 4. (A) Intracellular localization of CCs/siRNA and PCCs/siRNA complexes observed with confocal laser scanning microscopy (CLSM). Images of HepG2-Luc cells after 4 h of incubation with CCs-1/3/siRNA and PCCs-1/3/siRNA complexes at the N/P ratio of 5:1. Final concentration of siRNA was 50 nM. LysoTracker Green was used to stain lysosome (green), Hoechst 33342 was used to stain nuclei (blue), and siRNA was labeled with Cy5 (red); scale bar = 20 μm. (B) Relative Plk1 mRNA expression level in HepG2-Luc cells, determined by qRT-PCR and normalized to GAPDH mRNA. *$P < 0.05$. All the experiments were performed three independent times in replicates.

that PCCs-1/3/siRNA sustained much longer in the liver than CCs-1/3/siRNA complexes (Figure 5A,B). Moreover, the kidney also exhibited massive accumulation of the two complexes. Nevertheless, the naked siRNA group showed a much stronger signal intensity in the kidneys than the PCCs-1/3/siRNA and CCs-1/3/siRNA groups but a much weaker intensity in the livers (Figure 5C). This demonstrates that cerasomes, especially the PEGylated PCCs, could greatly enhance siRNA accumulation in liver. A previous study by D. T. Leong[42,43] demonstrated that nanomaterials trigger intracellular signaling cascades via specific interaction with VE-cadherin, resulting in nanomaterial-induced endothelial cell leakiness. The accumulation of PCCs-1/3/siRNA and CCs-1/3/siRNA might also be attributed to nanoparticles-induced endothelial leakiness. Cryosections of liver stained with Hoechst 33342 were utilized to further investigate siRNA intracellular localization. Confocal images (Figure 5D) and images with low magnification (Supporting Information, Figure S5) exhibited that the CCs-1/3/siRNA complexes were primarily phagocytized by Kupffer cells. In contrast, more PCCs-1/3/siRNA complexes were taken up by hepatocytes, since PEGylation protected nanoparticles from agglomeration

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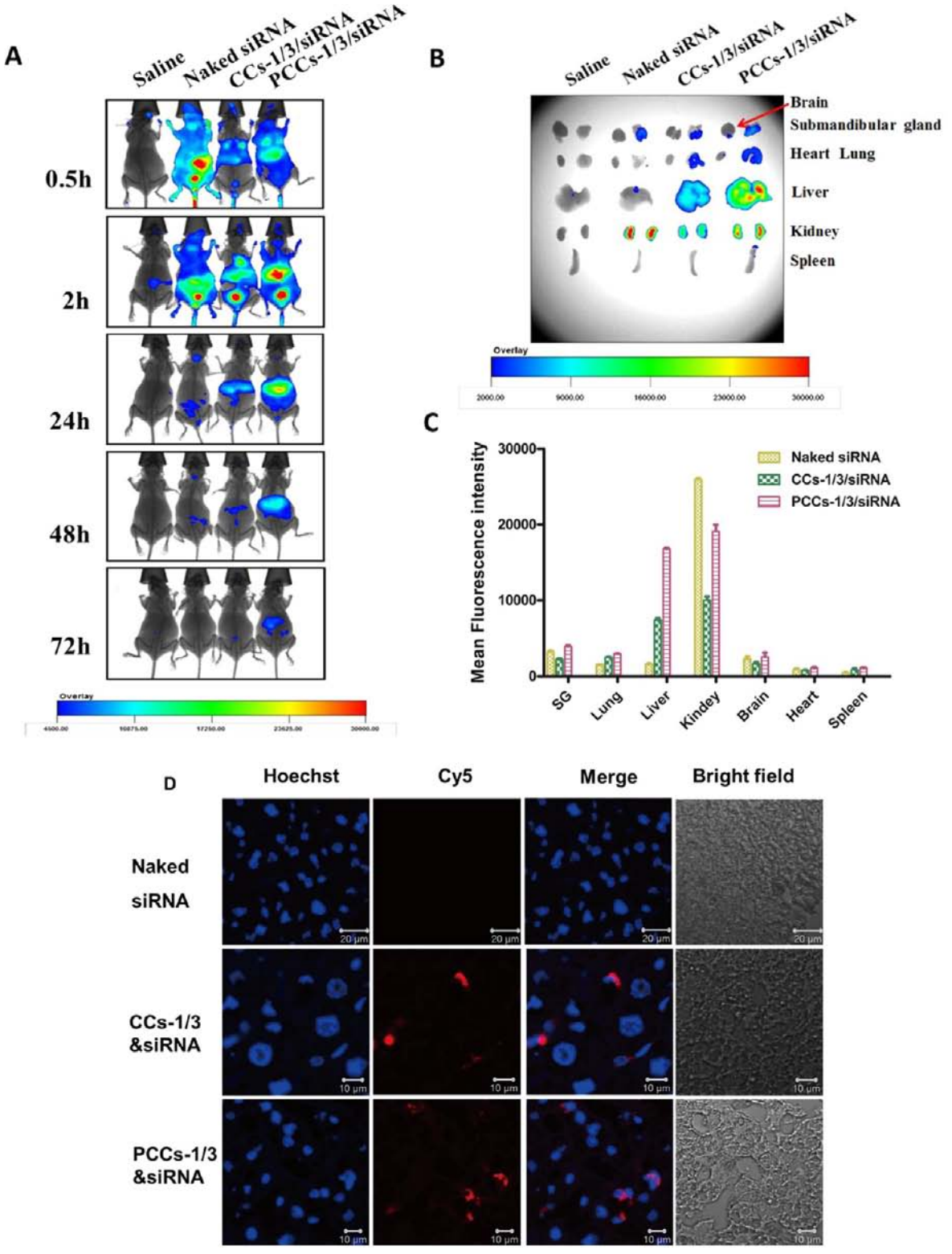

Figure 5. *In vivo* distribution of CCs-1/3/Cy5-siRNA and PCCs-1/3/Cy5-siRNA complexes in C57BL/6 mice (2.0 mg/kg for siRNA). (A) Whole-body imaging at given time points after administration via tail vein injection. (B) Fluorescence detection of isolated main organs of mice 3 days after tail vein injection. (C) Mean fluorescence intensities of Cy5-siRNA in isolated organs 3 days after tail vein injection. Data were normalized to corresponding organs from saline-treated animals. Each bar represents the mean ± SD of three independent experiments. (D) Confocal images of frozen sections of livers stained with Hoechst 33342.

and macrophage capture.[7] Visible Cy5 red signals were observed around the nuclei of hepatocytes, providing evidence that the siRNAs in PCCs-1/3/siRNA complexes could release into the cytoplasm, where the RNA interference occurred.[44]

Liver-Targeting Functional Study. Apolipoprotein B (ApoB) is the primary apolipoproteins of chylomicrons, very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL),[45] and is predominantly expressed in the jejunum and

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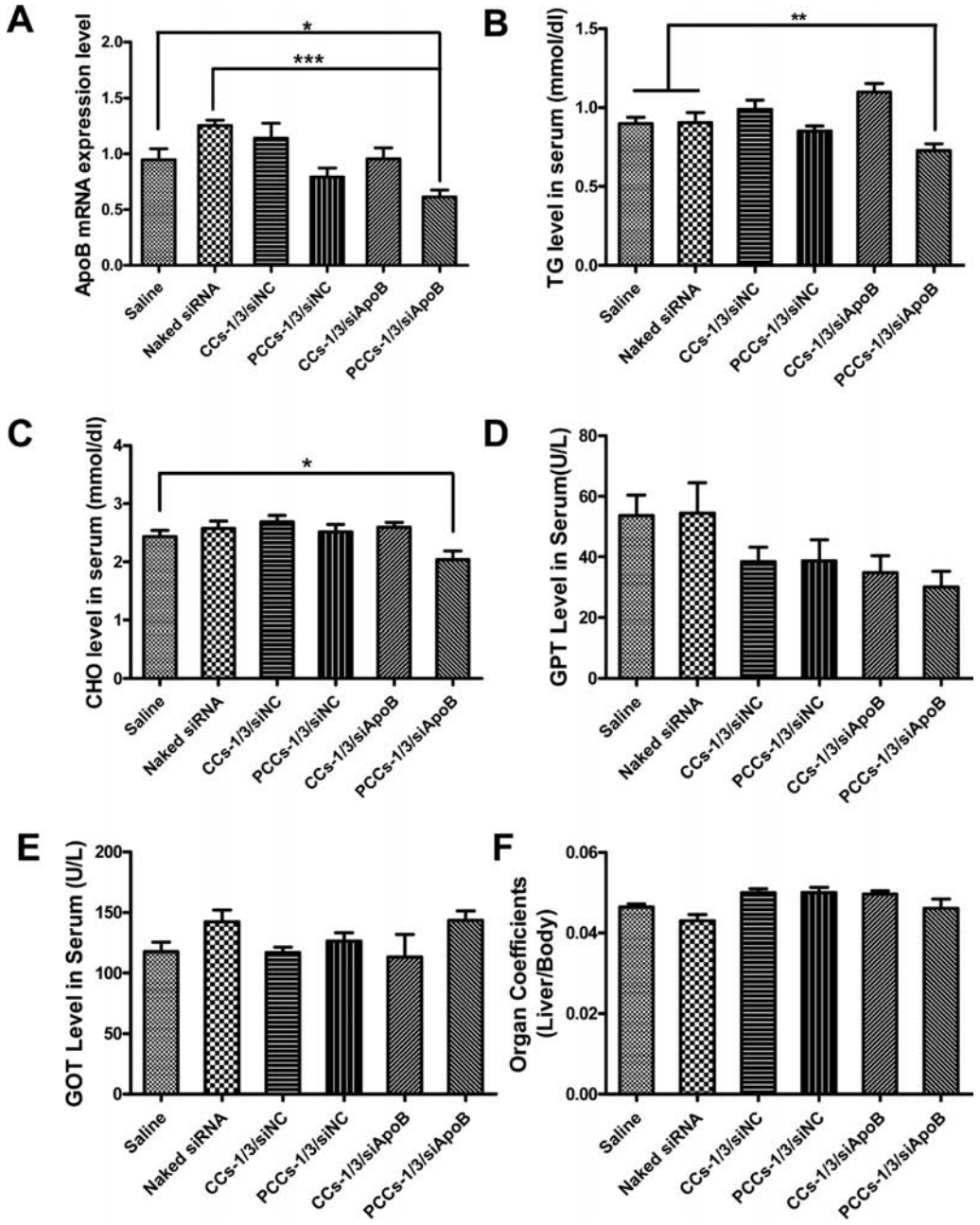

Figure 6. *In vivo* functional study with ApoB siRNA at the dose of 1.0 mg/kg in C57BL/6 mice. (A) ApoB mRNA level in livers of mice 3 days after tail intravenous administration. (B,C) TG and CHO concentrations were repressed in serum from mice administered CCs-1/3/siApoB and PCCs-1/3/siApoB complexes. (D,E) GPT and GOT levels in serum and (F) liver coefficients (liver weight normalized to body weight, g/g) manifested no significant toxicity observed in all mice. Each bar represents the mean ± SD (n = 6). *P < 0.05, **P < 0.01, and ***P < 0.001.

liver.[46] Therefore, we chose siApoB, a siRNA which can silence the expression of ApoB effectively, to investigate the ability of CCs- and PCCs-mediated gene silencing *in vivo*. Two groups of mice with injection of saline and naked siApoB were used as negative controls, respectively. The mice groups injected with CCs-1/3/siNC and PCCs-1/3/siNC complexes at the N/P ratio of 5:1 were employed to investigate the specificity of siApoB. CCs-1/3/siApoB and PCCs-1/3/siApoB complexes at the N/P ratio of 5:1 were used to detect the *in vivo* transfection efficiency. All of them were administered intravenously (i.v.) at a single dose of 1.0 mg/kg for siRNA. Mice were sacrificed at the end point to harvest their livers and blood for examination. Considering the important function of ApoB in liver, we chose three main components, i.e., VLDL, cholesterol (CHO), and triglycerides (TG), as functional items to investigate the quantity change of AopB in livers of mice with different treatments. Since ApoB is responsible for transporting VLDL, down-regulation of ApoB expression in liver will cause an increase of VLDL in liver and further block the VLDL secretion pathway from liver to circulation. Encouragingly, after systemic administration, the relative levels of CHO and TG in the serum of the PCCs-1/3/siApoB group were lower than in the other groups (Figure 6B,C), which could be attributed to siApoB-induced repression of ApoB expression. The mRNA level of ApoB in the liver of the treated mice was detected by qRT-PCR with liver tissue to investigate the silencing efficiency. GAPDH was used as internal control. The qRT-PCR data showed that the mRNA level in liver of PCCs−1/3/siApoB-treated mice

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was lower than that of the CCs-1/3/siApoB group and had remarkable differences with the saline-treated group and naked siRNA-treated group (Figure 6A). Together, all these results prove that PCCs can effectively deliver siRNA into liver, be taken up by hepatocytes, and silence the target gene effectively and efficiently. In addition, the concentrations of glutamic–oxaloacetic transaminase (GOT) and flutamic–pyruvic transaminase (GPT) in serum remained at normal levels (Figure 6C,D). Moreover, we observed no significant difference in liver coefficients (ratio of liver/body weight) among various groups (Figure 6E,F), demonstrating that PCCs-1/3/siRNA and CCs-1/3/siRNA did not induce extensive toxicity in the mice liver after tail i.v. injection.

CONCLUSION

In summary, this study represents the fabrication of PEGylated cationic cerasomes, or PCCs, for efficient siRNA delivery *in vivo*. PCCs have low cytotoxicity and remarkable stability when incubated under physiological conditions. *In vitro* studies showed that PEGylation of the cationic cerasome could enhance the siRNA delivery efficiency, and incorporation of the lipid containing a hydroxyl group could facilitate siRNA escape from the lysosome and release into the cytoplasm. Biodistribution and *in vivo* functional study revealed that PCCs have fulfilled the function of delivering siRNA to liver to regulate targeted genes which are related to diseases. Expression of each of the genes targeted by the siRNAs was shown to be repressed at the protein level. According to loading capacity, targeting capabilities, and potency of action, PCCs provide unique attributes as a delivery platform for therapeutic oligonucleotides.

MATERIALS AND METHODS

Materials. Dicyclohexylcarbodiimide (DCC), 2-(2-aminoethylamino)ethanol, and agarose gel were purchased from Sigma Aldrich, USA. Other organic reagents or solvents were of analytical grade. All the chemicals were used directly without further purification. 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(methoxy(polyethylene glycol)-2000 (DSPE-PEG2000) was purchased from Shanghai Advanced Vehicle Technology Pharmaceutical Ltd. The CFL was synthesized according to the method reported previously.[32] The synthesis procedure and characterization of HCL of N^1,N^1-dihexadecyl-N^4-(2-(2-hydroxyethylamino)ethyl)succinamide are shown in the Supporting Information. The siLuc (targeting firefly luciferase gene), siApoB (targeting Apolipoprotein B gene), etc. were supplied by Suzhou Ribo Life Science Co. (Jiangsu, China). The sequences were as follows: Cy5-NC-sense, 5′-Cy5-CCUUGAGGCAUACUUCAAAdTdT-3′; Cy5-NC-antisense, 5′-UUUGAAGUAUGCCUCAAGGdTdT-3′; siLuc-sense, 5′-CCCUAUUCUCCUUCUUCGCdTdT-3′; siLuc-antisense, 5′-GCGAAGAAGGAGAAUAGGGdTdT-3′; siPlk1-sense, 5′-UGAAGAAGAUCACCCUCCUUAdTdT-3′; siPlk1-antisense, 5′-CAGAAGUCAUCUUGAUAGAdTdT-3′; siRHoA-sense, 5′-UCUAUCAAGAUGACUUCUGdTdT-3′; siRHoA-antisense, 5′-UAAGGAGGGUGAUCUUCUUCAdTdT-3′; siApoB-sense, 5′-GUCAUCACACUGAAUACCAAUdTdT-3′; siApoB-antisense, 5′-AUUGGUAUUCAGUGUGAUGACACdTdT-3′. Hochest33342 was from Dojindo Molecular Technologies, Inc. LysoTracker Green and Lipofectamine2000 were purchased from Invitrogen. Dulbecco's modified Eagle medium (DMEM), MTT, Opti-MEM, penicillin/streptomycin, and trypsin were from Gibco (Grand Island, NY).

Preparation of PEGylated Cationic Cerasomes. The cationic cerasomes were prepared according to the method described by Bangham.[33] Briefly, HCL, CFL, and 5 mol % DSPE-PEG2000 were dissolved by 1−2 mL of chloroform, followed by removal of organic solvent by a rotary evaporator to obtain a thin film. After drying overnight in a vacuum oven, deionized water was added to achieve the final concentration of 3 mg/mL. The suspension was incubated at 55 °C for 30 min to fully hydrolyze the CFL to enable the self-assembly of cerasomes.[32] Upon sonication with a probe-type sonicator, cationic cerasomes with a reduced size distribution were formed. For comparative investigation, cationic cerasomes with no PEGylation were prepared with HCL and CFL using the same procedure.

Characterization of Cerasome/siRNA Complexes. Zeta-potential and size distribution in water were analyzed using a zeta and size analyzer (Brookhaven Instruments, USA). The samples were analyzed at room temperature, and each bar in the figures represents the mean ± SD of three independent experiments. For TEM measurements, the sample suspension was deposited onto a protonate-treated 400-mesh carbon-coated copper grid and absorbed for 1 min. The grid was washed with deionized water one or two times to remove unabsorbed samples and then dipped into 10 μL of uranyl acetate aqueous solution (0.2%, w/v) for 30 s for negative staining. The samples were imaged on a H9000NAR high-resolution TEM instrument at 300 kV accelerating voltage.

Preparation of Cerasome/siRNA Complexes and Gel Retardation Assay. For siRNA binding, cerasomes diluted at different concentrations with diethyl pyrocarbonate (DEPC) and water were mixed with the desired amount of siRNA solution (20 μM) by gentle pipetting and incubated at room temperature for 20 min before characterization. Electrophoresis was performed on 4% agarose gel containing 0.5 μg/mL ethidium bromide at a voltage of 90 V for 20 min in Tris-acetate-EDTA (TAE) running buffer solution. The nucleic acid bands were visualized by UV imaging equipment (ABI, GIS-2500).

***In Vitro* Gene Silencing.** All cells used were maintained in DMEM supplemented with 100 μg/mL streptomycin, 100 U/mL penicillin, and 10% FBS and incubated at 37 °C in a 5% CO_2 atmosphere. To access gene-silencing efficiency, Hela-Luc or HepG2-Luc cells were seeded in 24-well plates at 5.0×10^4 cells/well and transfected with various CCs/siRNA and PCCs/siRNA complexes. Lipofectamine2000 was used as positive control following the manufacturer's protocol. The final concentration of siRNA in each well was 50 nM, and the amount of carriers complied with the N/P ratios of 2.5:1 and 5:1. After another 24 h of culturing, the cells were lysed with passive lysis buffer for luminescence measurements. The relative light units were measured with a fluorometer (Synergy HT, BioTek, USA).

For qRT-PCR analysis, the cells were transfected using the method described above, and total cellular RNA was isolated using an RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. cDNA was synthesized using the PrimeScript First Strand cDNA synthesis kit (A3500, Promega, USA) with 2 μg of total RNA. The reaction mixture was then subjected to qRT-PCR, which was performed using an Applied Biosystems StepOne Real-Time PCR system. The housekeeping gene GAPDH was used as an internal standard. The

primers used in the qRT-PCR for Plk1, RHoA, and GAPDH were as follow: Plk1-forward, 5′-GCCCCTCACAGTCCT-CAATA-3′; Plk1-reversed, 5′-TACCCAAGGCCGTACTTG-TC-3′; RHoA-forward, 5′-ACTGGTGATTGTTGGTGATG-3′; RHoA-reversed, 5′-CATAAAGCCAACTCTACCTGC-3′; GAPDH-forward, 5′-AGAAGGCTGGGGCTCATTTG-3′; GAPDH-reversed, 5′-AGGGGCCATCCACAGTCTTC-3′.

Cytotoxicity Measurements. The cytotoxicity of the carriers and carrier/siRNA complexes was assessed via MTT viability assay against HepG2-Luc cells or Hela-Luc cells. Cells were seeded in 96-well plates at 10 000 cells/well in 100 μL of complete DMEM supplemented with 10% FBS and incubated at 37 °C in a 5% CO_2 atmosphere for 12 h, and then cells were separately incubated with different concentrations of carriers or carrier/siRNA complexes and further cultured for 24 h. Cells used as control received an equivalent volume of PBS treatment. Next, MTT stock solution was added into each well to achieve a final concentration of 1 g/L. Plates were incubated for an additional 4 h, after which all medium was removed, 100 μL of DMSO (cell degrade) was added into each well, and the plates were further incubated in the dark for 15 min. Finally, the absorbance was read at 570 nm with a reference wavelength of 630 nm using a microplate reader (Synergy HT, BioTek, USA). Cell viability was normalized to cells maintained in DMEM with PBS treatment. These experiments were performed in three independent replicates.

Cellular Internalization of Cerasomes/Cy5-siRNA. Cy5-labled siRNA was mixed with carriers following the procedure described above. Lyso Tracker specific antibody was used to stain lysosome, and Hoechst 33342 was used to stain nuclei. Both of them assisted the subcellular analysis. HepG2-Luc cells were seeded at a density of 2.0×10^5 cells/well in 6-well plates overnight. The cells were then transfected with CCs/siRNA and PCCs/siRNA complexes for 4 h at 37 °C in Opti-MEM, and the cellular location distribution of siRNA was recorded in living cells via a Zeiss confocal microscope (LSM700, Carl Zeiss, Germany).

***In Vivo* Distribution of Cerasomes/Cy5-siRNA.** The mice were maintained in Peking University Laboratory Animal Center (AAALAC-accredited experimental animal facility). All procedures involving experimental animals were done following the protocols approved by the Institutional Animal Care and Use Committee of Peking University. Male BALB/C nude mice, 6−8 weeks old, weighing 18−22 g, were purchased from Vital River Co. (Beijing, China). CCs/siRNA and PCCs/siRNA complexes were administered to mice via tail vein injection at a dosage of 2.0 mg/kg. Whole body fluorescence imaging was detected using a Kodak *in vivo* imaging system (Kodak *In-vivo* Imaging System FX Pro, Carestream Health, USA) at given time points. Mice were sacrificed at the end-point by cervical dislocation, and the major organs were isolated and examined. Tissues were embedded in optimum cutting temperature (OCT) compound and frozen in liquid nitrogen for ∼1 min until the OCT compound turned white and opaque. Subsequently, the specimens were cut into 6 μm sections and picked up on a glass slide, stained by Hoechst 33342 for viewing the nucleus. Finally, frozen sections were observed under the confocal microscope to validate the liver-targeting ability of cerasomes/siRNA complexes.

***In Vivo* Functional Study.** To assess the gene-silencing capability of the CCs and PCCs *in vivo*, anti-ApoB siRNA was used. Male C57BL/6 mice, 6−8 weeks old, weighing 18−22 g, were randomly divided into different groups and treated with various complexes by one i.v. injection at 1.0 mg/kg (n = 6). These mice were sacrificed 3 days later. Liver tissues and serum specimens were collected for further analysis. Total RNA in livers was isolated with Trizol reagent. cDNAs were acquired by reverse transcription with the PrimeScript First Strand cDNA synthesis kit, and then ApoB mRNA levels were evaluated employing the Applied Biosystems StepOne Real-Time PCR system. β-Actin (housekeeping gene) was used as an internal control. The primers used in the qRT-PCR for ApoB and β-actin were as follow: ApoB-forward, 5′-ATGGGAAGAAAC-AGGCTTGA-3′; ApoB-reversed, 5′-TTCTGTCCCACGAAT-TGACA-3′; β-actin-forward, 5′-ATCTGGCACCACACCTTC-TACAATG-3′; β-actin-reversed, 5′-CGTCACACTCCTGCT-TGCTGATCCACATCTGC-3′. The concentrations of CHO, TG, GOT, and GPT in serum were further assayed by using a SABA-18 automatic biochemical analyzer (Analyzer Medical System, Roma, Italy).

■ ASSOCIATED CONTENT

Supporting Information

Additional details about novel hybrid cationic cerasomes for siRNA delivery. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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