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Cholesterol derived cationic lipids as potential non-viral gene delivery vectors and their serum compatibility

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ABSTRACT

Cholesterol derivatives **M1**–**M6** as synthetic cationic lipids were designed and the biological evaluation of the cationic liposomes based on them as non-viral gene delivery vectors were described. Plasmid pEGFP-N1, used as model gene, was transferred into 293T cells by cationic liposomes formed with **M1**–**M6** and transfection efficiency and GFP expression were tested. Cationic liposomes prepared with cationic lipids **M1**–**M6** exhibited good transfection activity, and the transfection activity was parallel (**M2** and **M4**) or superior (**M1** and **M6**) to that of DC-Chol derived from the same backbone. Among them, the transfection efficiency of cationic lipid **M6** was parallel to that of the commercially available Lipofectamine2000. The optimal formulation of **M1** and **M6** were found to be at a mol ratio of 1:0.5 for cationic lipid/DOPE, and at a N/P charge mol ratio of 3:1 for liposome/DNA. Under optimized conditions, the efficiency of **M1** and **M6** is greater than that of all the tested commercial liposomes DC-Chol and Lipofectamine2000, even in the presence of serum. The results indicated that **M1** and **M6** exhibited low cytotoxicity, good serum compatibility and efficient transfection performance, having the potential of being excellent non-viral vectors for gene delivery.

Gene therapy is generally considered as a promising approach in the treatment of diseases such as degenerative disorders, cancer, and AIDS.[1–3] However, there are many challenges associated with the delivery of gene, such as enzymatic instability, low cellular uptake and inability to escape from the endosomes.[4] Therefore, finding a safe and effective vector for gene delivery is very important. Gene delivery vectors include viral and non-viral ones. Viral vectors showed highly efficient intracellular gene delivery, but the high cost, serve immune reactions and tumorigenic mutation risk limited their clinical use.[5,6] Thus, various non-viral vectors based on cationic lipids[7–9] or polymeric carriers[10,11] have been developed for delivery of gene to cells. Among non-viral vectors, cationic liposomes based on cationic lipids showed great potential for gene delivery with easily preparation, less toxicity, low immunogenicity, and high DNA carrying capacity.[12–14] Meanwhile, they could also protect DNA against enzymatic degradation and fulfill effective intracellular gene delivery.[15]

Cationic liposomes were consisted of cationic and neutral lipids, while cationic lipids mainly accounted for gene delivery. The cationic lipids were amphiphilic molecules and generally made of hydrophobic domain, positive charged head group and the linker between them.[16] The structure of cationic lipids might take important effect on the complexation and gene transfection activity. As cholesterol derived cationic lipids, such as commercially available DC-Chol (3-β-[*N*-(*N′*,*N′*-dimethylaminoethyl)carbamoyl]-cholesterol),[17] could improve gene transfection efficiency, cholesterol skeleton was developed as hydrophobic part for newly cationic lipids.[18–23] The hydrophobic domain and positive charged head group in cationic lipids could be linked via carbamate, amide, ester or ether bonds. It has been noticed that ether-linked lipids were more stable than other linkers, and could perform good gene transfection efficiency.[18,21,24] Positive charged head group was also related to gene delivery.[21] Generally, the head group could be divided into amines,[25] amino acids,[26] guanidiniums,[19,24] and heterocyclic groups,[21] and their different gene transfection ability might attribute to the substituent on nitrogen atom.[19]

In this Letter, we designed and synthesized cholesterol derived cationic lipids via ether or ester linkages with different head groups, and then cationic liposomes were obtained by introducing

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helper lipid DOPE. The cytotoxicity of the cationic liposome was evaluated and the liposome formulation for gene delivery was optimized. The relationship between chemical structure of cationic lipids and gene delivery efficacy were elucidated and the serum compatibility was also investigated through testing the transfection efficiencies in the presence of serum.

In view of the fact that chemical structure of head group and linkage between cholesterol backbone and head group play important roles on gene transfection profile, a series of cholesterol derivatives were prepared (see Supplementary information), in which natural cholesterol was employed as the hydrophobic domain, and different positive charged amines were served as cationic head groups. The hydrophobic domain and head group were connected through ester or ether bond as linkage and the efficiency comparison was further carried out. The synthetic route of the cholesterol-based cationic lipids **M1**–**M6** was showed in Figure 1. Cholesterol derivatives **M1**–**M5** were ether-modified compounds, while **M6** was synthesized by ester linkage. Briefly, cationic lipid **M1** was synthesized from natural cholesterol through three steps. Compounds **2** and **3** were synthesized in 86.6% and 78.2% yield through the method reported by our group.[27] Compound **3** reacted with HN_3 (1.3 equiv, 1 M in chloroform), DIAD (1.25 equiv) and PPh_3 (1.25 equiv) in THF to afford **M1** in 65.4% yield. Meanwhile, compound **3** was bromized to obtain the compound **4** in 96% yield. With the precursor **4** in hand, lipids **M2**–**M5** were further synthesized by conjugating corresponding amine head groups to **4** in a screw-top pressure tube. The yield of **M2**–**M5** was 65.4%, 27.0%, 61.8%, and 42.8%, respectively. Using cholesterol and Boc-aminohexanoic acid as starting materials, **M6** was synthesized through catalyzed condensation by DCC/NHS in THF at room temperature. All synthesized lipids were characterized and identified by ^{1}H NMR, MS and IR spectrum. Cationic lipids **M1**–**M6** showed obvious proton signal of cholesterol, including the proton signal of cholesterol skeleton falling at 2.51–0.67 ppm, and the signal at 5.35 ppm was attributed to the double bond of cholesterol. As for lipid **M1**, proton signals of $–CH_2–$ attached to the amine group showed obvious peaks at 2.7 ppm. Lipid **M2** showed obvious peaks at 2.20 ppm, which were identified as proton signals of the $–CH_3$ attached to the nitrogen atoms. Moreover, the signal of $–CH_2–$ group could also be confirmed by ^{1}H NMR spectrum (**M3** at 3.41–3.49, 3.66 and 4.09 ppm; **M4** at 3.02–3.16 ppm; **M5** at 3.02–3.16 and 3.42–3.46 ppm; **M6** at 2.27–2.32 and 2.75 ppm).

The helper lipid is one of the factors influencing transfection efficiency. The appropriate addition of neutral DOPE to the liposome formulation significantly enhanced the transfection of many cationic lipids in cells.[28] To form cationic liposome formulation, neutral lipid DOPE (1,2-dioleoyl-L-α-glycero-3-phosphatidylethanolamine) was used as helper lipid. Liposomes were prepared by film dispersion methods using cationic lipids **M1**–**M6** and DOPE at mol ratio of 1:1 (see Supplementary information). All lipids film re-dispersed in deionized water and formed stable liposome formulations. The liposomes were all optically clear, no precipitation or noticeable increase in turbidity was observed even stored at 4 °C under sterile conditions one month later. To elucidate the physicochemical properties of liposomes prepared from **M1**–**M6**, the size distribution and zeta potential were determined and shown as mean ± SD in Table 1. The particle size of liposomes prepared from **M1**–**M6** ranged from 110.4 to 170.6 nm, and the polydispersity index (PDI) of liposomes was from 0.178 to 0.243, indicating a good dispersion. Among them, lipid **M3** and **M5** showed lower particle size due to the quaternary ammonium structure. The surface charge of six liposome vesicles was all positive, varied from 36.7 to 64.0 mV. When the amine head group was same, ether-linked conjugate might bring greater positive surface charge than ester-linked one (liposomes **M1** > **M6**). Meanwhile, among liposomes obtained from ether-linked lipids **M1**, **M2** and **M4**, positive surface charge decreased as the introduction of substituent to nitrogen. The high zeta potential of liposomes **M5** might be due to the presence of quaternary ammonium. The liposome **M3** exhibited much lower zeta potential than **M5**, indicating the introduction of hydroxyl group to the amine head also had effect on physicochemical properties of liposomes.

Gel retardation assay was used to optimize the N/P (+/−) mol charge ratio of cationic liposomes and DNA. pEGFP-N1 was applied as pDNA, while the ratio of cationic lipid/DNA (N/P) was varied from 0.25 to 6. The gel electrophoresis results were shown in Figure 2. With regard to each formulation, when N/P ratio was low, the free DNA still exist and DNA migration could be observed. However, once reached certain N/P ratio, DNA migration would be completely retarded. So the DNA binding ability of cationic liposomes increased with an increase in the N/P ratio. When N/P ratio was 1, free DNA was still seen in **M2**–**M4**. The results also identified that the liposomes were capable of completely inhibiting the electrophoretic mobility of plasmid DNA when the N/P charge ratio was over 2:1.

In order to find out the most effective cationic lipids, the transfection of all synthesized lipids **M1**–**M6** were tested in 293T cell lines in our preliminary studies using the mol ratio of lipid/DOPE at 1:1 and charge ratio of N/P at 3:1. Moreover, two commercially available liposomal transfection reagents, DC-Chol and Lipofectamine2000 were chose as control. The transfection efficiency was evaluated through the percent of positive transfected cells and the mean fluorescence intensity (MFI) by flow cytometry (see Supplementary information) as shown in Figure 3. It has been observed that except for the cationic lipids **M3** and **M5**, **M1**, **M2**, **M4** and **M6** showed effective transfection performance compared with the naked DNA and displayed a close relationship between structure and efficiency. Lipids **M1** and **M6** with primary amine head groups gave much higher transfected efficiency than DC-Chol and other lipids. When compared with Lipofectamine2000, although MFI is low, the percent of positive transfected cells using **M6** was parallel to that of the commercially available Lipofectamine2000, showing high transfected efficiency. Interestingly, the ether and ester linkage between cholesterol and head groups in structure of lipid **M1** and **M6** might take little effect on their delivery efficiency. Meanwhile, lipids **M2** and **M4** with tertiary amine groups showed decreased transfection efficiency as the introduction of alkyl chain in the head. It has been reported that the transfection would increase by appending hydroxyl moiety. But when we synthesized **M3** based on **M2** by introduction of hydroxyethyl group, obviously decreased delivery efficiency was found, behaving a completely different manner. The results could also be confirmed by GFP expression observed under fluorescence microscope (Fig. 4). Lipid **M1** and **M6** gave much more fluorescence signals than other lipids and DC-Chol, which were in consistent to the data of flow cytometry. All the results indicated the transfection activities of cationic lipids were mainly affected by the head groups, and their transfection ability decreased from primary amine to quaternary ammonium, while the length of substituent alkyl chain on the nitrogen also affect the efficiency.

Neutral helper lipid also played important role to form cationic liposomes with high activity. In cationic liposomes formulations, the proper addition of DOPE can enhance the transfection efficiency in various cell types by destabilizing endosomal or plasma membranes.[29] As lipids **M1** and **M6** exhibited good transfection profile, we further optimized the ratio of cationic and neutral lipids for them. Different mol ratios of cationic lipids (**M1** and **M6**) and DOPE were performed for optimization. Both the percent of positive transfected cells and the mean fluorescence intensity were recorded by flow cytometry as shown in Figure 5A and B. Compared with the ratio of cationic and neutral lipid at 1:1 in preliminary test, when DOPE was absent in the liposome formulation,