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Asymmetric 1-alkyl-2-acyl phosphatidylcholine: A helper lipid for enhanced non-viral gene delivery

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ABSTRACT

Rationally designed asymmetrical alkylacyl phosphatidylcholines (APC) have been synthesized and evaluated as helper lipids for non-viral gene delivery. A long aliphatic chain (C22–C24) was introduced at the 1-position of glycerol backbone, a branched lipid chain (C18) at the 2-position, and a phosphocholine head group at the 3-position. The fusogenicity of APC depends on the length and degree of saturation of the alkyl chain. Cationic lipids were formulated with APC as either lipoplexes or nanolipoparticles, and evaluated for their stability, transfection efficiency, and cytotoxicity. APC mediated high *in vitro* transfection efficiency, and had low cytotoxicity. Small nanolipoparticles (less than 100 nm) can be obtained with APC by applying as low as 0.1% PEG-lipid. Our study extends the type of helper lipids that are suitable for gene transfer and points the way to improve non-viral nucleic acid delivery system other than the traditional cationic lipids optimization.

1. Introduction

The development of a safe, efficient system for gene delivery remains a main challenge for the scientific community to turn gene therapy into a successful therapeutic method. Tremendous efforts have been expended to optimize the cationic liposome system for gene transfer (Gao et al., 2007; Li and Szoka, 2007; Liu et al., 2003). However, most of attention has been paid to new cationic lipids (Stanton and Colletti, 2010), and less emphasis has been placed on the development of novel helper lipids. For maximal transfection efficiency, most cationic liposome formulations require a significant amount of helper lipid (Fasbender et al., 1997; Felgner et al., 1987; Hui et al., 1996), typically 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine (DOPE) or cholesterol. Although DOPE has been extensively formulated with cationic lipid to achieve enhanced *in vitro* cell transfection, DOPE-containing lipoplexes often showed a strongly reduced transfection activity in serum-containing media *in vitro*, as well as poor transfection *in vivo* (Audouy and Hoekstra, 2001; Sakurai et al., 2001a, 2001b). Thus it is necessary to design novel helper lipids to expand the choices of cationic liposome formulations.

DOPE has a cone shape, presents a smaller hydrophilic head group area than the hydrophobic tail area, resulting in natural negative curvature in aqueous solution. The role of DOPE in enhancing transfection is generally attributed to its ability to promote the lamellar to inverted hexagonal phase transition, which leads to the fusion of lipoplexes with endosome membranes resulting in the escape of DNA from endosome (ElOuahabi et al., 1997; Koltover et al., 1998). Partially fluorinated DOPE analogs have been introduced by Gaucheron and colleagues as helper lipids with promising transfection activity when formulated with conventional cationic lipids (Gaucheron et al., 2001). Zeisig and coworkers have demonstrated that the utilization of membrane-interacting alkyl phospholipids as helper lipids enhanced gene transfer (Zeisig et al., 2003). Enhanced transfection efficiency has also been observed with a new helper phospholipid bearing terminal benzyl group on the lipid chains (Prata et al., 2008). Recently, N-lauroylsarcosine was found to be a promising helper lipid among several transdermal penetration enhancers tested (Kurosaki et al., 2008). Although the exact mechanism of helper lipid effect is still not very clear (Hafez et al., 2001), it is believed that endosome membrane-destabilization is an important function of helper lipid (Xu and Szoka, 1996; Zuhorn et al., 2002). With this in mind, we have designed a series of asymmetric 1-alkyl 2-acyl glycerophosphatidylcholine (APC) as novel helper lipids. Here we report the rational design, synthesis and evaluation of these compounds.

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2. Materials and methods

2.1. Materials

Docosyl mesylate, erucyl mesylate, and nervonyl mesylate are from Nu-Chek Prep. Inc. (Elysian, MN). The phospholipids DOPE, 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), PEG-1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine (PEG-DSPE) conjugate, were purchased from Avanti Polar Lipids (Alabaster, AL). Thiocholesterol-based cationic lipids (TCL) were synthesized as previously described (see Fig. 1 for the structures of selected lipids). All other chemicals are from Aldrich (Milwaukee, WI). Plasmid luciferase (pLC0888) and β-galactosidase were generous gifts from Valentis, Inc. (Burlingame, CA, USA). PicoGreen reagent was from Invitrogen (Eugene, OR). CV1 and B16F10 cells, cell culture mediums, fetal bovine serum (FBS), phosphate buffered saline (PBS) and antibiotics were obtained from the UCSF Cell Culture Facility.

2.2. General methods

^{1}H NMR (400 MHz) spectra were recorded on a Varian 400 MHz instrument. Chemical shifts are expressed as parts per million using tetramethylsilane as internal standard. *J* values are in Hertz. Mass spectra were obtained at the Mass Spectrometry Facility, University of California at San Francisco. TLC analyses were performed on 0.25-mm silica gel plates. Lipids were detected on TLC plates by exposure to iodine vapor, by heating after spraying with cerium-molybodic acid, or by spaying with phosphorus stain (to identify phosphorus). High performance flash chromatography (HPFC) was carried out on a Biotage (Charlottesville, VA) Horizon™ HPFC™ system with pre-packed silica gel columns (60 Å, 40–63 μm). Unless noted otherwise, the ratios describing the composition of solvent mixtures represent relative volumes. APCs of various alkyl chains were synthesized by the same method as shown in Scheme 1, and only details of representative synthetic procedures were reported here.

2.3. Synthesis of APC

2.3.1. 4-(Docosyloxymethyl)-2,2-dimethyl-1,3-dioxolane (**1a**)

A mixture of solketal (3.3 g, 25 mmol) and powdered potassium hydroxide (6.5 g) in toluene (100 mL) was refluxed for 1.5 h under nitrogen in a round-bottomed flask fitted with a Dean-Stark head. Docosyl mesylate (5 g, 12.4 mmol) in toluene (50 mL) was added dropwise into the mixture within 30 min and the refluxing

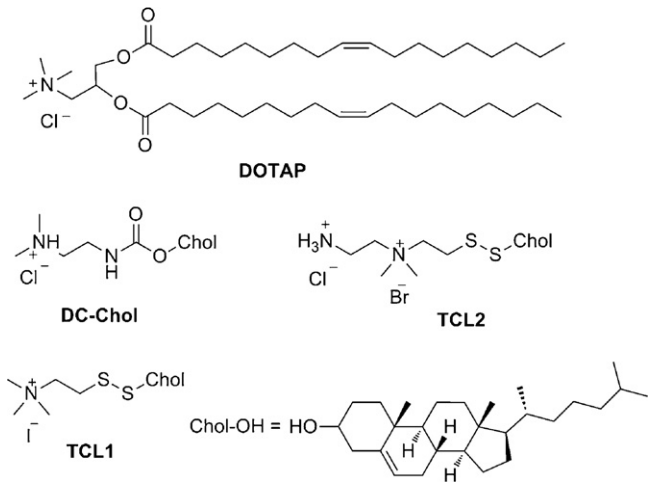
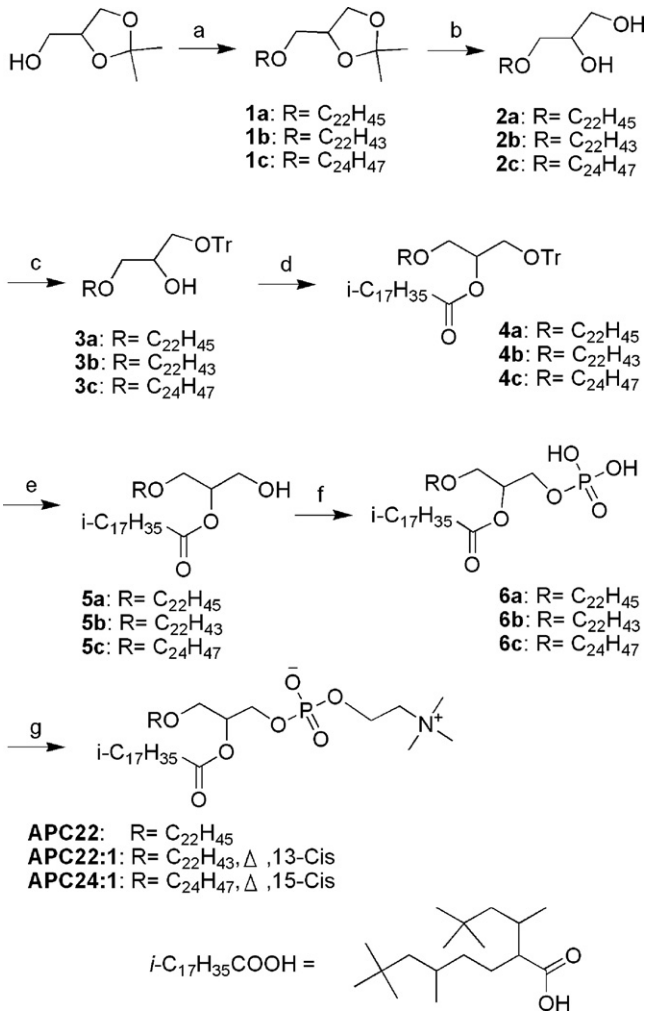


Fig. 1. Structures of cationic lipids TCL1, TCL2, DC-Chol and DOTAP.



Scheme 1. Synthesis of APC: (a) toluene, KOH, $ROSO_2Me$, 4 h, reflux; (b) MeOH, 4 M HCl, 12 h, r.t.; (c) Py, trityl chloride (1.2 equiv.), 12 h, 50 °C; (d) CH_2Cl_2, i-$C_{17}H_{35}COOH$, DCC, DMAP, 18 h, r.t.; (e) $CHCl_3$/MeOH, $BF_3.Et_2O$, 1 h, 0 °C; (f) THF, Py (2 equiv.), $POCl_3$ (1.1 equiv.), 3 h, 0 °C; g) Py, TPS, choline teraphenylborate, 16 h, r.t.

was continued for another 6 h. After cooling down to the room temperature, 100 mL water was added to the mixture. The organic phase was collected. The aqueous phase was extracted with ether (100 mL × 2). The organic layers were combined and evaporated to dryness. The crude product showed a major spot on TLC, and was used directly for next step reaction. R_f = 0.58 (toluene-ether, 9/1).

2.3.2. 3-(Docosyloxy)propane-1,2-diol (**2a**)

The crude **1a** was dissolved in methanol (100 mL) and hydrochloric acid (4 M, 15 mL). The mixture was stirred at r.t. for 12 h. After the addition of 100 mL water, the mixture was extracted with diethyl ether (100 mL × 2). The ether layer was then washed with water (40 mL × 3) and dried over anhydrous sodium sulfate, filtered, and evaporated to dryness. Recrystallization from hexane (120 mL) at 4 °C afforded 4.6 g white crystal. Total yield for two steps: 92.5%. R_f = 0.18, (hexane-ethyl acetate, 2/1). MALDI-MS calcd for $C_{25}H_{53}O_3^+$ $[M+H]^+$ 401.40, found 401.35 ($M+H^+$).

2.3.3. 1-(Docosyloxy)-3-(trityloxy)propan-2-ol (**3a**)

Trityl chloride (3.48 g, 12.48 mmol) and **2a** (4.54 g, 11.35 mmol) in 25 mL anhydrous pyridine were stirred for 24 h at 50 °C under argon. Then 4 mL water was added to the mixture to stop the reaction. Solvents were evaporated under reduced pressure. The residue was purified by HPFC (2–11% ethyl acetate in hexane). Yield

5.5 g, 75%. R_f = 0.72, (hexane-ethyl acetate, 2/1). MALDI-MS calcd for $C_{44}H_{67}O_3^+$ $[M+H]^+$ 643.51, found 643.48 ($M+H^+$).

*2.3.4. 1-Docosyl-2-iso-stearoyl-3-trityl glycerol (**4a**)*

To a mixture of **3a** (3.22 g, 5 mmol) and *iso*-stearic acid (2.84 g, 2 equiv.) in anhydrous methylene chloride (40 mL), were added DCC (2.27 g, 2.2 equiv.) and DMAP (1.22 g, 2 equiv.) at 0 °C. The mixture was then stirred at r.t. for 24 h. The reaction mixture was extracted with water (10 mL), dried over anhydrous sodium sulfate, filtered and evaporated. The residue was purified by HPFC (0–8% ethyl acetate in hexane). White solid (4.4 g, 97% yield) was obtained. R_f = 0.54, (hexane-ethyl acetate, 10/1). ^{1}H NMR ($CDCl_3$), δ 0.84–1.05 (m, 31H); 1.26 (m, 40H); 1.40-1.60 (5H, m); 2.19 (m, 2H); 3.25 (t, J = 4, 2H); 3.36 (d, J = 4, 2H); 3.56 (d, J = 4, 2H); 5.18 (m, 1H); 7.26 (m, 9H); 7.43 (m, 6H). MALDI-MS calcd for $C_{62}H_{101}O_4^+$ $[M+H]^+$ 909.77, found 909.78 ($M+H^+$).

*2.3.5. 1-Docosyl-2-iso-stearoyl-glycerol (**5a**)*

To a solution of **4a** (1.82 g, 2 mmol) in anhydrous methylene chloride (15 mL), was added boron trifluoride diethyl etherate (1.25 mL) at 0 °C. The mixture was stirred at 0 °C for 1 h, then partitioned between chloroform–water (30 mL/10 mL). The organic layer was dried over anhydrous sodium sulfate, filtered, and evaporated. The residue was dried over phosphorous pentoxide under high vacuum for 4 h and used directly for next step reaction.

*2.3.6. 1-Docosyl-2-iso-stearoyl-3-glycero-phosphatidic acid (**6a**)*

A solution of the above crude product **5a** and anhydrous pyridine (0.31 mL) in 10 mL anhydrous THF was added dropwise into freshly distilled phosphorus oxychloride (0.22 mL) in 4 mL anhydrous THF with stirring at 0 °C. After the addition, the mixture was kept at 0 °C for another 3 h. Then 4 mL 10% sodium carbonate was added at once, and the mixture was stirred at 0 °C for 15 min. The mixture was poured into 20 mL ice-water, acidified with conc. hydrochloric acid, extracted with diethyl ether consecutively (80 mL × 2). The ether layer was dried, evaporated to dryness. The residue was dried azeotropically with toluene, and used directly for subsequent reaction. The major product had an R_f value of 0.05 on TLC (chloroform-methanol-ammonium hydroxide, 65/25/4).

*2.3.7. 1-Docosyl-2-iso-stearoyl-3-glycero-phosphatidyl choline (**7a**, APC22)*

The above crude product **6a** (0.67 g, ca. 0.7 mmol), choline tetraphenyl borate (0.59 g, 2 equiv.), and 2,4,6-triisoproylbenzene sulfonyl chloride (0.53 g, 2.5 equiv.) were dissolved in anhydrous pyridine (15 mL) with brief warming, then stirred at room temperature for 16 h. After the addition of water, the solvents were removed by rotary evaporation. The residue was extracted with diethyl ether twice. The ether extract was evaporated, and the crude product was purified by HPFC (chloroform to chloroform–methanol–ammonium hydroxide 65/25/4). Waxy solid (0.42 g, 70% yield) was obtained. R_f = 0.34, (chloroform–methanol–ammonium hydroxide, 65/25/4). ^{1}H NMR ($CDCl_3$), δ 0.84–1.05 (m, 31H); 1.26 (m, 40H); 1.40–1.60 (5H, m); 2.19 (m, 2H); 3.39 (s, 11H); 3.57 (d, J = 6, 2H); 3.86 (m, 2H); 3.93 (m, 2H); 4.36 (d, J = 6, 2H); 5.12 (m, 1H). MALDI-MS calcd for $C_{48}H_{99}NO_7P^+$ $[M+H]^+$ 832.72, found 832.70.

*2.3.8. 1-Erucyl-2-iso-stearoyl-3-glycero-phosphatidyl choline (**7b**, APC22:1)*

Compound **7b** was synthesized according to the method of **7a.** ^{1}H NMR ($CDCl_3$), δ 0.84-1.05 (m, 31H); 1.26 (m, 32H); 1.40–1.60 (5H, m); 2.01 (m, 4H); 2.19 (m, 2H); 3.39 (s, 11H); 3.60 (d, J = 6, 2H); 3.88 (m, 2H); 3.96 (m, 2H); 4.39 (d, J = 6, 2H); 5.12 (m, 1H), 5.36 (t, J = 4, 2H). MALDI-MS calcd for $C_{48}H_{97}NO_7P^+$ $[M+H]^+$ 830.70, found 830.66.

*2.3.9. 1-Nervonyl-2-iso-stearoyl-3-glycero-phosphatidyl choline (**7c**, APC24:1)*

Compound **7c** was synthesized according to the method of **7a.** ^{1}H NMR ($CDCl_3$), δ 0.84–1.05 (m, 31H); 1.26 (m, 36H); 1.40-1.60 (5H, m); 2.01 (m, 4H); 2.19 (m, 2H); 3.39 (s, 11H); 3.60 (d, J = 6, 2H); 3.88 (m, 2H); 3.96 (m, 2H); 4.39 (d, J = 6, 2H); 5.12 (m, 1H), 5.36 (t, J = 4, 2H). MALDI-MS calcd for $C_{50}H_{101}NO_7P^+$ $[M+H]^+$ 858.73, found 858.75.

2.4. Preparation of liposomes and lipoplexes

Liposomes and lipoplexes were prepared as previously described (Huang et al., 2005). Briefly, a chloroform solution of lipids was evaporated in a glass tube under reduced pressure, placed under high vacuum for 4 h, hydrated in pH 7.4 10 mM HEPES buffer with 140 mM NaCl under argon. TCL/helper lipids were hydrated at 60 °C and all other lipids were hydrated at room temperature. The hydrated lipids were sonicated for 15 min under argon to form a translucent liposome suspension. Lipoplexes of various charge ratios were prepared by adding an equal volume of plasmid solution dropwise into the cationic lipid solution with constant mixing on a vortex mixer over a 2 min period. After the addition of DNA, the mixtures were incubated at room temperature for at least 5 min and no longer than 30 min before transfection. When the lipoplexes were used for transfection, both plasmid DNA and the cationic lipid were diluted in the cell growth medium before adding them to the cultured cells.

2.5. Preparation of nanolipoparticles (NLP)

NLP was formulated by mixing DNA and lipids in an aqueous-ethanol monophase (Hayes et al., 2006). The NLP lipids comprised of 50 mole% cationic lipid, x mol% PEG-DSPE, and $(50 - x)$ mol% help lipid, wherein: x ranging from 0.1 to 10. The plasmid DNA was complexed with lipids in 50% ethanol at a fixed charge ratio 3/1 (+/−) between the cationic lipids and the negatively charged DNA. NLP was obtained after removing ethanol from the complex by membrane dialysis. The efficiency of DNA encapsulation was measured by PicoGreen assay as previously described (Li et al., 2005).

2.6. Particle diameter measurement

The diameter of liposomes, lipoplexes and NLP were measured on a Malvern Zetasizer 3000 Dynamic Light Scattering Instrument (Malvern Instruments Ltd., Worcestershire, UK). The particle diameter was analyzed by the PCS 1.32a software.

2.7. Transfection experiments

CV-1 cells were grown in DME H-21 medium containing 10% FBS and antibiotics (100 units/ml penicillin and 0.1 mg/mL streptomycin). B16F10 cells were maintained in MEM Eagle's with EBSS medium containing 10% fetal bovine serum, 1% MEM non-essential amino acids, 1% sodium pyruvate 11 mg/mL, and 1% penicillin–streptomycin, and 0.1 μm of sterile filtered. Cells were seeded onto the 96-well plate at 10,000 cells/well in a humidified atmosphere containing 5% CO_2 at 37 °C 1 day before transfection. The growth medium was refreshed before transfection. A solution of freshly prepared lipoplex or NLP was added to each well and the final volume per well was kept at 125 μL. The cells were incubated at 37 °C for 4 h, and then the growth medium was refreshed. After an additional 48 h incubation, the medium was removed and the cells were washed with PBS twice (100 μL per well). Then, the cells

were lysed in 50 μL reporter lysis buffer (Promega, Madison, WI) using the freeze–thaw method. After the completion of cell lysis, the plate was centrifuged for 5 min at 4 °C at 50 × *g*. The supernatant was then used for the standard β-galactosidase assay or luciferase assay. Similar protocol was used for 24-well plate but at larger scale.

2.8. Cytotoxicity of lipoplexes

The effect of the lipoplexes on the cell growth during the transfection was assessed with CellTiter-Blue assay (Promega, technical bulletin No.317). Cells were transfected according to the 96-well protocol mentioned above at various charge ratios. After 48 h incubation, 20 μL CellTiter-Blue reagent was added to each well, and the mixture was incubated at 37 °C with 5% CO_2 for 2.5 h. Fluorescence at 560 nm (excitation)/590 nm (emission) were read and the relative cell viability was calculated by comparing treated cells with untreated cells.

3. Results and discussion

3.1. Synthesis of APC

As shown in Scheme 1, APC with different alkyl chains were synthesized in a straightforward route with typical phospholipid synthesis strategy (Paltauf and Hermetter, 1994). The long alkyl chain was first introduced to the 1-position of glycerol backbone by Williamson etherization. Then the iso-stearoyl chain was coupled to the 2-position with the 3-hydroxyl protected by trityl group. The removal of trityl group by boron trifluoride at 0 °C resulted in non-detectable acyl migration (Paltauf and Hermetter, 1991). Phosphorylation of the 3-hydroxyl group with phosphorus oxychloride led to the corresponding phosphatic acid with minor side product. The yield of the attachment of choline to the phosphatic acid was significantly improved by the use of choline tetraphenylborate compared with choline tosylate.

3.2. Design of APC

On the rationale design of APC, we emphasize three factors: (1) the geometry of the whole molecule; (2) the use of choline as the head group; (3) the asymmetry and rigidity of the hydrophobic tail. All APC (Scheme 1) have the choline as the head group, a long alkyl chain at 1-position, and the highly branched iso-stearoyl group at 2-position. Contrary to the small head group area of phosphatidylethanolamine (PE), phosphatidylcholine (PC) has a relatively large head group area. To obtain a cone or cylinder shape of APC as other helper lipids generally have (Ulrich, 2002), we include the bulky branched iso-stearoyl group in the molecule to generate a big tail area. We then use the long alkyl chain to tune the geometry of APC by varying the chain length and introducing the double bond. Lipoplexes formulated with DOPE as helper lipid tend to induce hemagglutination and potential microinfarction (Eliyahu et al., 2002; Sakurai et al., 2001a, 2001b). Most *in vivo* lipoplexes formulations employed cholesterol as the helper lipid but with compromised transfection efficiency (Sakurai et al., 2001b; Xu and Anchordoquy, 2008). DOPE was also reported to activate lipid mixing with lipoprotein resulting in poor *in vivo* transfection while lyso-PC inhibited such mixing (Tandia et al., 2005). The use of choline as the head group of APC may help formulate long-circulating and effective lipid-based gene delivery system since PC is the major lipid in our body and has been widely applied in the liposomal drug delivery. In addition to the geometry of the neutral lipid, bending rigidity of the bilayer is also important for the helper lipid effect (Safinya and Koltover, 1999). It is well known that long symmetric diacyl PC generally cannot enhance the transfection efficiency of cationic liposomes due to the stable lamellar phase (Fasbender et al., 1997). However, the addition of short chain co-surfactants like pentanol, hexanol, and heptanol to membranes of lamellar phase with a mole ratio of between two to four leads to a significant decrease of bending rigidity (Safinya et al., 1989). Wang et al. reported the enhanced gene transfer mediated by the combination of dilauroyl (C12 chain) and dioleoyl (C18 chain) homologues of O-ethylphosphatidylcholine in contrast to the poor transfection ability of the individual cationic lipid (Wang and MacDonald, 2004). There are also increasing interest in designing asymmetric cationic lipids to improve transfection efficiency (Chandrashekhar et al., 2011; Koynova et al., 2009; Nantz et al., 2010). Some fusogenic cationic lipids can mediate efficient transfection without helper lipids (Cherezov et al., 2002; Koynova et al., 2009). Clearly, it is the overall property of the lipoplexes that determines transfection efficiency of the specific formulation. For many traditional symmetric cationic lipids, asymmetric helper lipids may be a good match for the formulation. Here, we introduce two highly asymmetric hydrophobic chains in the tail to make APC less rigid and hard to pack compactly so that the destabilization of the lamellar phase would be favored when the phase transition is triggered, preferably in the endosome.

3.3. Formulation of APC liposome and lipoplex

Diacyl-based lipid DOTAP and cholesterol-based lipid DC-Chol, TCL1, and TCL2 (Huang et al., 2005) (Fig. 1) were used as model cationic lipids for the evaluation of APC due to their high transfection activity when formulated with DOPE. Similar to DOPE, APC24:1 itself cannot form stable liposome but aggregated upon hydration (Fig. 2A, curve c, aggregated particles reported as 1000 nm since they settled at the bottom of cuvette). When mixed with TCL1 at 1:1 mole ratio, APC24:1 can form relatively stable cationic liposomes (Fig. 2B, curve c). However, the lipoplexes of TCL1/APC24:1/plasmid were not stable even at charge ratio 3/1 (+/−), they aggregated within 30 min (Fig. 2C, curve c). Helper lipids with shorter alkyl chain (APC22 and APC22:1) can form relatively stable liposomes either alone or in the presence of TCL1 (Fig. 2, curves a and b). Most liposomes had polydispersity index 0.1–0.3, and 0.4–0.6 for lipoplexes. The overall properties of lipoplexes are determined by many factors such as the type of cationic lipids and helper lipids, the ratio of lipid components, charge ratio, and the method of preparation. Here we chose formulations based on our previous knowledge and the commonly used lipids and charge ratios to investigate the influence of APC on the formulation. By monitoring the particle diameter change over time, we can see a trend of increasing destabilization of APC containing liposomes in the presence of longer alkyl chain or double bond.

3.4. In vitro transfection

3.4.1. Transfection of APC lipoplexes

To evaluate the helper lipid effect of APC, we formulated them with cationic lipids at 1:1 mole ratio, and used these liposomes to condense plasmid at charge ratio 3/1 (+/−) for the transfection of cells in the presence of 10% fetal bovine serum. Interestingly, all three APC significantly enhanced β-galactosidase transfection on CV-1 cells when formulated with cholesterol-based cationic lipid TACS, but not with the diacyl-based cationic lipid DOTAP (Table 1). However, high transfection activity of plasmid luciferase on B16F10 cells was observed for both types of cationic lipids formulated with APC (Fig. 3). Formulations containing APC22 showed least variation in transfection activity compared with the two order of difference in APC24:1 formulations. This may relate to the increasing instability of the formulation introduced by APC24:1. It seems that the helper lipid effect not only depends on the helper lipid itself, but also the overall biophysical properties of the formulation, and the

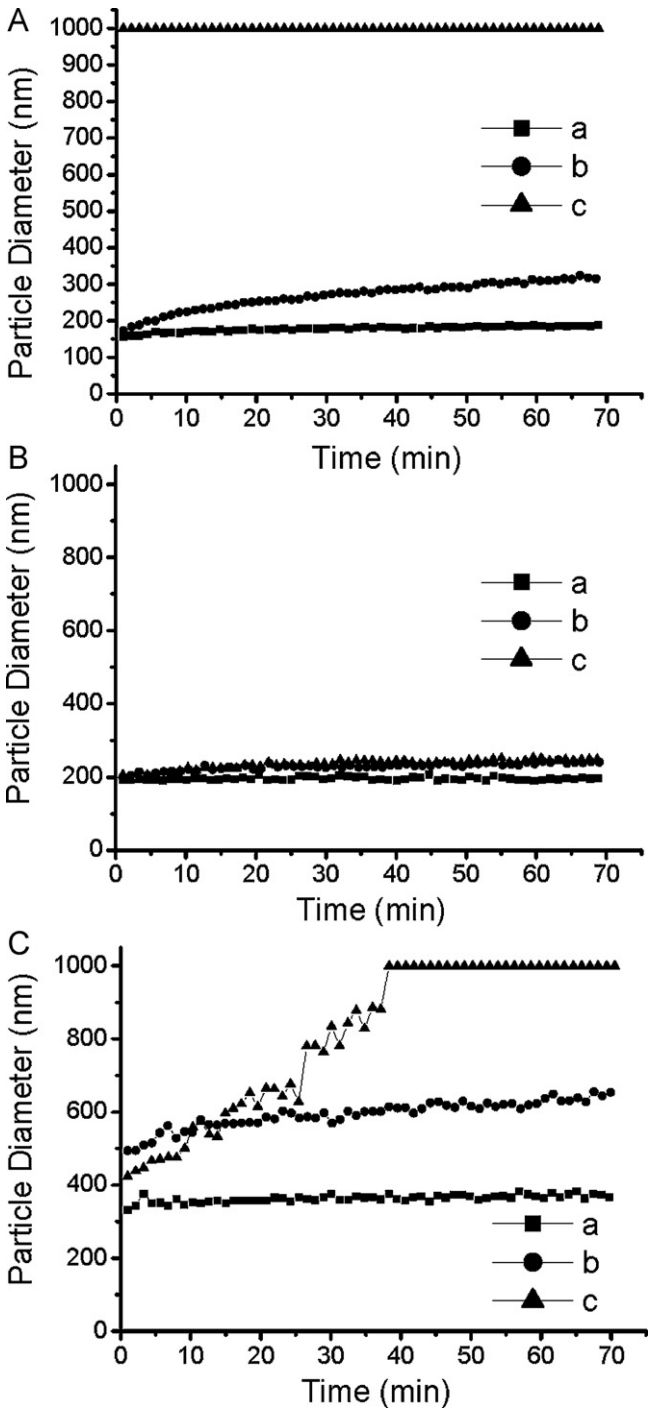


Fig. 2. Stability of particle diameter over time. Diameters no less than 1000 nm (most of time aggregation) are shown as 1000 nm: (A) liposomes formed by APC alone; (B) liposomes composed of TCL1/APC (1:1 mole ratio); (C) lipoplexes of TCL1/APC (1:1) and β-galactosidase plasmid at charge ratio 3/1 (+/−); curve a: APC22; curve b: APC22:1; curve c: APC24:1. Liposome diameters were monitored immediately after 10 min bath sonication at r.t. Diameters of lipoplexes were measured 1 min after their preparation.

cell type. Systematic study is needed to figure out the exact mechanism to help choose appropriate helper lipid for given formulation. At least, high transfection efficiency was observed in both cell lines for cholesterol-based cationic lipids formulated with APC. Based on this observation, we chose TCL1 as the model cationic lipid for NLP formulation and transfection study.

Table 1
β-Galactosidase transfection activity on CV-1 cells (munit/mg protein).[a]

Cationic lipids	DOPE	APC22	APC22:1	APC24:1
DOTAP	863	66	34	17
TCL1	2602	2072	944	1539
TCL2	1701	802	566	472
DC-Chol	463	235	410	191

[a] Cationic lipids were formulated with different helper lipids at charge ratio 3/1 (+/−). Cells were transfected in 96-well plate with 0.5 μg plasmid/well. Results are the average of triplicate experiments with a variation less than 10%. The transfection activity of free plasmid DNA is around 0 munit/mg protein.

3.4.2. Transfection of APC nanolipoparticles

Although lipoplexes are effective for *in vitro* cell transfection, the application of them for systemic *in vivo* gene transfer is complicated, less effective and mainly confined to the lung (Audouy et al., 2002; Zhang et al., 2008). Their high cationic charge density and tendency to form large aggregates result in quick clearance from the circulation, and subsequent accumulation in the 'first pass' organs such as the liver, the spleen, and particularly the lungs (Liu et al., 1997; Uyechi et al., 2001; Zhang et al., 2008). Due to the nature of lipoplexes, it is also difficult to correlate the *in vitro* data to the *in vivo* results (McNeil et al., 2010). We have reported a method to prepare DNA-containing nanolipoparticles (NLP) through sequential assembly of TCL1/DOPE/PEG-DSPE/DNA system (Huang et al., 2005). These surface tunable NLP could be useful for the *in vivo* gene delivery. Here we want to know whether APC can be used to form DNA-containing NLP. Small diameter NLP of TCL1/APC22/PEG-DSPE was successfully prepared by the ethanol dialysis method (Hayes et al., 2006) with most of pLC0888 plasmid encapsulated inside (Table 2). It is noticeable that the diameter of APC22 NLP was less than 100 nm in the presence of only 0.1% PEG-lipid, and around 60 nm with 10% PEG-lipid while DOPE NLP required higher percentage of PEG-lipid to reach small diameter. APC22 NLP showed good luciferase transfection activity with small amount of PEG-lipid in the formulation. However, the transfection activity dropped dramatically with increasing percentage of PEG-lipid. The inclusion of excessive inverted cone shaped PEG-lipid in the formulation may make the NLP too stable to escape out of the endosome in time. This problem may be addressed by incorporating pH-cleavable PEG-lipid in the formulation to facilitate endosome escape (Guo and

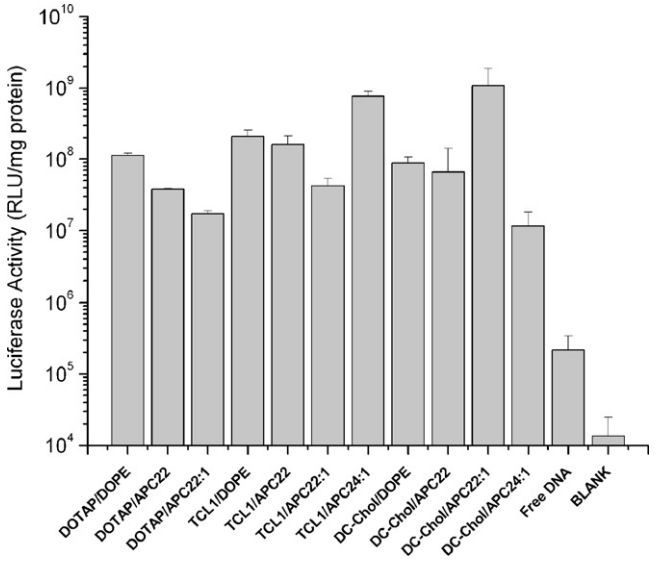


Fig. 3. Plasmid luciferase transfection activity on B16F10 cells. Cationic lipids were formulated with different helper lipids at charge ratio 3/1 (+/−). Cells were transfected in 24-well plate with 2 μg plasmid per well.

Table 2
NLP[a] containing different percentage of PEG-lipid.

PEG-lipid (mole%)	10		6		3		1.5		0.5		0.1	
Sample[b]	A	D	A	D	A	D	A	D	A	D	A	D
NLP diameter (nm)	57.7	67.7	60.3	73.9	74.7	126.2	85.2	146.7	93.6	194.1	93.3	213.7
DNA encapsulation ratio (%)[c]	87.0	84.8	91.5	89.0	95.8	91.0	96.6	88.0	96.3	81.4	97.1	89.3
Relative luciferase activity (RLU/mg protein)	3.5E2	2.0E5	5.6E3	6.8E5	1.6E5	1.2E6	4.5E5	3.3E7	1.5E7	1.1E9	2.4E8	1.3E9

[a] NLP composition: x %PEG-DSPE, 50% TCL1, $(50-x)$ % APC22 or DOPE with 2.5 μmole total lipids. Charge ratio 3/1 (+/−).
[b] A: APC22; D: DOPE. All data are based on triplicate measurements.
[c] DNA encapsulation ratio was determined by Picogreen assay.

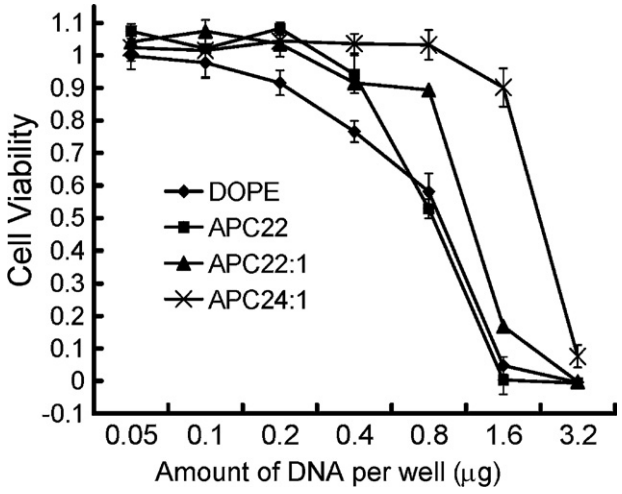


Fig. 4. Cytotoxicity of APC lipoplexes on CV-1 cells.

Szoka, 2003; Li et al., 2005). DNA-containing APC NLP could be carefully tailored for systemic gene delivery by varying the ratio of components and tuning the surface characteristics (Huang et al., 2005).

3.5. Cytotoxicity of APC lipoplexes

We further studied the cytotoxicity of APC lipoplexes (Fig. 4) on CV-1 cells. APC lipoplexes are almost nontoxic in the range of low to high dose (0.5 μg DNA/well in 96-well plate). Toxicity was observed only under very high dose condition (1.6 or 3.2 μg DNA/well). According to our previous study, NLP showed much lower cytotoxicity than lipoplexes (Li et al., 2005). APC-containing NLP, although not tested here, is expected to be less toxic than APC lipoplexes. The low toxicity makes APC the promising helper lipids for cationic lipids based non-viral delivery system.

4. Conclusion

In summary, we have developed a series of asymmetric helper lipids (APC) through *de novo* synthesis with a rationale design. APC significantly enhance the gene transfer efficiency of cationic lipids with low toxicity. They can further be formulated into DNA-containing nanolipoparticles by the ethanol dialysis method. Through the development of APC, we have demonstrated a strategy other than modifying cationic lipids to improve the non-viral gene delivery efficiency. Further systematic work on the exact mechanism of helper lipid effect may help develop more sophisticated neutral lipids to enhance the gene transfer. Additionally, the inclusion of a targeting ligand onto the APC NLP may lead to systemic targeted gene therapy.

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