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Novel macrocyclic and acyclic cationic lipids for gene transfer: Synthesis and in vitro evaluation

William P. D. Goldring [a,*,†], Emile Jubeli [b,†], Rachael A. Downs [a,†], Adam J. S. Johnston [a], Nada Abdul Khalique [b], Liji Raju [b], Deena Wafadari [c], Michael D. Pungente [c,*,†]

[a] School of Chemistry and Chemical Engineering, Queen's University Belfast, David Keir Building, Stranmillis Road, Belfast, Northern Ireland, BT9 5AG, United Kingdom
[b] Research Division, Weill Cornell Medical College in Qatar, Education City, PO Box 24144, Doha, Qatar
[c] Premedical Unit, Weill Cornell Medical College in Qatar, Education City, PO Box 24144, Doha, Qatar

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ABSTRACT

The synthesis and in vitro evaluation of four cationic lipid gene delivery vectors, characterized by acyclic or macrocyclic, and saturated or unsaturated hydrophobic regions, is described. The synthesis employed standard protocols, including ring-closing metathesis for macrocyclic lipid construction. All lipoplexes studied, formulated from plasmid DNA and a liposome composed of a synthesized lipid, 1,2-dimyristoyl-*sn*-glycero-3-ethylphosphocholine (EPC), and either 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) or cholesterol as co-lipid, exhibited plasmid DNA binding and protection from DNase I degradation, and concentration dependent cytotoxicity using Chinese hamster ovary-K1 cells. The transfection efficiency of formulations with cholesterol outperformed those with DOPE, and in many cases the EPC/cholesterol control, and formulations with a macrocyclic lipid (+/− 10:1) outperformed their acyclic counterparts (+/− 3:1).

Gene therapy has significant potential for the treatment of inherited and acquired life threatening diseases, such as cancer, AIDS, cardiovascular diseases, and certain autoimmune disorders.[1] Viral vectors are the most widely used gene delivery vehicle due to their high transfection efficiency. However, the use of viral based therapeutics is restricted by drawbacks including immunogenicity, biological safety, and size restrictions on the plasmid DNA (*p*DNA) cargo. For these reasons, non-viral synthetic gene delivery reagents represent an attractive, alternative approach to gene delivery that offers many advantages over viral vectors. Liposomes based on cationic lipids have been the subject of considerable interest as non-viral delivery vectors,[2,3] which, in general, exhibit excellent biocompatibility, low immunogenicity,[4] low toxicity,[5,6] well defined physical and chemical composition, large nucleic acid payload capacity, and the potential to transfect numerous tissue and cell types.[7] Furthermore, cationic lipids are characterised by their simplicity of production and ease of application.

The typical structure of cationic lipid vectors includes a positively charged polar (hydrophilic) headgroup connected through to a hydrophobic domain via a linker unit (e.g., an ester) and a backbone, typically glycerol (Fig. 1). The two most common hydrophobic domain architectural constructs are composed of either; (i) two flexible (non-rigid) hydrophobic tails, mainly as saturated or mono-unsaturated fatty acid chains containing typically 14 to 18 carbon atoms, where 1,2-dimyristoyl-*sn*-glycero-3-ethylphosphocholine (EPC) in Figure 1 is a representative example, or (ii) a rigid cholesteryl moiety, exemplified by 3β-[*N*-(*N*′,*N*′-dimethylaminoethyl)-carbamoyl]cholesterol (DC-Chol). A common empirical

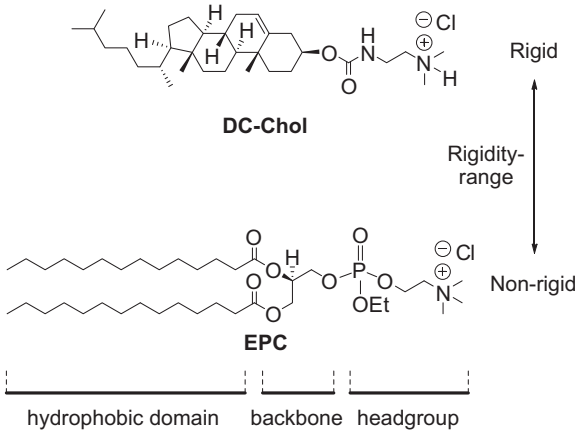

Figure 1. Lipid structural features and the rigidity range associated with the hydrophobic domain of common cationic lipids DC-Chol and EPC.

* Corresponding authors. Tel.: +44 (0) 28 9097 4414; fax: +44 (0) 28 9097 6524 (W.P.D.G.); tel.: +974 4492 8216; fax: +974 4492 8222 (M.D.P.).
E-mail addresses: w.goldring@qub.ac.uk (W.P.D. Goldring), mdp2001@qatar-med.cornell.edu (M.D. Pungente).
† These authors have contributed equally to this work.

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approach to improving transfection efficiency and reducing cellular toxicity has focused on synthetic modifications to the positively charged headgroup and/or linker functionality, while less attention has been given to the role of the hydrophobic domain. Our recent research efforts have focused on structural modifications to the lipid headgroup, but also an investigation of the rigidity-range (Fig. 1) associated with the hydrophobic core of lipid vectors towards enhancing nucleic acid delivery,[8,9] where the lipids EPC and DC-Chol represent two extremes along the rigidity-range.

Macrocyclic archaebacteria lipids[10] have attracted much attention because they form liposomes that demonstrate enhanced stability to oxidative stress, high temperature, alkaline pH and the action of serum proteins.[11] It has been previously reported that these lipids form more tightly packed vesicles than their acyclic counterparts.[12] Furthermore, Benvegnu and co-workers[13] have reported the use of novel, archaeobacterial-like cationic lipids for in vitro gene transfection for the purpose of modulating the lipidic membrane fluidity of the complexes they form with DNA. Such reports have led us to hypothesize that macrocyclic cationic lipids may package *p*DNA sufficiently different than their acyclic lipid analogues, possibly giving rise to enhanced protection of the *p*DNA cargo from degradative enzymes, and ultimately enhanced gene transfer. To the best of our knowledge there are no reports on the use of cationic lipids containing a macrocyclic hydrophobic domain as gene carriers. The synthesis of a macrocyclic lipid as a 'perspective' molecule for gene transfer was previously described in the literature.[14] Reports on the use of cyclic structures in the headgroup have appeared in the literature, including applications of; liposomes containing varying amounts of surfactin, a biosurfactant containing a cyclic peptide in the headgroup,[15] a novel, single-chain lipid containing a macrocyclic polyamine (Cyclen) headgroup,[16] and a cationic glycolipid containing a cyclic galactose-based headgroup.[17] Finally, examples of gene carriers which incorporate a macrocyclic structure in the backbone include structures which possess a calix[4]arene[18] or a cyclodextrin.[19]

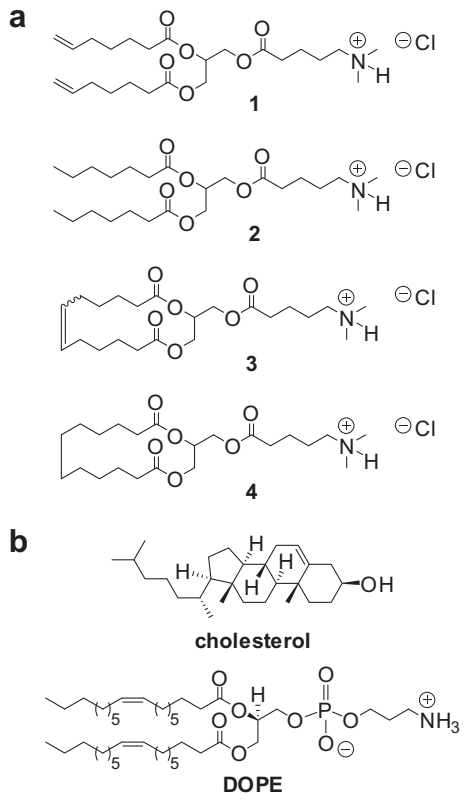
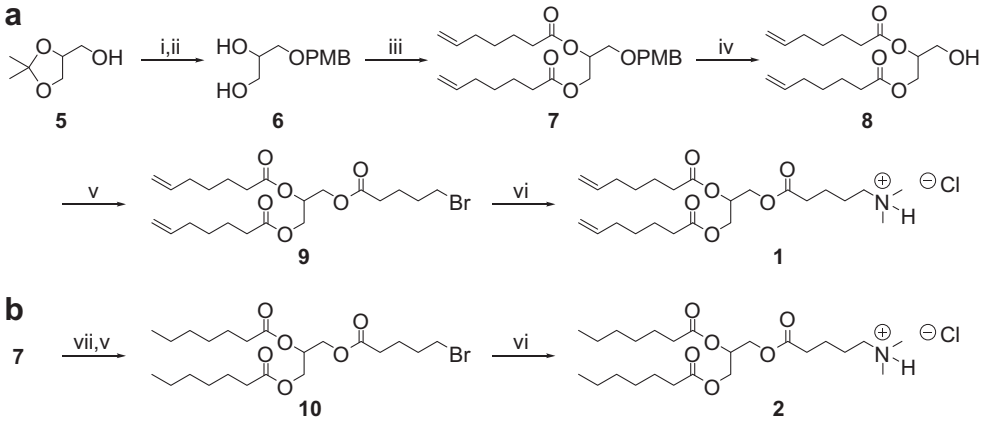


Figure 2. The structures of lipids employed in this study: (a) the acyclic and macrocyclic cationic lipids, **1**–**4**; and (b) the neutral co-lipids cholesterol and DOPE.

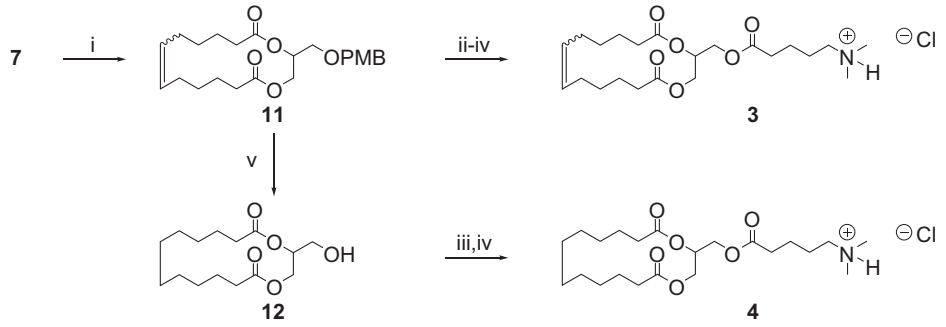
In the present work, four novel cationic lipids, **1**–**4**, were synthesized as gene delivery reagents (Fig. 2a), and liposomes were prepared from the synthetic lipids mixed with the commercial cationic lipid EPC together with a neutral co-lipid, either 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) or cholesterol (Chol) (Fig. 2b). An equal molar proportion of the cationic lipid EPC was included in each lipoplex formulation to aid in the solubility of the synthetic lipids **1**–**4**. Furthermore, the formulations contained either the neutral lipid DOPE or Chol to assess the impact these co-lipids had on gene delivery. Lipid/*p*DNA complexes (lipoplexes) were formulated by associating varying amounts of liposomes with a constant amount of *p*DNA. The interaction of liposomes with *p*DNA was investigated via gel electrophoresis and the protective character of the cationic lipids within these structures was assessed via a degradation assay. The set of four related cationic lipids possess identical amine headgroups, and comprise two pairs; each of which contains, in the hydrophobic domain, an acyclic and a cyclic counterpart. One pair, **1** and **3**, is composed of lipids containing one or two alkene units in the hydrophobic region, and the other pair, **2** and **4**, is composed of the corresponding saturated lipids. An evaluation of the in vitro cytotoxicity and gene transfer structure-activity relationships were conducted with Chinese hamster ovary-K1 (CHO-K1) cells.

Our synthesis of a cationic lipid library began with the protection of commercially available solketal (**5**), using 4-methoxybenzyl chloride and potassium hydroxide in dimethyl sulfoxide (DMSO), followed by treatment of the cyclic acetal with 10% aqueous acetic acid to give the diol **6** in 50% yield over two steps (Scheme 1a). Esterification of the diol **6** with two equivalents of 6-heptenoic acid using *N*,*N*′-dicyclohexylcarbodiimide (DCC) and 4-(dimethylamino)pyridine (DMAP) gave the diene **7** in 58% yield. The diene **7** was used as a common building block from which all of the required cationic lipids were elaborated. For example, construction of the unsaturated acyclic cationic lipid **1** began with deprotection of the benzyl ether in **7** using 2,3-dichloro-5,6-dicyano-*p*-benzoquinone (DDQ) to give the crude alcohol **8**, which was used in the subsequent step without further purification. Our attempts to purify the alcohol **8** by flash column chromatography on silica, and other related structures such as the alcohol **12** (Scheme 2), led to an inseparable mixture of acyl shift products, which included the corresponding symmetrical constitutional isomer.[20] Next, installation of the cationic headgroup began with esterification of the crude alcohol **8** using 5-bromovaleric acid, in the presence of DCC and DMAP, to give the tri-ester **9** in 17% yield over two steps. We were disappointed to discover this reaction, and others such as the construction of **10**, suffered from the formation of a symmetrical tri-ester, which made up the remainder of the material balance, resulting from secondary to primary hydroxyl acyl shift, followed by esterification of the resulting secondary alcohol with 5-bromovaleric acid. Finally, the bromide **9** was displaced with a 10-fold excess of dimethylamine (2 M in tetrahydrofuran (THF)), followed by treatment of the resulting amine with a 2 M solution of hydrochloric acid in diethyl ether to give the cationic unsaturated acyclic lipid **1** in 99% yield over two steps.[21]

Construction of the cationic saturated acyclic lipid **2**, which followed a similar sequence of steps to those described above for **1**, started from the common building block **7**. Hydrogenation and hydrogenolysis of **7** using hydrogen gas and 10% palladium on carbon, followed by esterification of the resulting crude alcohol gave the tri-ester **10** in 67% yield over two steps (Scheme 1b). In a two-step sequence, involving amination and subsequent treatment with acid, the bromide **10** was converted into the cationic lipid **2** in 99% yield over two steps.



Scheme 1. Reagents and conditions: (i) PMBCl, KOH, DMSO, rt; (ii) 10% AcOH, water, 90 °C, 50% (2 steps); (iii) 6-heptenoic acid (2 equiv.), DCC, DMAP, CH_2Cl_2, rt, 58%; (iv) DDQ, water/CH_2Cl_2 (1:10), rt; (v) 5-bromovaleric acid, DCC, DMAP, CH_2Cl_2, rt, 17% (**9**) or 67% (**10**) (2 steps); (vi) a. 2 M $(CH_3)_2NH$ in THF, THF, rt; then b. 2 M HCl in diethyl ether, rt, 99% (2 steps); (vii) H_2, 10% Pd-C, AcOH/EtOH (1:10), rt. PMB = *p*-methoxybenzyl.



Scheme 2. Reagents and conditions: (i) Grubbs' 2nd generation pre-catalyst, CH_2Cl_2, 40 °C, 73% (3:1, *E*:*Z*); (ii) DDQ, water/CH_2Cl_2 (1:10), rt; (iii) 5-bromovaleric acid, DCC, DMAP, CH_2Cl_2, rt, 44% (to **3**) or 52% (to **4**); (iv) a. 2 M $(CH_3)_2NH$ in THF, THF, rt; then, b. 2 M HCl in diethyl ether, rt, 99% (**3**) or 93% (**4**); (v) H_2, 10% Pd-C, AcOH/EtOH (1:10), rt.

The common building block **7** was employed to elaborate the macrocyclic cationic lipids **3** and **4**, corresponding to the cationic acyclic lipids **1** and **2**, respectively. Ring-closing metathesis (RCM) of the diene **7** using Grubbs' second generation pre-catalyst, under high dilution conditions, gave an inseparable 3:1 mixture of the macrocyclic alkene isomers **11** in 73% combined yield (Scheme 2). Using proton NMR we were unable to conclusively determine which of the alkene isomers of **11** was the major product. Molecular mechanics calculations, together with a Monte Carlo conformational search, were separately performed on the individual isomeric macrocyclic products **11**, each of which led to a number of low energy conformations. Using the energy difference between the global minimum conformation of each geometric isomer a 3:1 (*E*)-**11** to (*Z*)-**11** ratio was calculated.[22] Next, the macrocyclic lipid **11** was converted into the unsaturated macrocyclic cationic lipid **3** in 44% yield over three steps, which included: DDQ deprotection, esterification with 5-bromovaleric acid, and then conversion of the alkyl bromide into the ammonium salt **3**. During the conversion of **11** into **3**, all synthetic intermediates and the cationic lipid **3** were formed as inseparable 3:1 mixtures of geometric isomers. Finally, using a similar sequence of steps the saturated macrocyclic cationic lipid **4** was constructed from **11**, via the crude alcohol **12**, in 48% yield over three steps.

Lipoplexes were prepared from liposomes that contained one of the four synthetic cationic lipids, **1**–**4**, combined with *p*DNA. The liposomes were formulated first by combining ethanolic stock solutions of a cationic lipid, **1**–**4**, along with EPC and a neutral co-lipid, DOPE or cholesterol, in a 1:1:2 molar ratio. The cationic liposomes were prepared by sonication of hydrated thin films of the combined lipids formed upon evaporation of ethanol under reduced pressure. Liposome particle sizing was obtained for all liposome formulations (with DOPE or Chol as co-lipid) using dynamic light scattering and revealed a range in diameter between 150 and 530 nm. It is interesting to note, all of the formulations using the unsaturated lipids, **1** or **3**, with the exception of **1**/EPC/Chol, were approximately three times larger than their saturated counterparts. Subsequently, combining the various cationic liposomes with negatively charged *p*DNA resulted in lipoplex formation, initially mediated by electrostatic interactions and subsequently by hydrophobic effects.[23–25] The lipoplexes assembled into nano-sized particles with diameters in the range 290–900 nm.

Lipoplex formation upon association of liposomes with *p*DNA was investigated over a range of cationic lipid:DNA (+/−, or nitrogen/phosphorous (N/P)) molar charge ratios using a gel retardation assay (Fig. 3). Similar retention trends were observed in formulations with either DOPE or cholesterol as the co-lipid. Liposome formulations with high N:P molar charge ratios revealed the highest level of *p*DNA association, evident from Figure 3. Lipoplexes containing **2**/EPC/DOPE, **4**/EPC/DOPE, **2**/EPC/Chol and EPC/Chol revealed complete retention at a N:P molar charge ratio of 10:1. All other formulations showed incomplete retention, even at the higher molar charge ratios, however most lipoplexes exhibited some degree of retention from a N:P molar charge ratio of 3:1. The minor differences observed for *p*DNA association between lipoplexes using the same co-lipid and at the same molar charge ratio, were likely due to the differences in the hydrophobic domain of the cationic lipids, **1**–**4**, each of which possess the same headgroup.

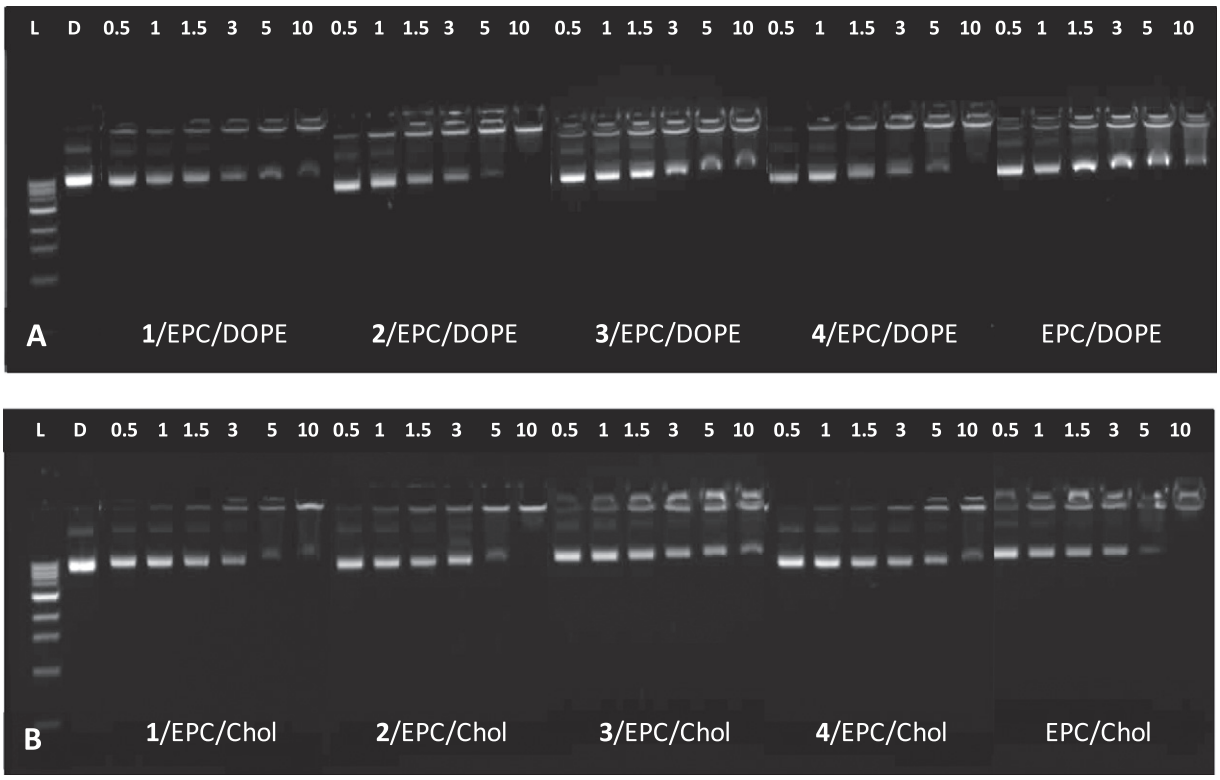


Figure 3. Gel retardation assays of liposomes formulated with a cationic lipid, **1**–**4**, EPC, and either DOPE (A) or cholesterol (B) as co-lipid, combined with *p*DNA at various N:P (+/−) molar charge ratios, ranging from 0.5:1 to 10:1, and run through a 1% agarose gel impregnated with the *p*DNA gel stain, ethidium bromide. Lanes L and D denote the 1 kb DNA ladder and *p*DNA, respectively.

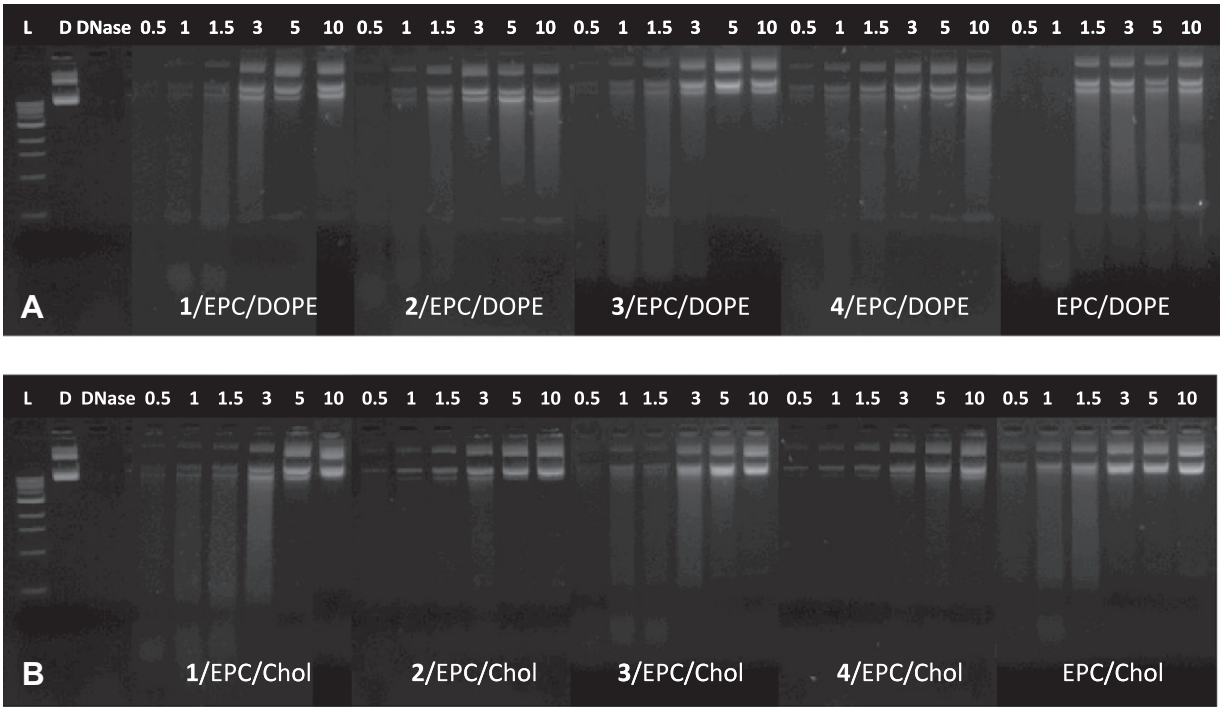


Figure 4. DNase I degradation assays of liposomes formulated with a cationic lipid, **1**–**4**, EPC, and either DOPE (A) or cholesterol (B) as co-lipid, combined with *p*DNA at various N:P (+/−) molar charge ratios, ranging from 0.5:1 to 10:1, and run through a 1% agarose gel impregnated with the *p*DNA gel stain, ethidium bromide. Lanes L and D denote the 1 kb DNA ladder and *p*DNA, respectively.

A set of DNase I degradation assays were performed to assess the protective character the novel cationic vectors offer the nucleic acid cargo from degradative enzymes. This assay involved incubation of each lipoplex with DNase I, which cleaves unbound and/or unprotected *p*DNA into linear fragments, followed by quenching of the DNase and incubation of the lipoplexes with the surfactant sodium dodecyl sulfate (SDS) to release any residual, intact DNA from the lipoplex which was subsequently visualized by gel electrophoresis.

DNase I protection experiments were carried out by complexing *p*DNA with liposomes containing a cationic lipid, **1**–**4**, at N:P (+/−) molar charge ratios ranging from 0.5:1 to 10:1 and then incubating with DNase I for 1 h at 37 °C. Figure 4 shows the naked *p*DNA (lane D) followed by the naked *p*DNA incubated with DNase I, resulting in complete digestion (lane DNase) as indicated by the absence of the intense band for naked *p*DNA. Lipoplexes with N:P molar charge ratios ⩾3:1 generally revealed greater protection of the nucleic acid cargo. A comparison between the lipoplexes formulated with DOPE (Fig. 4A) versus those with cholesterol (Fig. 4B) as co-lipid indicated that formulations using cholesterol led to a modest improvement in the protective character of the lipoplex. However, it should be noted that a comparison between the various lipoplex formulations within either co-lipid series did not indicate a significant difference in the ability to protect the *p*DNA cargo.

The cytotoxicity associated with lipoplex formulations at N:P (+/−) molar charge ratios ranging from 0.5:1 to 10:1 was determined using the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium) assay. CHO-K1 cells were incubated with different formulations for 48 h at 37 °C in the presence of 5% CO_2. The results illustrated in Figure 5 show a concentration-dependent cytotoxicity associated with all lipoplex formulations.

Lipoplexes formulated with lipids **1**–**4**, EPC and DOPE as co-lipid revealed a general trend of higher toxicity than the control lipoplexes containing only EPC and DOPE at all but the lowest molar charge ratio of 0.5:1 (Fig. 5A). With the exception of **3**/EPC/DOPE and EPC/DOPE, less than 70% cell viability was observed in the DOPE-containing formulations with a N:P molar charge ratio higher than 1:1. However, formulations of the cationic lipids **1**–**4** with EPC and cholesterol as co-lipid showed comparable or better cell tolerance than the control lipoplexes containing only EPC and cholesterol (Fig. 5B). The formulations with a N:P molar charge ratio up to and including 3:1 revealed approximately >80% cell viability. Moreover, lipoplexes containing the macrocyclic cationic lipids (**3** and **4**) with cholesterol as co-lipid revealed cell viability approximately >60% for all N:P molar charge ratios examined in this study.

In order to evaluate the *p*DNA transfection efficiency of lipoplexes formulated with the cationic lipids **1**–**4**, cells were transfected with lipoplexes for 4 h, then replaced with complete growth media, followed by incubation for a further 48 h at 37 °C in the presence of 5% CO_2 before assaying β-galactosidase expression using the Beta-Glo® Assay System (Promega).

All lipoplex formulations containing compounds **1**–**4** formulated with EPC and either DOPE or cholesterol as co-lipids demonstrated some level of gene induction evidenced by the luciferin luminescence as a result of β-galactosidase gene transfer to cells (Fig. 6). Cells incubated with the free soluble *p*DNA did not show any detectable transfection (data not shown). The transfection efficiency of lipoplexes containing cholesterol as co-lipid was generally found to be greater than those formulated with DOPE, with the exception of the lower N:P (+/−) molar charge ratios assayed (e.g., 0.5:1 and 1:1).

Aside from the lipoplex **3**/EPC/DOPE at a N:P molar charge ratio of 10:1, all lipoplex formulations with DOPE as co-lipid showed lower transfection efficiency when compared with the control lipoplex EPC/DOPE (Fig. 6A). Within this series of lipoplexes, those composed of unsaturated lipids (**1** or **3**) revealed greater transfection than their saturated counterparts (**2** or **4**) regardless of the

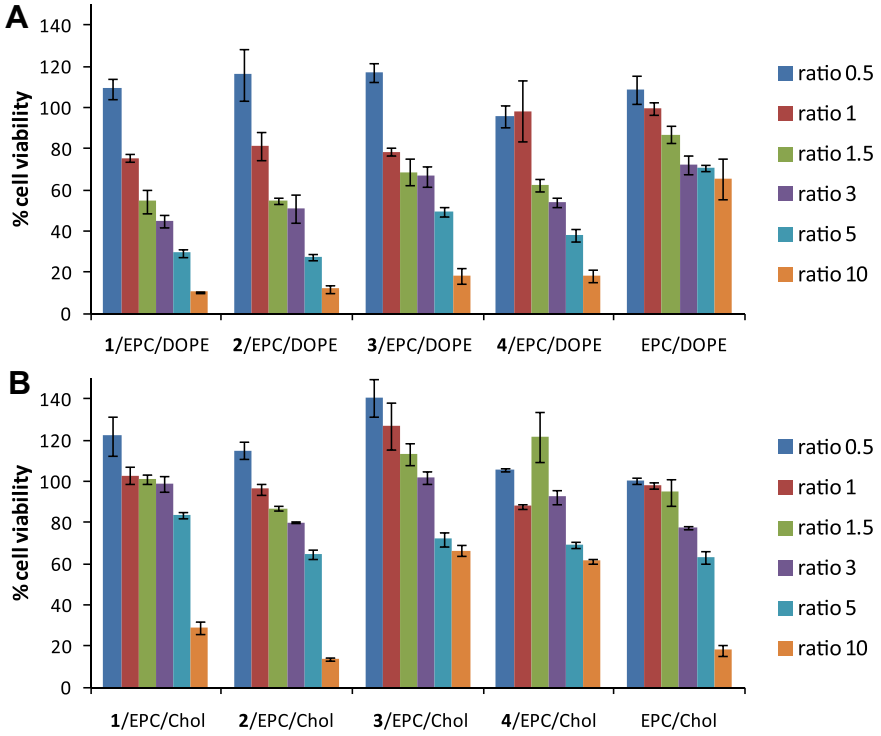


Figure 5. Cytotoxicity of liposomes formulated with a cationic lipid, **1**–**4**, EPC, and either DOPE (A) or cholesterol (B) as co-lipid, complexed with *p*DNA at various N:P (+/−) molar charge ratios, ranging from 0.5:1 to 10:1, 48 h after transfection of CHO-K1 cells. The percentage of viable cells was calculated as the absorbance ratio of treated to untreated cells. The data reported was the average of two experiments, each performed in triplicate, and expressed as mean ± S.E.

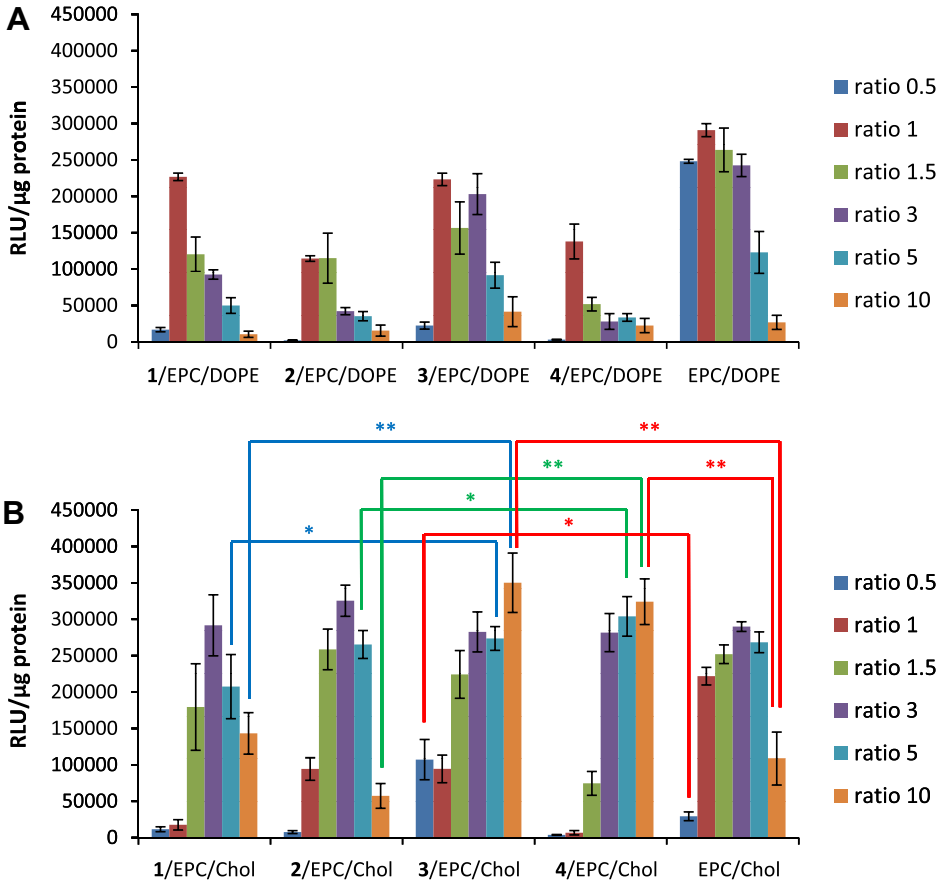


Figure 6. Transfection efficiency of liposomes formulated with a cationic lipid, **1**–**4**, EPC, and either DOPE (A) or cholesterol (B) as co-lipid, complexed with *p*DNA at various N:P (+/−) molar charge ratios, ranging from 0.5:1 to 10:1, 48 h after transfection of CHO-K1 cells. Luciferin luminescence for the *p*DNA treated cells was normalized to total protein. The data reported was the average of two experiments, each performed in triplicate, and expressed as mean ± S.E. $^{*}p$ <0.05, $^{**}p$ <0.01 (Student's *t* test).

acyclic or cyclic nature of the hydrophobic domain. This result was consistent with similar reports found in the literature.[26–28] In general, optimum efficiency was observed at a N:P molar charge ratio of 1:1 for all lipids **1**-**4** formulated with DOPE as co-lipid.

Lipoplex formulations containing lipids **1**–**4** formulated with cholesterol as co-lipid revealed, in most cases, a similar level of transfection when compared with the control lipoplex EPC/Chol (Fig. 6B). Three of the lipoplex formulations composed of the macrocyclic cationic lipids **3** and **4** performed significantly better than the control, as illustrated by the red correlation lines. Interestingly, formulations containing the cationic acyclic lipids **1** and **2** showed optimal transfection efficiency at a N:P molar charge ratio of 3:1, while those containing the macrocyclic lipids **3** and **4** exhibited a general concentration dependence up to a N:P molar charge ratio of 10:1. In general, the lipoplexes formulated with the lipids **1**–**4**, EPC and cholesterol as co-lipid exhibited higher transfection efficiency than the corresponding formulations containing DOPE as co-lipid.

For N:P (+/−) molar charge ratios of 5:1 and 10:1, lipoplexes formulated with the macrocyclic compounds **3** or **4**, EPC, and cholesterol as co-lipid revealed a trend towards better transfection than lipoplexes composed of their acyclic counterparts **1** or **2**. Among the formulations included in the present study, the lipoplex composed of the unsaturated macrocyclic cationic lipid **3** exhibited the highest transfection efficiency at the N:P molar charge ratio of 10:1.

Taken into consideration that the lipoplexes composed of **3** or **4** did not show better association with *p*DNA or better protection against DNase I than their acyclic counterparts **1** or **2**, it is possible this enhancement of transfection may be due to an improvement of *p*DNA delivery inside the cell. Indeed, it is well documented that transfection using mixed cationic lipids resulting in the liposomal incorporation of aliphatic chains of varying lengths, as is the case with a mixture of EPC and cationic lipids **1**–**4**, can improve transfection efficiency potentially by promoting endosomal escape.[29–31] MacDonald and co-workers[32] have demonstrated that when mixtures of cationic lipids, particularly with significantly different structural properties, are used together, the system is no longer in a single fully miscible phase. That is, the lipids pack poorly and thus constitute a defect, resulting in fusion with bilayers of the endosome membrane, a key step for DNA release from the lipoplexes.[33,34] This may be the case in the current study where the length of the hydrophobic domain of our novel lipids, **1**–**4**, differs from that of EPC. This effect is pronounced with cholesterol and particularly with the cyclic compounds that have a more rigid hydrophobic domain enabling the formation of such structural defects, and may result in a more effective release of the DNA, and ultimately better transfection efficiency. This hypothesis will be evaluated through further studies on the next generation of cyclic compounds whose synthesis is already in progress.

We report for the first time the successful application of cationic lipids containing a macrocyclic hydrophobic domain as gene delivery vectors. Four novel cationic lipids with short hydrophobic domains, including two acyclic (**1** and **2**) and two macrocyclic compounds (**3** and **4**), were designed and synthesized. In association with EPC and either DOPE or cholesterol as co-lipid the synthesized lipids **1**–**4** achieved efficient plasmid DNA complexation and protection from DNase I degradation, with no significant difference

observed between the cationic lipids. All lipoplex formulations generally exhibited molar charge ratio dependent cytotoxicity. The formulations which contained cholesterol generally exhibited marginally to significantly higher cell viability and significantly higher in vitro transfection into CHO-K1 cells than those formulated with DOPE as co-lipid. In some cases the observed transfection efficiency was greater than the EPC/Chol control, a formulation widely used for gene transfer. Within the cholesterol formulation series, the highest transfection efficiency was achieved at N:P 3:1 for lipoplexes composed of the acyclic lipids, **1** or **2**, and at N:P 10:1 for the macrocyclic lipids, **3** or **4.** However, were the macrocyclic lipids **3** or **4** to be employed for gene therapy at this molar charge ratio (N:P 10:1) it would be at the expense of low cell viability. In this context, the matched characteristics of high cell viability (approximately >80%) and high transfection efficiency was achieved at a N:P molar charge ratio of 3:1 for all lipoplexes formulated with a cationic lipid (**1**-**4**), EPC and cholesterol.

The results of these studies are very encouraging and support our hypothesis that macrocyclic lipids possess interesting properties as non-viral gene delivery vectors, including enhanced transfection efficiency. Further work, including the optimization of the lipid synthetic route, an investigation of different hydrophobic domains and headgroups, as well as an investigation of the intracellular trafficking of these lipoplexes are in progress and will be disclosed in due course.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.05.080.

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21. Selected data recorded for the amine lipids **1**–**4**:
*3-((5-(Dimethylamino)pentanoyl)oxy)propane-1,2-diyl bis(hept-6-enoate) (**1**):* ν_{max} (film)/cm^{-1} 3072, 2963, 2919, 2855, 1739, 1606, 1462, 1415, 1261, 1072 and 802; δ_H (300 MHz, $CDCl_3$) 5.85–5.72 (2H, m, H_2C=C*H*CH_2), 5.30–5.23 (1H, m, OC*H*), 5.04–4.93 (4H, m, H_2C=CHCH_2), 4.33–4.27 (2H, m, OCH_2), 4.14 (1H, dd, *J* 11.9 and 6.0, OC*H*H), 4.13 (1H, dd, *J* 11.9 and 6.0, OCH*H*), 2.40–2.27 (8H, m, C(O)CH_2 and CH_2N$(CH_3)_2$), 2.30 (6H, s, CH_2N$(CH_3)_2$), 2.10-2.03 (4H, m, H_2C=CHCH_2), 1.68–1.58 (2H, m, CH_2), 1.47–1.39 (2H, m, CH_2), 1.32–1.20 (8H, m, CH_2); δ_C (100 MHz, $CDCl_3$) 173.13, 173.09, 172.7, 138.3, 114.8, 68.9, 62.2, 62.1, 59.0, 45.3, 34.0, 33.8, 32.8, 30.1, 30.0, 29.3, 28.2, 27.0, 24.3, 24.2, 22.7; *m*/*z* (ES) 440.3013 (M^++H, 100%, $C_{24}H_{42}O_6N$ requires 440.3012).
*3-((5-(Dimethylamino)pentanoyl)oxy)propane-1,2-diyl diheptanoate (**2**):* ν_{max} (film)/cm^{-1} 2961, 2927, 2856, 1734, 1456, 1415, 1261, 1098 and 800; δ_H (300 MHz, $CDCl_3$) 5.30–5.23 (1H, m, OC*H*), 4.30 (1H, dd, *J* 11.9 and 4.3, OC*H*H), 4.29 (1H, dd, *J* 11.9 and 4.3, OCH*H*), 4.15 (1H, d, *J* 11.9, OC*H*H), 4.13 (1H, d, *J* 11.9, OCH*H*), 2.39-2.26 (8H, m, C(O)CH_2 and CH_2N$(CH_3)_2$), 2.27 (6H, s, CH_2N$(CH_3)_2$), 1.67–1.50 (8H, m, CH_2), 1.34–1.21 (12H, m, CH_2), 0.90–0.83 (6H, m, CH_3); δ_C (100 MHz, $CDCl_3$) 173.3, 173.0, 172.9, 68.8, 62.2, 62.1, 59.2, 45.3, 34.2, 34.0, 33.8, 32.8, 32.7, 30.0, 29.4, 28.75, 28.70, 27.1, 25.6, 22.7, 22.4, 14.1, 14.0; *m*/*z* (ES) 444.3336 (M^++H, 100%, $C_{24}H_{46}O_6N$ requires 444.3325).
*(E)- and (Z)-(5,16-Dioxo-1,4-dioxacyclohexadec-10-en-2-yl)methyl 5-(dimethylamino)-pentanoate (**3**):* Data for the major (*E*)-isomer; ν_{max} (film)/cm^{-1} 2961, 2928, 2857, 1732, 1635, 1461, 1262, 1099, 1016 and 808; δ_H (300 MHz, $CDCl_3$) 5.31-5.21 (3H, m, O*CH* and *CH*=*CH*), 4.31–4.11 (4H, m, OCH_2), 2.37–2.26 (8H, m, C(O)CH_2 and CH_2N$(CH_3)_2$), 2.25 (6H, s, CH_2N$(CH_3)_2$), 2.10–2.02 (4H, m, C=CHCH_2), 1.71–1.38 (8H, m, CH_2), 1.36–1.21 (4H, m, CH_2); δ_C (100 MHz, $CDCl_3$) 173.2, 172.9, 172.8, 131.0, 130.9, 68.9, 62.6, 62.1, 59.1, 45.2, 33.8, 32.0, 31.9, 30.3, 30.1, 29.4, 28.04, 27.99, 26.8, 24.8, 22.7; *m*/*z* (ES) 412.2701 (M^++H, 100%, $C_{22}H_{38}O_6N$ requires 412.2699).
*(5,16-Dioxo-1,4-dioxacyclohexadecan-2-yl)methyl 5-(dimethylamino)pentanoate (**4**):* ν_{max} (film)/cm^{-1} 2927, 2856, 1741, 1457, 1260, 1129 and 735; δ_H (300 MHz, $CDCl_3$) 5.32–5.26 (1H, m, OC*H*), 4.39–4.26 (2H, m, OCH_2), 4.21–4.12 (2H, m, OCH_2), 2.39–2.31 (8H, m, C(O)CH_2 and CH_2N$(CH_3)_2$), 2.29 (6H, s, CH_2N$(CH_3)_2$), 1.94–1.60 (8H, m, CH_2), 1.35–1.29 (12H, m, CH_2); δ_C (100 MHz, $CDCl_3$) 173.5, 173.05, 173.00, 69.1, 62.8, 62.1, 59.2, 45.4, 33.8, 33.72, 33.69, 31.9, 30.0, 29.4, 27.1, 27.0, 26.1, 26.02, 25.95, 25.90, 22.7; *m*/*z* (ES) 414.2853 (M^++H, 100%, $C_{22}H_{40}O_6N$ requires 414.2856).
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