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RESEARCH ARTICLE

On the mechanism whereby cationic lipids promote intracellular delivery of polynucleic acids

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The mechanism whereby cationic lipids destabilize cell membranes to facilitate the intracellular delivery of macromolecules such as plasmid DNA or antisense oligonucleotides is not well understood. Here, we show that cationic lipids can destabilize lipid bilayers by promoting the formation of nonbilayer lipid structures. In particular, we show that mixtures of cationic lipids and anionic phospholipids preferentially adopt the inverted hexagonal (H_{II}) phase. Further, the presence of 'helper' lipids such as dioleoylphosphatidylethanolamine or cholesterol, lipids that enhance cationic lipid-mediated transfection of cells also facilitate the formation of the H_{II} phase. It is suggested that the ability of cationic lipids to promote nonbilayer structures in combination with anionic phospholipids leads to disruption of the endosomal membrane following uptake of nucleic acid–cationic lipid complexes into cells, thus facilitating cytoplasmic release of the plasmid or oligonucleotide. Gene Therapy (2001) **8,** 1188–1196.

Keywords: *lipoplexes; transfection; lipid polymorphism; gene therapy*

Introduction

The cell membrane presents a major barrier to the intracellular delivery of macromolecules such as plasmids, antisense oligonucleotides and therapeutic proteins. A number of approaches for enhancing the intracellular delivery of nucleic acids and proteins have been investigated, including the use of liposomes,[1] membrane-translocating peptides,[2] cationic peptides,[3]cationic proteins,[4] cationic polymers[5] and cationic lipids.[6] Cationic lipids provide a particularly powerful tool for the introduction of polynucleic acids into cells. For example, cationic lipid–nucleic acid complexes or 'lipoplexes' can promote the intracellular delivery of plasmid DNA,[6] RNA,[7] antisense oligonucleotides,[8] transcription factor decoy oligonucleotides,[9] ribozymes[10] and transcription factors.[11]

Cationic lipid in complexes with plasmids or other polynucleic acids performs at least three functions. First, by electrostatic association with the plasmid, the cationic lipid coats and partially condenses the plasmid to form the transfection-competent particle. Second, the presence of cationic lipid at levels that give rise to an overall positive charge leads to enhanced association of the lipoplex with negatively charged cell surfaces[12] leading to cellular uptake via endocytosis.[13,14] Third, following uptake, the cationic lipid plays a role in destabilizing the endosomal membrane,[15–17] thus facilitating cytoplasmic delivery of the plasmid. The mechanism whereby cationic lipid destabilizes the endosomal membrane remains ill-defined and is the subject of this investigation.

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Previous work has shown that cationic lipids interact strongly with systems containing anionic lipids. For example, cationic liposomes fuse with liposomes containing anionic phospholipids[12,18,19] and addition of anionic lipids to cationic lipid–nucleic acid complexes displaces cationic lipid from the nucleic acid to form ion pairs of cationic and anionic lipid and 'free' nucleic acid.[20–22] Here, we characterize the structural preferences of the ion pairs formed from mixtures of cationic lipids and anionic phospholipids using ^{31}P NMR techniques. We show that these mixtures preferentially adopt the nonbilayer inverted hexagonal (H_{II}) phase and that 'helper' lipids such as dioleoylphosphatidylethanolamine (DOPE) or cholesterol further facilitate formation of such nonbilayer structure. These results suggest that cationic lipids disrupt the endosomal membrane by inducing formation of nonbilayer lipid structures.

Results

Mixtures of cationic lipids and anionic phospholipids adopt the hexagonal H_{II} phase

In previous work we have shown that mixtures of the cationic lipid N,N-dioleyl-N,N-dimethylammonium chloride (DODAC) with the anionic lipid cholesteryl hemisuccinate (CHEMS) can form nonbilayer structures such as the hexagonal H_{II} phase under conditions of neutral surface charge.[23] Similarly, mixtures of the cationic lipid 3α-[N-(N′,N′-dimethylaminoethane)-carbamoyl] cholesterol hydrochloride (DC-Chol) and dioleoylphosphatidic acid (DOPA) also exhibit H_{II} phase preferences.[23] Initial studies were conducted to determine whether this behaviour is a general property of mixtures of cationic and anionic lipids. In the first set of experiments,

dioleoylphosphatidylserine (DOPS) was used as the anionic lipid and was mixed with equimolar amounts of a variety of cationic lipids, including DODAC, N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA),[6] 1,2-dioleoyloxy-3-(trimethylammonio) propane (DOTAP)[12] and DC-Chol.[24] The phase behaviour of these dispersions could be investigated using ^{31}P NMR techniques due to the presence of the phosphate group on the phospholipids. As shown in Figure 1a, a dispersion of the anionic phospholipid DOPS prepared at pH 7.4 reveals the characteristic asymmetric ^{31}P NMR lineshape with a low field shoulder and a high field peak associated with bilayer structure.[25] However, the samples composed of equimolar mixtures of DOPS and DODAC, DOTMA and DOTAP (Figure 1b–d, respectively) all gave rise to ^{31}P NMR spectra that are a factor of two narrower and exhibit reversed asymmetry compared with the 'bilayer' lineshape and are characteristic of phospholipids organized in the inverted hexagonal (H_{II}) phase.[25,26] It may be noted that the ^{31}P NMR spectrum obtained for the equimolar mixture of DC-Chol/DOPS (Figure 1e) is consistent with the co-existence of both H_{II} and bilayer phases.

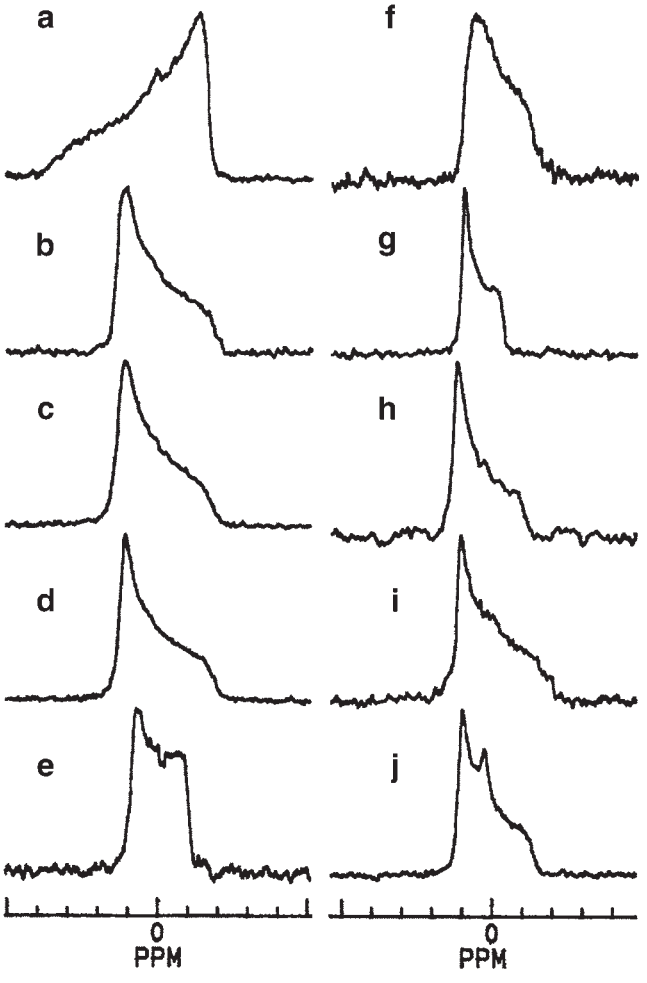

Figure 1 *Equimolar mixtures of cationic lipids and anionic phospholipids adopt the hexagonal H_{II} phase. ^{31}P NMR spectra obtained from aqueous dispersions of (a) DOPS, (b) DODAC/DOPS, (c) DOTMA/DOPS, (d) DOTAP/DOPS, (e) DC-Chol/DOPS, (f) DODAC/DOPA, (g) DODAC/LBPA, (h) DODAC/CL (1:2; molar ratio), (i) DODAC/PI, (j) DODAC/POPG.*

The second set of experiments, performed to establish the generality of the observation that mixtures of cationic and anionic lipids adopt H_{II} phase structure, examined the structural preferences of mixtures of DODAC with a variety of different species of phospholipids. These included DOPA, lysobisphosphatidic acid (LBPA; a major component of late endosomal membranes[27]), tetraoleoylcardiolipin (CL), liver-derived phosphatidylinositol (PI) and 1-palmitoyl-2-oleoyl phosphatidylglycerol (POPG). As shown in Figures 1f, g, h, i and j for mixtures of DODAC with DOPA, LBPA, CL, PI and POPG, respectively, ^{31}P NMR spectra consistent with lipids organized into the H_{II} phase were observed for all these samples. For these experiments, anionic phospholipids were mixed with an equimolar amount of DODAC, with the exception of cardiolipin, a bivalent lipid, which was mixed with two molar equivalents of DODAC. The remarkable potency of DODAC and, by extension, other cationic lipids for inducing H_{II} phase structure is inherent in the fact that PS, PA, CL, PI and PG all adopt the bilayer organization in isolation at neutral pH and all can stabilize nonbilayer lipids such as DOPE into a bilayer organization.[28]

Effect of 'helper' lipids

Plasmid DNA–cationic lipoplexes used for transfection are usually derived from mixtures of plasmid with liposomes composed of cationic lipids and 'helper' lipids such as DOPE[6,15,29,30] or cholesterol.[31–34] These helper lipids give rise to improved transfection potencies of the resulting complex. If the mechanism of action of cationic lipids relies on an ability to induce H_{II} phase or related nonbilayer structure to destabilize target membranes such as the endosomal membrane it would be expected that these helper lipids would also promote H_{II} organization. Conversely, it would be expected that lipids such as dioleoylphosphatidylcholine (DOPC), which give rise to reduced levels of transfection when present in complexes,[6,35,36] would hinder the ability of cationic lipids to induce nonbilayer structure. That this is the case is illustrated in Figure 2. As shown in Figure 2a, aqueous dispersions of DOPS/DOPC (1:1; molar ratio) adopt the bilayer structure. As shown in Figure 2b for a lipid dispersion containing DODAC/DOPS/DOPC (1:1:1; molar ratio), the addition of an equimolar amount of DODAC (with respect to DOPS) does not result in induction of H_{II} phase structure. Thus the presence of DOPC prevents the DODAC–DOPS ion pairs from adopting the H_{II} phase they would otherwise adopt.

This behaviour contrasts with the properties of lipids used as helper lipids. As shown in Figure 2c, aqueous dispersions of DOPS/DOPE (1:1; molar ratio) adopt the bilayer phase, consistent with the ability of DOPS to stabilize DOPE in the lamellar organization as noted previously.[37] The addition of equimolar amounts of DODAC (with respect to DOPS) results in a complete transition to H_{II} phase organization as shown in Figure 2d for a DODAC/DOPS/DOPE (1:1:1; molar ratio) dispersion. Furthermore, the addition of DOPE to the DOPC-containing lipid dispersion of Figure 2b results in a transition to H_{II} phase organization as shown in Figure 2e for a DODAC/DOPS/DOPC/DOPE (1:1:1:1; molar ratio) lipid dispersion. This is consistent with the preference of DOPE for the H_{II} phase in isolation[26] and again points

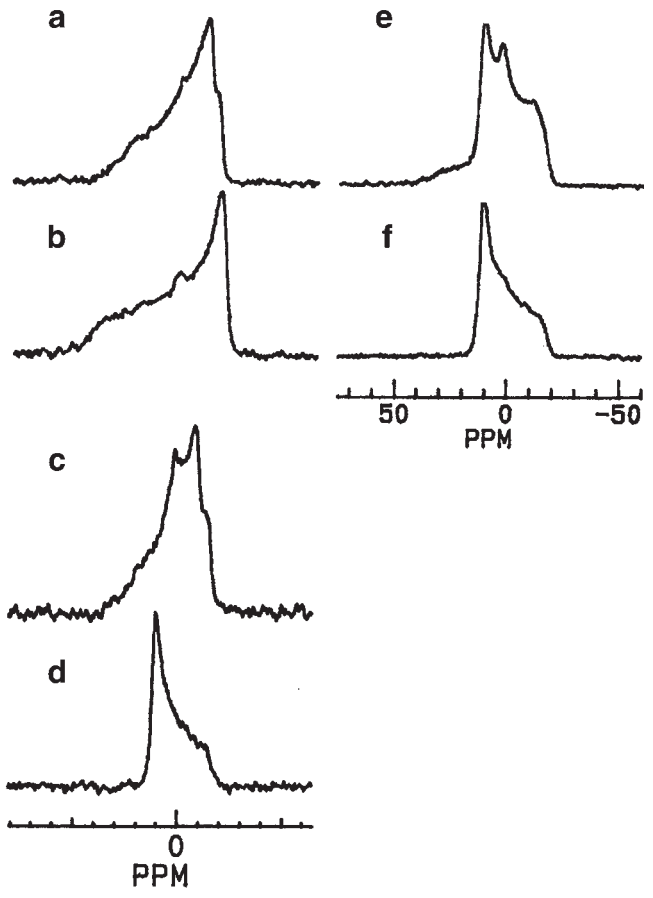

Figure 2 *The 'helper' lipids DOPE and cholesterol promote the formation of the hexagonal H_{II} phase structure in lipid mixtures containing cationic and anionic lipids. ^{31}P NMR spectra obtained from aqueous dispersions of (a) DOPS/DOPC (1:1; molar ratio), (b) DODAC/DOPS/DOPC (1:1:1; molar ratio), (c) DOPS/DOPE (1:1; molar ratio), (d) DODAC/DOPS/DOPE (1:1:1; molar ratio), (e) DODAC/DOPS/DOPC/DOPE (1:1:1:1; molar ratio), (f) DODAC/DOPS/DOPC/Chol (1:1:1:1; molar ratio).*

to the ability of the helper lipid DOPE to facilitate H_{II} phase organization.

Cholesterol has similar abilities to promote H_{II} phase organization. This is illustrated by the influence of cholesterol on the structure of the DODAC/DOPS/DOPC (1:1:1; molar ratio) system which, as previously indicated, adopts the bilayer organization (Figure 2b). As shown in Figure 2f for a DODAC/DOPS/DOPC/cholesterol (1:1:1:1; molar ratio) lipid dispersion, the inclusion of cholesterol induces H_{II} phase structure. This is consistent with the previously noted ability of cholesterol to promote the H_{II} phase in a variety of mixed phospholipid systems.[38–40]

Influence of cationic lipid structure on the polymorphism of mixtures of cationic and anionic lipids

It is well known that the chemical structure of cationic lipids can strongly influence the transfection potency of plasmid DNA–cationic lipid complexes formed from them. Although a detailed correlation between the transfection potency of complexes formed from different cationic lipids and the polymorphic phase preferences of these lipids in mixtures with anionic lipids is outside the scope of this study, it is important to point out that the ability of cationic lipids to adopt H_{II} phase structure does offer the possibility of obtaining a measurable parameter to correlate with transfection potency. Briefly, the relative tendency of lipids to adopt H_{II} phase organization is reflected by parameters such as the intrinsic radius of curvature which is a measure of the radius of the H_{II} phase cylinders formed in the absence of other constraints.[41] A related parameter is the bilayer-to-H_{II} transition temperature (T_{BH}).[25,26] Lipids that adopt the H_{II} phase more avidly exhibit lower T_{BH} values, and it is of interest to show that cationic lipids that give rise to lower T_{BH} values in mixtures with anionic lipids exhibit more potent transfection properties. In this regard, the transfection potency of cationic lipids has been found to decrease as the saturation of the cationic lipid is increased.[29,42] We therefore investigated the bilayer-to-H_{II} phase transition temperatures of a series of dialkyl-dimethylammonium chlorides, namely di-C18:1(Δ9) (DODAC), C18:1(Δ9)/C18:0 (OSDAC) and di-C18:0 (DSDAC), in equimolar mixtures with DOPS. It was found that an equimolar mixture of DODAC/DOPS exhibits a T_{BH} between 1 and 6°C (Figure 3a). The introduction of a single saturated alkyl chain on the cationic lipid resulted in an increase in T_{BH} to 25–31°C for OSDAC/DOPS (1:1; molar ratio) (Figure 3b), whereas for DSDAC/DOPS (1:1; molar ratio) a T_{BH} between 43 and 48°C was observed (Figure 3c). These data are therefore consistent with the hypothesis that the enhanced transfection efficiency observed for unsaturated cationic lipids[42] is due to their enhanced ability to promote nonbilayer structure in the presence of anionic cellular phospholipids.

A wide variety of cationic lipids with different headgroups have been synthesized, however, no general correlation between transfection potency and type of headgroup structure has been achieved.[29,43,44] Here, we only point out that differing cationic headgroups can lead to different abilities to promote H_{II} organization as indicated by the T_{BH} parameter. In particular, it was found that both DOTAP/DOPS and DOTMA/DOPS mixtures exhibited similar T_{BH} values between 7 and 13°C (Table 1). As indicated above, the mixture of DODAC/DOPS (1:1; molar ratio) had a T_{BH} in the range of 1–6°C. It will be of considerable interest to determine whether determi-

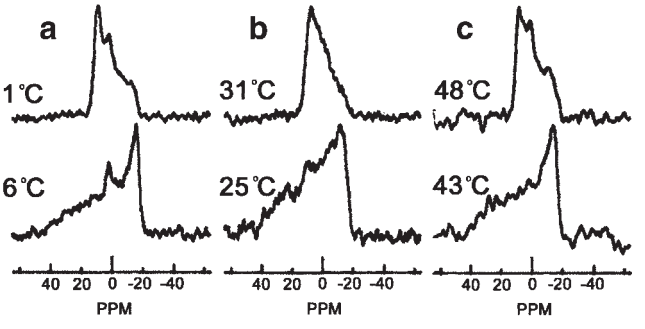

Figure 3 *The hydrocarbon chain composition of cationic lipids modulates the bilayer to hexagonal H_{II} phase transition temperature (T_{BH}) of mixtures of cationic lipids with DOPS. ^{31}P NMR spectra obtained from aqueous dispersions of (a) DODAC/DOPS (1:1; molar ratio), (b) OSDAC/DOPS (1:1; molar ratio), (c) DSDAC/DOPS (1:1; molar ratio). Spectra were collected at the temperatures indicated. A 50 Hz line broadening was applied. For other acquisition parameters see Materials and methods.*

Table 1 Bilayer to hexagonal (H_{II}) phase transition temperatures (T_{BH}) of equimolar (with respect to charge) mixtures of cationic and anionic lipid

Cationic lipid	*Anionic lipid*	T_{BH}
DSDAC	DOPS	43–38°C
OSDAC	DOPS	25–31°C
DODAC	DOPS	1–6°C
DODAC	DPPS	43–49°C
DODAC	DOPA	<–3°C
DODAC	CL	<–3°C
DODAC	PI	12–17°C
DOTMA	DOPS	7–13°C
DOTAP	DOPS	7–13°C

nation of the T_{BH} parameter offers a method for rational design of more potent cationic lipids.

Influence of anionic lipid structure on the polymorphism of mixtures of cationic and anionic lipids

In the same way that the hydrocarbon chain composition and headgroup structure of cationic lipids can influence the propensity of their mixtures with an anionic lipid for H_{II} phase, it would be expected that the structure of the anionic lipid could influence the polymorphism of these mixtures. This has important implications as it could explain the differing susceptibility of cells to transfection as due to differences in the acyl chain or headgroup composition of endogenous anionic lipids. As shown in Table 1, increases in the saturation of the anionic lipid species causes a marked increase in the T_{BH} in mixtures with DODAC, with T_{BH} increasing from between 1 and 6°C for the DODAC/DOPS mixture to more than 43°C for a DODAC/dipalmitoylphosphatidylserine (DPPS) mixture. As shown in Table 1, the T_{BH} is also sensitive to the type of anionic headgroup exhibited by the phospholipid, with transition temperatures ranging from less than –3°C for mixtures of DODAC with DOPA and CL to between 1 and 6°C for DOPS. Mixtures of DODAC with PI exhibit a higher T_{BH} between 12 and 17°C, however, this cannot be unambiguously attributed to the influence of the inositol headgroup, as the acyl chain composition of the liver-derived PI is a heterogeneous mixture of saturated and unsaturated lipid species.

Equimolar mixtures of DODAC with POPG and LBPA did not exhibit a well-defined T_{BH} value, as narrow 'isotropic' ^{31}P NMR spectra were observed between –3°C and 22°C, with H_{II} phase spectra at temperatures above 26°C (data not shown). Such isotropic spectra have been associated with nonbilayer structures such as cubic phases,[45] which basically consist of three-dimensional intercalated networks of lipid tubules or inverted micelles,[46] and, like the H_{II} phase, have been associated with lipid intermediates occurring during membrane fusion.[47]

Addition of liposomes containing anionic lipids to liposomes containing cationic lipids or plasmid DNA–cationic lipid complexes results in hexagonal H_{II} phase formation

In the preceding experiments cationic lipids were mixed with anionic phospholipids in organic solvent, dried to a film and then hydrated by vortex mixing before examining structural preferences employing ^{31}P NMR spectroscopy. If the behaviour observed is related to the ability of cationic lipids to facilitate intracellular delivery, it is important to show that similar behaviour is observed in aqueous media when liposomes containing anionic lipids are mixed with liposomes containing cationic lipids or with plasmid DNA–cationic lipid complexes. In order to perform these experiments, liposomes in the form of large unilamellar vesicles (LUV) of 100 nm diameter were employed to facilitate physical mixing between the liposomes and between the liposomes and the complexes. DOTAP, DODAC, DODAC/DOPE (1:1) and DOPS LUV were generated as indicated in Materials and methods. Complexes were formed by incubating DOTAP LUV with pCMVluc plasmid at a 3:1 positive-to-negative charge ratio. It should be noted that DOTAP LUV are standard transfection reagents and the properties of plasmid DNA–DOTAP complexes have been well characterized.[36,48]

The addition of DOPS LUV to LUV-containing cationic lipids immediately resulted in the transformation of the translucent LUV preparations to milky dispersions characteristic of the presence of larger particles. This was accompanied by changes in structure as monitored by ^{31}P NMR. As shown in Figure 4a, DOPS LUV exhibit an isotropic ^{31}P NMR lineshape characteristic of phospholipid in small vesicular structures. The addition of LUV composed of DODAC/DOPE, DOTAP and DODAC to these DOPS LUV (see Figure 4b, c and d, respectively) results in lipid dispersions that exhibit ^{31}P NMR spectra with the asymmetric lineshape characteristic of the H_{II} phase.[26]

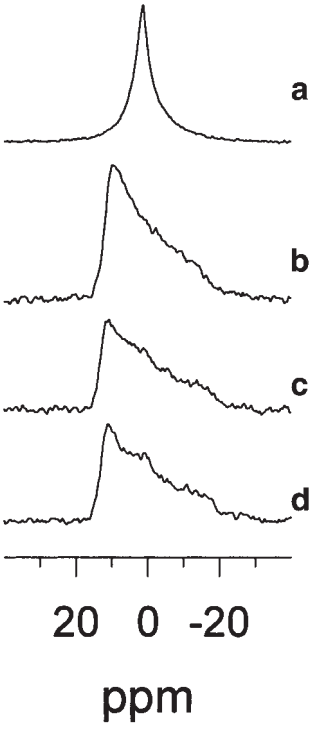

Figure 4 *Equimolar mixtures of LUV composed of cationic lipids and LUV composed of anionic lipids adopt the hexagonal H_{II} phase. ^{31}P NMR spectra are illustrated for (a) DOPS LUV, (b) DOPS LUV mixed with DODAC/DOPE LUV, (c) DOPS LUV mixed with DOTAP LUV, (d) DOPS LUV mixed with DODAC (1:1) LUV. All mixtures contained equimolar DOPS with respect to cationic lipid. Experiments were performed in 5 mM Hepes, 5 mM sodium chloride buffer at pH 7.5.*

The ^{31}P NMR behaviour of plasmid DNA–DOTAP complexes during formation and following addition of DOPS LUV is illustrated in Figure 5. As shown in Figure 5a, the nucleotide phosphates of the plasmid give rise to an isotropic ^{31}P NMR lineshape (spectrum 1) in aqueous media, which disappears (spectrum 2) on addition of DOTAP LUV to form complexes. This disappearance can be attributed to reduced motion of the plasmid in complexes, thus causing the plasmid ^{31}P NMR signal to become considerably broader. The subsequent addition of DOPS LUV at a 0.83 molar ratio with respect to the DOTAP in the complexes results in the appearance of an isotropic component superimposed on a component characteristic of the H_{II} phase (spectrum 3). The H_{II} phase component of spectrum 3 may be attributed to the formation of H_{II} phase DOPS-DOTAP ion pairs. This conclusion is supported by the fact that the addition of DOPS LUV to DOTAP LUV (in the absence of plasmid DNA) in the same aqueous buffer employed for the complexes gives rise to an H_{II} phase profile (spectrum 4).

The isotropic component of spectrum 3 does not arise from 'free' DOPS LUV as removal of such LUV by centrifugation of the sample giving rise to spectrum 3 followed by re-suspension of the pellet in aqueous buffer results in a sample that exhibits a similar spectrum (spectrum 5). This component could arise from plasmid DNA or phospholipid in phases allowing isotropic motion, such as the cubic phase.[45] It would be expected that formation of DOPS–DOTAP ion pairs following addition of DOPS LUV would result in dissociation of the DOTAP from the plasmid. This was tested by assaying the accessibility of the plasmid in the complexes before and after addition of the DOPS LUV, employing the PicoGreen assay described in Materials and methods. As shown in Figure 5b, access of the PicoGreen to the plasmid DNA following addition of DOPS LUV to plasmid DNA–DOTAP complexes is considerably increased. This result indicates release of plasmid DNA from the cationic lipid following addition of DOPS, consistent with previous results.[20,22]

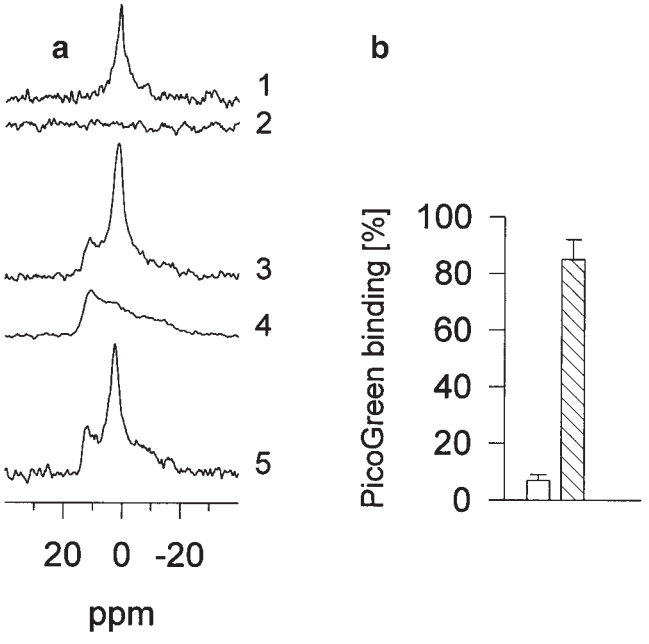

Figure 5 *Addition of LUV composed of anionic lipids to plasmid DNA–cationic lipid complexes results in formation of H_{II} phase lipid structure and concomitant release of plasmid DNA. (a) ^{31}P NMR spectra of (1) pCMVluc at 1 mg/ml in 5 mM Hepes buffer pH 7.5, (2) pCMVluc-DOTAP complexes (1 mg/ml plasmid) prepared at a 3:1 positive-to-negative charge ratio in 5 mM Hepes buffer, (3) pCMVluc-DOTAP complexes, separated from excess cationic liposomes by centrifugation and re-suspended in HBS, following addition of DOPS LUV (0.83 DOPS/DOTAP molar ratio), (4) DOPS LUV mixed with DOTAP LUV (1:1, molar ratio) in HBS (in the absence of pDNA) and (5) the sample of spectrum 3 after removal of 'free' DOPS LUV by centrifugation, washing, centrifugation and re-suspension of the pellet in HBS. (b) Accessibility of plasmid to the fluorescent DNA-binding dye PicoGreen in pCMVluc-DOTAP complexes before and after addition of DOPS LUV. Complexes were prepared at a positive-to-negative charge ratio of 3:1 and contained 50 μg/ml pCMVluc. DOPS LUV were added to achieve a 1:1 DOTAP/DOPS molar ratio.*

Discussion

The results presented here show that cationic lipids used for transfection exhibit, as a general property, the ability to induce nonbilayer H_{II} phase structure in lipid systems containing anionic phospholipids. Furthermore, helper lipids such as DOPE and cholesterol also promote H_{II} phase organization. These results suggest that the ability of cationic lipids to facilitate the intracellular delivery of macromolecules arises, at least in part, from an ability to destabilize endosomal or plasma membranes by inducing nonbilayer lipid structures. Three aspects of these findings that are of particular interest concern the molecular mechanism whereby cationic lipids are able to combine with anionic lipids to promote H_{II} phase organization, the relationship between these findings and a model of intracellular delivery of macromolecules, and finally, how one may use this understanding to design improved intracellular delivery systems. We discuss these areas in turn.

As discussed elsewhere,[23] the ability of equimolar mixtures of cationic and anionic lipids, both of which adopt the bilayer organization in isolation, to adopt H_{II} phase structure in combination can be attributed to the formation of ion pairs in which the net headgroup area is substantially reduced in comparison to the sum of the headgroup area of the cationic and anionic lipid species in isolation. Using the 'shape' arguments employed to rationalize the phase properties of lipids, this corresponds to a transition from charged lipids with a cylindrical shape compatible with the bilayer organization to neutral ion pairs with a 'cone' shape that preferentially adopt inverted lipid phases such as the hexagonal H_{II} phase.[25] Using the formalism of Gruner *et al*,[49] this corresponds to a substantial decrease in the intrinsic radius of curvature in comparison to the cationic or anionic lipid species in isolation.

The influence of hydrocarbon chain unsaturation, headgroup composition and temperature on the polymorphism of the mixtures of cationic and anionic lipids detailed here is fully consistent with the large body of literature describing lipid polymorphism.[25,49–51] Briefly, it is well established that increased unsaturation leads to increased preference for the H_{II} organization, which can be attributed to an increase in the cross-sectional area in the hydrocarbon chain region and increased 'cone' shape character. In the case of the mixtures of cationic and anionic lipids, this is reflected by the decrease in T_{BH} from ~48°C to less than 6°C as the alkyl chain composition of the dialkylammonium chloride (DAC) component is varied from distearyl to dioleyl in DAC/DOPS lipid mixtures. Very similar behaviour is observed when the acyl

chain composition of the anionic lipid component is varied, with T_{BH} decreasing from more than 43°C to less than 6°C as the PS acyl chain composition is changed from dipalmitoyl to dioleoyl in DODAC/PS systems. Although the data set is not extensive, the influence of the anionic headgroup on T_{BH} can be rationalized in a similar manner, as the phospholipids with the smaller headgroups (and thus a more pronounced cone shape) such as CL and PA exhibit the lowest T_{BH} values in mixtures with DODAC. Finally, as previously mentioned the ability of 'helper' lipids such as DOPE and cholesterol to adopt or promote H_{II} organization in mixed lipid systems has been well described elsewhere.[26,38,40]

The ability of cationic lipids to induce inverted nonbilayer structures in combination with anionic lipids leads naturally to a model describing intracellular delivery of nucleic acid polymers mediated by cationic lipids. The model is shown in Figure 6 and is an extension of the model previously proposed by Szoka and co-workers. Briefly, these authors[20,21] demonstrated that anionic phospholipids such as PS could displace cationic lipids from plasmids, thus assisting in the release of 'free' plasmid following uptake of complexes into cells.[20,22] As detailed here, the additional ability of the cationic–anionic ion pairs thus generated to induce H_{II} phase structure suggests a direct role in destabilizing the endosomal membrane, therefore facilitating escape of the plasmid from the endosome into the cytoplasm (Figure 6, step 4). It may be noted that electron micrographs of COS cells transfected with gold labelled plasmid DNA–cationic lipid complexes exhibit similar structures to those depicted in step 4 of Figure 6.[13] A highly ordered pattern of regularly packed tubules in endosomes was observed, visually similar to an H_{II} lipid phase. As depicted in the model shown in Figure 6, the presence of the cationic lipid–anionic lipid ion pairs results in the disruption of the endosomal membrane by complete induction of nonbilayer structure. Clearly, this requires delivery of enough cationic lipid that sufficient ion pairs are generated to induce H_{II} phase organization for the entire endosomal membrane lipid. Some simple calculations were carried out to determine if this is possible.

A first calculation was made to determine the smallest size of complex required to deliver sufficient cationic lipid to form ion pairs with all the anionic lipid in the endosome. We assume that the complex has an internal repeat structure consisting of lipid bilayers of 4 nm thickness sandwiching plasmid DNA in an interlamellar water space 2.5 nm thick as indicated by X-ray studies[52] and that the cationic lipid is mixed with equimolar amounts of DOPE. Assuming that the endosome is of 200 nm diameter and contains 18 mol% anionic lipid,[27] it is straightforward to show that a complex of a diameter ~90 nm contains sufficient cationic lipid to form ion pairs with all the anionic lipid in the endosome. Although complexes exhibit a heterogeneous size distribution, most reports indicate an average size of 100 nm diameter or larger.[13,29,53] As a second step, we calculate the percentage of non-bilayer lipid in the complex-endosome mixture following uptake of this putative 90 nm complex, mixing of the complex and endosomal lipids and formation of the cationic–anionic lipid pairs. The lipid composition of early endosomes has been reported to be 47 mol% PC, 23% PE, 9 mol% sphingomyelin (SM) and 18 mol% anionic lipids.[27] Previous work has shown that SM is a bilayer-forming lipid.[54] Assuming that the endosome contains equimolar (with respect to phospholipid) amounts of cholesterol, it may then be calculated that the proportions of 'non-bilayer' lipid, bilayer lipid, and cholesterol in the endosome before and after uptake of the complex are 0.3/1.0/1.3 and 1.4/1.0/1.8, respectively. Given previous reports that DOPE/DOPC/cholesterol (1:1:2) mixtures preferentially adopt the H_{II} phase,[38] these results indicate that relatively complete induction of nonbilayer structure in endosomal lipids is possible following uptake of a lipoplex. It should also be noted that plasmid release may only require a transitory local disruption of the endosomal membrane rather than the complete disruption of the endosomal membrane that would be associated with large-scale induction of non-bilayer structure.

One other aspect of the model presented in Figure 6 that needs further discussion concerns the trigger that initiates contact between the complex and the endosomal membrane. In the early endosome anionic lipid is prim-

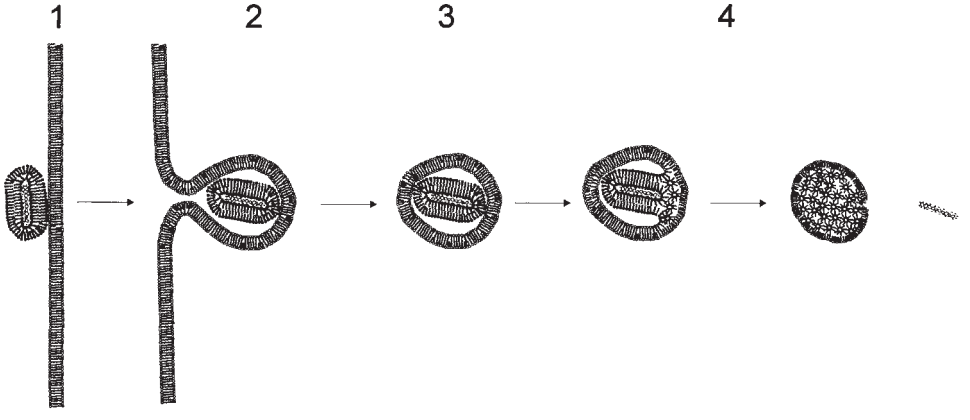

Figure 6 *Model of the intracellular delivery of plasmid mediated by cationic lipid in plasmid DNA–cationic lipid complexes (lipoplexes). Following binding (step 1) and endocytosis (step 2) into a target cell, the lipoplexes are transferred to endosomal compartments (step 3). The membrane of the lipoplex then fuses with the endosomal membrane due to the tendency of positively charged and negatively charged membranes to adhere and fuse, leading to lipid mixing between lipoplex and endosomal lipids. Anionic lipids from the endosomal membrane then displace cationic lipids from the plasmid DNA, leading to release of plasmid and further formation of non-bilayer structures (step 4).*

arily located on the exterior of the endosome due to lipid asymmetry in the plasma membrane, where anionic lipids such as PS are located on the cytoplasmic leaflet.[55] It is possible that small amounts of lumenal anionic lipid may be sufficient to initiate interactions with the cationic lipid of the complex and trigger events leading to membrane disruption. Alternatively, as the endosome matures, relatively large proportions of the unusual anionic lipid LBPA appear within the late endosome.[27] This lipid would be expected to be available to interact with cationic lipid.

The ability of cationic lipids to promote formation of nonbilayer structure and the correlation between this property and intracellular delivery of macromolecules such as plasmid DNA offers the possibility of rational design of intracellular delivery systems. In this regard, considerable effort has gone into the synthesis of a large number of cationic lipids to improve transfection properties.[29,43,56,57] This effort is largely phenomenological. It will be of considerable interest to determine whether optimum transfection for a given target cell is provided by a cationic lipid that exhibits maximum ability to induce H_{II} phase organization in appropriate lipid mixtures. Alternatively, it is well known that a variety of factors promote H_{II} phase organization in mixed lipid systems containing anionic lipid. These include elevated temperatures, low hydration, increased acyl chain unsaturation and the presence of divalent cations, particularly Ca^{2+}.[58–60] Recent results have shown that Ca^{2+} can stimulate the transfection potency of lipoplexes[61] suggesting that the presence of factors that promote H_{II} phase can act synergistically with cationic lipid to potentiate intracellular delivery. A final point is that it is possible that other cationic transfection agents such as cationic polymers[5] and cationic peptides[3,62] may induce membrane disruption and intracellular delivery by a related mechanism. In particular, cationic polymers such as polylysine can induce H_{II} phase structure in lipid mixtures containing anionic lipids[63] as can other cationic peptides.[64]

In summary, the studies presented here demonstrate that cationic lipids have, as a general property, the ability to promote nonbilayer hexagonal H_{II} phase structure in lipid mixtures containing anionic lipids. This suggests that the ability of cationic lipids to promote the intracellular delivery of macromolecules such as plasmids relies on an ability to destabilize cell membranes such as the endosomal membrane by inducing nonbilayer lipid structure. This is supported by the fact that helper lipids such as DOPE and cholesterol that enhance cell transfection using cationic lipids also promote H_{II} organization in mixed lipid systems. These results may provide a method for the rational design of improved intracellular delivery systems.

Materials and methods

Materials

The cationic lipid N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) was synthesized as previously described.[35] Dioleyldimethylammonium chloride (DODAC), N-oleyl,N-stearyl-N,N-dimethylammonium chloride (OSDAC) and distearyldimethylammonium chloride (DSDAC) were provided by Dr S Ansell, Inex Pharmaceuticals (Burnaby, BC, Canada). 1,2-dioleoyloxy-3-(trimethylammonio) propane (DOTAP) and 3α-[N-(N′,N′-dimethylaminoethane)-carbamoyl] cholesterol hydrochloride (DC-Chol) and the phospholipids, dioleoylphosphatidylserine (DOPS), dioleoylphosphatidic acid (DOPA), 1-palmitoyl-2-oleoylphosphatidylglycerol (POPG); phosphatidylinositol-liver derived (PI); tetraoleoylcardiolipin (CL), *sn*-(3-oleoyl-2-hydroxy)-glycerol-1-phospho-*sn*-3′-(1′-oleoyl-2′-hydroxy)-glycerol (lysobisphosphatidic acid; S,R isomer), dioleoylphosphatidylcholine (DOPC) and dioleoylphosphatidylethanolamine (DOPE) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Dipalmitoylphosphatidylserine (DPPS) was obtained from Genzyme (Cambridge, MA, USA). Cholesterol was obtained from Sigma Chemical Company (St Louis, MO, USA). The plasmid pCMVluc (5650 bp) was obtained from Inex Pharmaceuticals. The DNA-binding dye PicoGreen was purchased from Molecular Probes (Eugene, OR, USA). All other reagents used in this study were of analytical grade.

Sample preparation

Lipids were dissolved in chloroform and stored at –20°C. Phospholipid concentrations were determined by established methods.[65] Three different lipid systems were used in this study, including aqueous dispersions of lipid, LUV and plasmid DNA–cationic lipid complexes. Aqueous dispersions of mixtures of cationic and anionic lipids were obtained by hydrating a lipid film, which was prepared by co-dissolving lipids to desired molar ratios and drying under a stream of N_2. Typically 20 μmol of total phospholipid was used. Lipid films were further dried under high vacuum to remove residual solvent. Dried lipid films were hydrated with 20 mM Hepes, pH 7.4 with five freeze–thaw cycles (liquid N_2/room temperature). Large unilamellar vesicles (LUV) were prepared from cationic or anionic lipids as required and were generated by extrusion through two stacked 100 nm filters (10 passes) after hydration of a lipid film in 5 mM Hepes buffer at pH 7.5 containing 5 mM sodium chloride, employing five freeze–thaw cycles. LUV sizes were determined by dynamic light scattering using a NICOMP 370 particle sizer (Nicomp Particle Sizing, Santa Barbara, CA, USA). Sizes were obtained from the experimental correlation functions by a cumulant fit. All LUV formulations exhibited diameters between 70 and 100 nm.

Plasmid DNA–cationic lipid complexes were prepared in 5 mM Hepes buffer at pH 7.5 by mixing equal volumes of plasmid DNA and DOTAP LUV at the indicated positive-to-negative (cationic lipid-to-nucleotide) charge ratios. The plasmid was rapidly added to the cationic liposomes while vortexing. The positively charged complexes used for ^{31}P NMR studies were prepared at a final plasmid concentration of 330 μg/ml. Excess cationic LUV were removed by centrifugation. Briefly, complexes were pelleted employing centrifugation at 20000 *g* for 10 min using a CEI Eppendorf centrifuge and the supernatant containing excess cationic liposomes was removed. It has been shown elsewhere that this procedure results in complexes that exhibit equal transfection potency but reduced toxicity.[36] The complexes were washed with Hepes buffer, pelletted again and resuspended in HBS in concentrated form to achieve the high plasmid concentrations (approximately 1 mg/ml) required for the ^{31}P NMR experiments.

Accessibility of plasmid DNA in complexes

The DNA-binding dye PicoGreen was used to determine plasmid DNA accessibility in complexes.[66] Plasmid DNA–DOTAP complexes were prepared at 50 μg/ml pCMVluc in 5 mM pH 7.5 Hepes buffer at a positive-to-negative charge ratio of 3. Before addition of DOPS LUV to the complexes the salt concentration of the aqueous medium was adjusted to 150 mM NaCl (physiological salt concentration). Binding of PicoGreen was determined before and after addition of DOPS LUV by measuring fluorescence emission at 520 nm with an excitation wavelength of 480 nm and 10 nm excitation and emission slit widths, respectively. The amount of plasmid accessible to PicoGreen was calculated employing the relationship $P_a(\%) = (F - F_b)/(F_{tot} - F_b) \times 100$, where P_a is the amount of accessible plasmid, F is the PicoGreen fluorescence of the complexes in the absence or presence of DOPS LUV, F_b the background fluorescence (PicoGreen in buffer) and F_{tot} the fluorescence after addition of 10% Triton X-100 at a final concentration of 0.5%. The amount of pCMVluc associated with the complexes was determined after solubilization of the complexes in Triton X-100 or alternatively, after extraction of the lipid by the procedure of Bligh and Dyer.[67]

^{31}P NMR spectroscopy

Proton decoupled ^{31}P NMR spectra were obtained using a Bruker MSL-200 spectrometer operating at 81.02 MHz. Acquisition parameters included 50 or 60° pulses, a spectral width of 10–20 kHz with 4k data points and a 1 s interpulse delay time. The temperature was regulated using a Bruker VT-100 temperature controller. If not otherwise indicated the temperature was maintained at 25°C. An exponential multiplication corresponding to 50 Hz line broadening was applied to the free induction decays before Fourier transformation. The chemical shift was referenced to external 85% phosphoric acid (H_3PO_4).

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