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Topical Review

On the Mechanism of Cationic Amphiphile-mediated Transfection. To Fuse or not to Fuse: Is that the Question?

I.S. Zuhorn, D. Hoekstra
Department of Membrane Cell Biology, Faculty of Medical Sciences, University of Groningen, A. Deusinglaan 1, 9713 AV, Groningen, the Netherlands

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Abstract. Due to charge interaction, cationic lipids spontaneously associate with nucleic acids, resulting in the formation of so-called lipoplexes. Lipoplexes are membranous structures that are capable of transducing genes into cells, eventually leading to expression of the genes (transfection). The mechanism involved in the cellular uptake of lipoplexes is most likely endocytosis, which occurs after nonspecific charge-mediated binding to cellular receptors. An important step in the transfection process following the actual internalization of lipoplexes is the release of the lipoplex and/or its DNA into the cytoplasm in order to evade lysosomal degradation. Here, the membranous nature of the lipoplex seems to be crucial in that it allows the exchange of lipids between the endosomal membrane and the lipoplex, which results in membrane perturbations that are a prerequisite in the endosomal escape of DNA. Interestingly, a hexagonal phase of the lipoplexes has been correlated with efficient transfection and it can be envisaged that such a phase could be instrumental in the creation of membrane perturbations. Subsequent to its release into the cytoplasm, the DNA has to be transferred into the nucleus. The nuclear import of DNA is most likely a protein-mediated process. In addition, the nuclear uptake of DNA may be facilitated at the time of nuclear envelope disassembly during mitosis. Currently, cationic liposomes are widely used as gene carrier system to deliver nucleic acids into cells in culture to study the cell-biological functioning of genes plus accompanying proteins. Ultimately, cationic lipids may be used in gene therapeutic protocols.

Key words: Transfection — Cationic lipid — Lipoplex — Endocytosis — Trafficking — Hexagonal

Correspondence to: D. Hoekstra; email: d.hoekstra@med.rug.nl

Introduction

Since Felgner and colleagues exploited the cationic lipid N-[l-(2,3-dioleyloxi)propyl]-N,N,N-trimethylammonium chloride (DOTMA)[1] as vehicle for the transfer of DNA into eukaryotic cells (Felgner et al., 1994), many new cationic lipids have been synthesized with the ultimate goal to obtain efficient nonviral carrier systems for in vitro and in vivo gene delivery. However, due to a lack of insight in the process of cationic lipid-DNA assembly, the mechanism of cationic lipid-mediated transfection of cells, and, more importantly, the relation between the structure and activity of cationic lipid-DNA complexes, the synthesis of new compounds for transfection remains largely empirical.

The purpose of this review is to give an overview of current knowledge about the assembly of lipoplexes and trafficking of lipoplexes in cells, describing the cellular barriers that impede nuclear delivery of DNA. A better understanding of the individual steps that are involved in transfection allows a systematic improvement of the gene delivery system. For in vivo applications of lipoplexes, not so much the cellular barriers as the barriers to biodistribution have to be clarified and overcome. It is unlikely that a single gene delivery system can be utilized for treatment of all genetic disorders. An approach that is followed by several research groups is to screen for the natural

Abbreviations: DOTMA, dioleyltrimethylammonium chloride; DOTAP, N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulphate; DODAC, dioleyldimethylammonium chloride; DOPE, dioleoylphosphatidylethanolamine, DOPC, dioleoylphosphatidylcholine; PS, phosphatidylserine; PG, phosphatidylglycerol, RES, reticulo-endothelial system; NLS, nuclear localization signal; MHC-I, major histocompatibility complex class I; HA, haemagglutinin; TFO, triplex-forming oligonucleotide; H_{II}, hexagonal; L_{α}, lamellar; SAXS, small angle X-ray scattering; EM, electron microscopy; SAINT, synthetic amphiphile international.

homing capacity of the gene delivery system, followed by an attempt to treat a disease that is localized at this particular homing site (e.g., delivery of cationic lipoplexes to the lung to treat cystic fibrosis (CF)). However, assuming that most cationic lipid-based vectors share their preferential site for accumulation, there is need for constructing vectors that can be targeted to any specific site in the body and thereby treat in principle, any disorder. This calls for a thorough understanding of both the extracellular and the intracellular barriers to cationic lipid-mediated gene delivery.

A Rationale for the Application of Nonviral Vectors

The rationale for developing nonviral vectors, such as formulations based on synthetically prepared cationic lipids, stems from the following considerations. Although viral vectors are still superior in their transfection ability (MacLachlan, Cullis & Graham, 1999), their application raises a number of safety concerns (Ilan et al., 1997; Rubanyi, 2001), including possible mutagenesis and immunological responses, apart from limitations of large-scale production of appropriate viral vectors. Furthermore, incubation of cells in culture with naked plasmid DNA generally results in poor transfection efficiency. Naked DNA is not able to efficiently transfer across the negatively charged plasma membrane of cells. This is not unexpected given the hydrophilic nature of DNA in conjunction with the negative charge imposed by its phosphate backbone. However, it has been reported that direct injection of an antigen-encoding plasmid DNA into muscle in vivo generates an antibody response. Moreover, it was recently shown that intravenous administration of naked Epstein-Barr virus-based plasmids in mice results in efficient gene expression in the liver. It was suggested that certain cell surface receptors could mediate the uptake of naked plasmid DNA into hepatocytes (Cui et al., 2001). Apparently, such receptors are not active in vitro. Recently, injection of two plasmids, one encoding the therapeutic gene together with pieces of a transposon, the other the enzyme transposase, was shown to elicit long-term transgene expression in mice (Yant et al., 2000). In spite of the positive outcome of these experiments, the prevailing data indicate that successful expression following injection of naked DNA seems to be restricted to muscle and liver tissue (Ferber, 2001). Delivery can be further enhanced by the use of hydrostatic pressure, e.g., through the use of a blood-pressure cuff (Cui et al., 2001; Ferber, 2001). To improve the in vitro transfection efficiency with naked DNA, transient pores can be generated in cells by means of electroporation, which allows the diffusion of DNA across the plasma membrane and, presumably, the nuclear membrane. Nevertheless, transfection accomplished in this manner, both in vivo and in vitro is relatively low, while electroporation is accompanied by considerable cell toxicity.

Therefore alternative means for effective gene delivery are actively explored and current interest in designing appropriate delivery vehicles is very much focussed on nonviral carriers like polymers and cationic lipids. Indeed, complexes of DNA with both cationic lipids and polymers, so-called lipoplexes and polyplexes respectively, have been shown to act as highly effective alternatives for free DNA, although gene delivery is still relatively low compared to the gene transfer capacity of viral vectors. Yet, recent progress in defining some common structural requirements for several different cationic lipid formulations, and emerging insight into the pathway of entry of such complexes, may represent important steps that should lead to a further improvement of cationic lipid-mediated transfection.

Parameters that Govern Cationic Lipid-DNA Interaction

Fluidity of Cationic Lipids

In general, the transfection activity of cationic lipids increases when the chain length and degree of saturation of the hydrophobic alkyl or acyl chains decreases (Felgner et al., 1994). Such molecular changes cause a downward shift in the solid to liquid-crystalline phase transition temperature (T_m) of the pure cationic lipid, implying that the relative fluidity of the cationic lipid bilayers increases. Accordingly, this raises the question whether efficient transfection thus might depend on the state of lipoplex fluidity, or that for appropriate complex assembly, the cationic lipid requires to interact with the DNA in its fluid state. In fact, it was reported that fluidity determines the final size of lipoplexes and thereby transfection efficiency (Akao et al., 1996; Regelin et al., 2000; *see* also below). However, the fluidity of the cationic lipid per se does not necessarily predict the fluidity of the cationic lipid-DNA complex (Bennett et al., 1996). Interestingly, it has also been shown that the addition of DNA may display a fluidizing effect on solid cationic bilayers composed of DOTAP (N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulphate). It was suggested that at a temperature below the T_m of the lipids the interaction of the negatively charged DNA backbone with the positively charged lipid head groups could lead to a loosening of the tightly packed head-group organization, thereby increasing the distance between lipid molecules in the membrane and facilitating the relative hydration. When added to fluid bilayers, addition of DNA tends to diminish their relative fluidity. Indeed, a tight as-

sociation of the cationic lipids with the polyanion DNA upon their electrostatic interaction seems to decrease the lipid's lateral diffusion rate (Regelin et al., 2000). Overall, these observations indicate that electrostatic interactions may lead to water displacement from the lipid head group, which causes a relative solidifying effect. The relative fluidizing effect of DNA suggests that at least partial 'penetration' of nucleic acids into the cationic lipid membrane may occur, in a sense that the physical effect bears reminiscence of that occurring upon inclusion of cholesterol in solid membranes (Leonard & Dufourc, 1991).

However, there is no clear consensus yet as to the effects of DNA-cationic lipid interaction on the physical phase of the lipids, largely due to a lack of detailed studies thus far. In mixed cationic/zwitterionic lipid membranes, the binding of DNA was reported to rigidify the complex (Zantl et al., 1999; Huster et al., 2000). In contrast, in mixed bilayers consisting of DOTAP analogs and DOPE, the interaction with DNA causes only minor changes in the fluidity of such bilayers (Regelin et al., 2000; Hirsch-Lerner & Barenholz, 1999). In this context, the nature and state of hydration of the 'helper' lipid could be highly relevant. Thus, a relatively poorly hydrated lipid like phosphatidylethanolamine (PE), compared to phosphatidylcholine (PC), may be more effective in 'promoting' membrane rigidity, particularly when taking into account that salt formation between the phosphate of the PE head group and the quaternary amine of DOTAP and/or the facilitation of the removal of cationic lipid-associated counterions further facilitate dehydration (Hirsch-Lerner & Barenholz, 1999; Zuidam et al., 1999a). It has been suggested that cationic lipids that facilitate a decrease in the relative hydration of adjacent polar helper lipids, thereby promoting a relative enhancement of hydrophobic domain destabilization, display the highest transfection efficiency (Bennett et al., 1997). It is as yet unclear whether the enhanced transfection activity could be due to an enhanced internalization of the lipoplexes and/or due to an improved ability to fuse with or perturb the endosomal membrane, necessary for allowing the plasmid to gain cytosolic access. On the other hand, membrane fluidity may influence the DNA structure, which in turn may have consequences for the efficiency of processes like transcription (Akao et al., 1996). However, the generality of such an effect of the physical properties of the cationic lipid on DNA structure requires further investigation (Balasubramanian et al., 1996), since next to an effect of overall fluidity other cationic lipid-specific parameters may be involved as well. Such parameters may include lipid phase miscibility in case of mixed cationic lipid/helper lipid systems and its consequence on lipid-DNA interaction, or the triggering of such phases upon lipid-DNA interaction.

Higher radii of curvature (promoted when the relative cross-sectional areas of lipid head group and that occupied by the hydrophobic region do not match) have been correlated with higher in vitro transfection efficiencies (Xu et al., 1995; Bennett et al., 1997). More specifically, a hexagonal conformation of lipoplexes, favored by a small lipid head group and bulky tail group, appears to promote efficient DNA delivery to cells (Koltover et al., 1998; Lin et al., 2000; Smisterova et al., 2001). Among others, it has been proposed that non-bilayer lipid structures might play a direct role in transfection by promoting fusion between lipoplex and target membrane or a membrane destabilization that allows the plasmid's release/dissociation (Koltover et al., 1998; Hirsch-Lerner & Barenholz, 1999). Since the kinetics of drug release is strongly dependent on the liquid crystalline phase of the lipidic delivery system (Farkas et al., 2000) a hexagonal conformation of the lipoplex could possibly play a role in facilitating DNA release from the lipoplex as well as facilitating its endosomal escape, as will be discussed below.

Lipid-Domain Formation

Besides fluidity of the membrane, lateral domain formation of lipid phases distinctly enriched in certain lipids may co-determine lipoplex assembly and complex morphology, and, thus, transfection efficiency. Addition of DNA to liposomes composed of a mixture of cationic and zwitterionic lipids may give rise to phase separations and, hence, to lipid-microdomain organization, due to preferential interactions between DNA and the cationic lipid. At relatively low DNA/cationic lipid charge ratios (<1) the negatively charged DNA condenses the positively charged lipid headgroups, thereby inducing formation of cationic lipid-enriched domains. The ensuing membrane boundary defects at the lipid-water interface, caused by the exposure of the upper hydrocarbon region of the hydrophobic tails to water, leads to instability and aggregation of lipoplexes. At DNA/cationic lipid charge ratios ≥1, the condensing effect of DNA on the cationic lipids is less pronounced, involving homogeneous lateral condensation of all the lipids (Mitrakos & MacDonald, 1997; Hirsch-Lerner & Barenholz, 1998; MacDonald et al., 2000).

Upon formation of lateral domains, particularly when the properties of interfaces are modulated by water displacement upon electrostatic interactions, boundary phases will arise where adjacent lipids may mismatch. Such defects create membrane instability and may result in an enhanced permeability, while these regions are also potential target sites for exogenous molecular insertion (e.g., peptides).

Condensation of DNA

Next to a preferential requirement for a net positive charge ratio of cationic lipid over DNA, an effective condensation of the nucleic acid is also thought to be essential for an efficient and high transfection ratio. DNA condensation depends on the electrostatic interaction of DNA with the cationic lipid and the lipid packing parameters (Lasic & Templeton, 1996). Reports have been published that showed a lack of an ordered DNA phase when the lipid is kept below T_m, implying that fluidity of the membrane is essential for DNA condensation (Spector & Schnur, 1997). Indeed, also work in our laboratory indicated that complexes prepared in the presence of solid SAINT lipids lead to complexes with an appearance of 'beads (i.e., liposomes) on a string (i.e., the plasmid).' Although it seems obvious to assume that tightly ordered plasmids, i.e., as compact supercoiled structures, may more easily acquire access into cells (via endocytosis, followed by cytosolic escape and nuclear delivery) than unraveled, extended structures, the precise role of cationic lipids in condensation of plasmid DNA remains unclear. A careful analysis by atomic force microscopy of SAINT-2/DNA interaction revealed essentially no effect of the cationic lipid-plasmid interaction in terms of DNA condensation, the plasmid being essentially organized in a supercoiled structure before and after addition of the cationic lipid (Oberle et al., 2000). Interestingly, in addition to potential structural constraints for intracellular processing of the plasmid when the supercoiled structure is perturbed, the degree of DNA supercoiling has also been reported to be important in facilitating transcription and replication (Weintraub, Cheng & Conrad, 1986; Akao et al., 1996; Prazeres et al., 1999). Accordingly, these observations may hint to the possibility that transfection per se may not necessarily properly reflect the effectiveness of plasmid delivery into the nucleus.

Lipoplex Assembly

Because of their positive charge, liposomes prepared from cationic lipids readily associate with negatively charged DNA, thereby forming cationic lipid-DNA complexes. The hydrophilic interior of the liposomes is lost due to collapse of the lipid vesicles onto the DNA. As readily determined by lipid mixing assays (Hoekstra & Klappe, 1993) lipoplex assembly involves extensive mixing of the lipids, caused by either individual lipid recruitment by the plasmid, and/or possibly membrane fusion events when the vesicles enwrap the plasmids (Huebner et al., 1999; Oberle et al. 2000).

The driving force for lipoplex formation is the removal of counterions from the lipid surface by the DNA (Wagner et al., 2000). Inclusion of helper lipids such as DOPE in the liposomal formulation may facilitate this removal by weakening the binding of the ions to the cationic surface (Zuidam & Barenholz, 1998; Zuidam et al., 1999a). Moreover, inclusion of a helper lipid, provided its good miscibility with the cationic lipid, brings about a better matching in the density of surface charge distribution between the liposomes and DNA, resulting in a more complete and balanced packaging of DNA by the lipids (Zuidam et al., 1999a). This is also reflected, as visualized by atomic force microscopy, by the smooth surface of complexes composed of mixed lipids, as opposed to a ruffled surface when preparing complexes with the cationic lipid alone (Oberle, Bakowsky & Hoekstra, 2002).

Apart from facilitating complex formation, an additional effect of the helper lipid may rely on its effect on complex stability. While DOPC does not affect complex formation and stability in DODAC-DNA lipoplex assembly, the addition of DOPE affected both. This is likely due to the ability of hydrogen bonding between the PE headgroup and the cationic head group of DODAC (Wong et al., 1996; Bally et al., 1999), whereas the strongly hydrated head group of PC will exert repelling intermolecular hydration forces. Accordingly, in contrast to DOPC, DOPE will directly influence DODAC/DNA binding, an effect that will obviously affect issues such as DNA-lipoplex dissociation, which represents an important step in overall transfection.

The size instability of lipoplexes, which is presumably (partly) dictated by their lipid phase (in)stability, has been correlated with a high transfection activity in vitro (Escriou et al., 1998; Barenholz, 2001; Simberg et al., 2001). The formation of large lipoplex aggregates may be indicative of the presence of a hexagonal phase of the lipids. During a hexagonal transition, the hydrophobic part of the lipids becomes exposed to the aqueous phase, a thermodynamically highly unfavorable condition. As a result, the hexagonal phase transforms into an aggregate with a low surface-to-volume ratio. Besides negative curvature strain, provided by lipids that adopt the hexagonal phase, phase boundary defects or positive curvature strain are sources of bilayer destabilization that could result in aggregation of lipoplexes (Epand, 1996). Thus, precipitation of lipoplex due to colloidal instability does not necessarily imply the presence of a hexagonal phase, and turbidity measurements of lipoplex formulations alone do not suffice to support the presence of lipoplex with a hexagonal conformation, i.e., transfection-active lipoplexes. In addition, investigations of the lipoplex morphology by experimental approaches such as small angle X-ray (SAXS) or electron microscopy (EM) technologies are necessary for proper determination of the lipoplex conformation. It should be

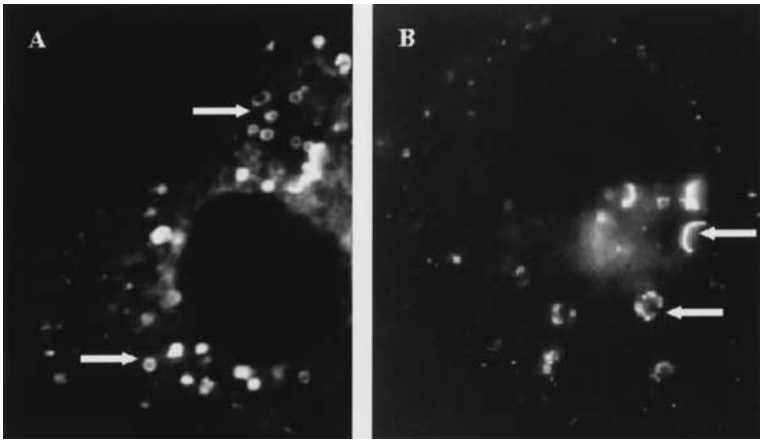

Fig. 1. Serum proteins affect lipoplex stability, thereby preventing the exchange of lipids between the lipoplex and the endosomal membrane. (*A*) Cells were incubated with N-Rh-PE-containing lipoplexes in the absence of serum. Continuous fluorescence can be seen in vesicular structures, presumably belonging to the endocytic pathway, indicating translocation of lipids between lipoplex and the endosomal/lysosomal membranes. (*B*) In contrast, when lipoplexes were prepared in the presence of serum, accumulation of intact lipoplexes at endosomal/lysosomal membranes is seen, without the occurrence of lipid redistribution. In this case, a strongly reduced level of transfection is observed (for details, *see* Zuhorn et al., 2002c).

noted, however, that the timing of the hexagonal conformation is crucial, implying that for effective transfection to occur, the complex may acquire such properties only when localized in the endosomal compartment, the presumed site of plasmid entry into the cytosol. (Hafez & Cullis, 2001; Zuhorn et al., 2002b).

The colloidal properties of lipoplexes are largely determined by the cationic lipid/DNA charge ratio and less so by the lipid composition (type of helper lipid). Therefore, transfection-inactive (lamellar) as well as transfection-active (hexagonal) complexes can show similar colloidal properties. As revealed by cryoEM, the transfection-active lipoplexes (DOTAP/DOPE) have a greater incidence of high-radius structures than transfection-inactive lipoplexes (DOTAP/DOPC) (Xu et al., 1999).

Contributing much to the frustration of properly defining and rationalizing lipoplex properties in terms of transfectability are the numerous parameters that govern lipoplex assembly and, thereby, the eventual morphology and physicochemical characteristics of the resulting cationic lipid-DNA structure (Kennedy et al., 2000). Among others, the vesicle size, concentration of lipid and DNA at the time of mixing, the mixing rate, order of addition, ionic strength of the mixing buffer, and the cationic lipid/DNA charge ratio have been shown to represent a minimal set of parameters that affects lipoplex characteristics. However, when the same reagents and same concentrations, but different formulation procedures are used, the resulting thermodynamic and biological stability of lipoplexes may again differ greatly (Hofland, Shephard, Sullivan, 1996; Thierry et al., 1997; Ferrari et al., 2001). Some generally applicable features to obtain transfection-active lipoplexes appear to be, however, (i) a proper dispersion of the reactants, (ii) injection of DNA into lipid rather than vice versa, (iii) the presence of salt to facilitate hexagonal phase formation, and (iv) a positive molar charge ratio of cationic lipid to DNA (Rosenzweig et al., 2000).

Extracellular Barriers in vitro and in vivo

In vitro, serum is usually present in the cell culture medium and transfection with lipoplexes in the presence of serum is often less efficient than in its absence. It was suggested that binding of serum proteins to the lipoplexes prevents their interaction with the cell surface and/or their internalization, thereby inhibiting transfection (Lewis et al., 1996; Zelphati et al., 1998). However, no correlation has been found between the cellular uptake of lipoplexes and their transfection efficiency. Recently, it was shown that artificial rupture of endosomes could enhance the transfection efficiency of lipoplexes, prepared in the presence of serum. Apparently, serum may affect the ability of the lipoplex to interact with the endosomal membrane. It was proposed that depending on the chemical nature of the amphiphile, serum proteins may affect lipoplex stability by virtue of their ability to penetrate into the complex, thereby stabilizing its lamellar phase which, as noted above, may interfere with the cytosolic release and dissociation of the complex (Fig. 1; Zuhorn et al., 2002c).

In vivo, additional parameters that constitute significant extracellular barriers for transfection are readily apparent. In addition to serum interference and DNA degradation, opsonization of lipoplexes by serum proteins may result in their clearance by the reticulo-endothelial system (RES). Serum protein binding is often associated with increases in plasma elimination rates, nonspecific cell binding, enhanced phagocytotic uptake by macrophages, perturbations of the membrane structure, and complement activation (Bally et al., 1999).

Optimal in vivo gene transfer requires a lower cationic lipid/DNA charge ratio than that used in cell culture (Bally et al., 1999). Uptake by macrophages and/or lodging of lipoplexes in capillary beds in, e.g., lung, liver, and spleen can be prevented when relatively small (< 200 nm) and neutral or charge-shielded particles are administered (Bally et al., 1999). While posi-

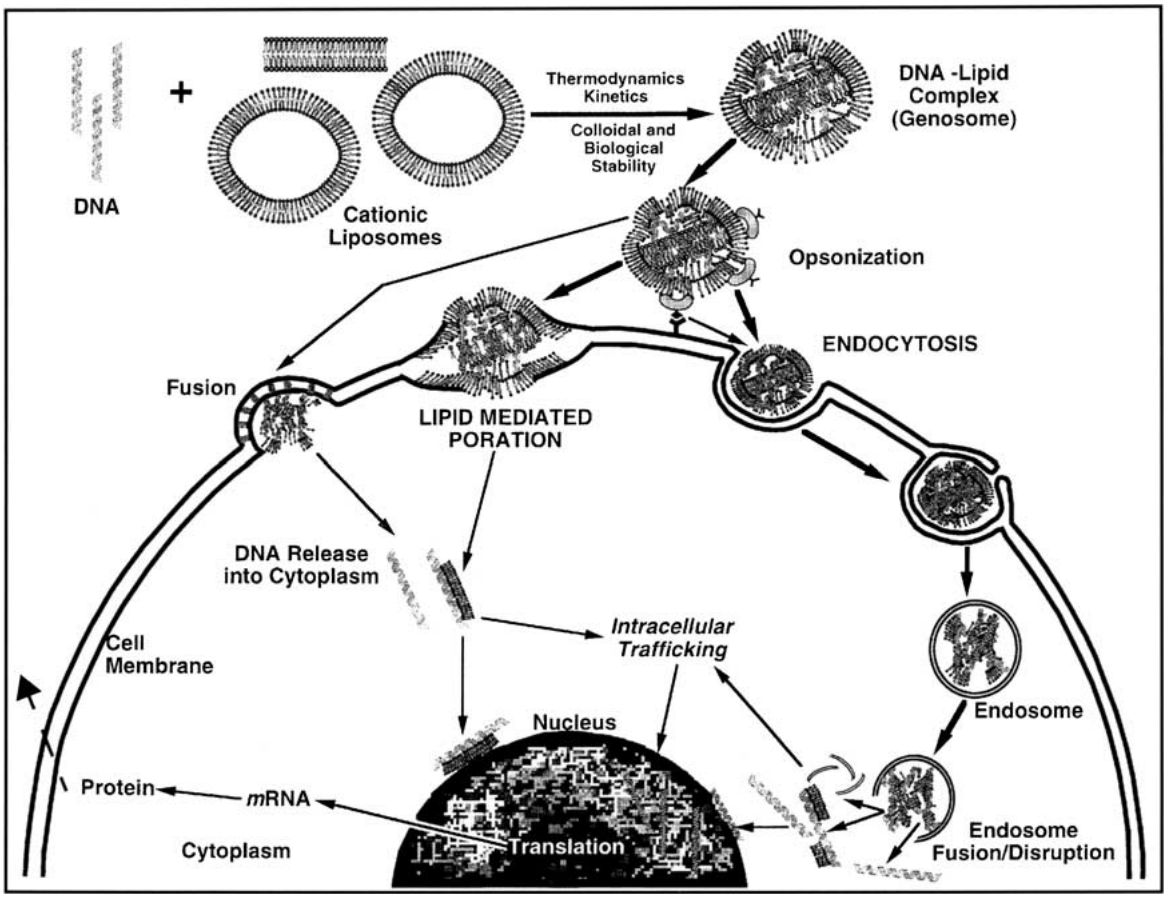

Fig. 2. Barriers to lipofection. Three possible modes for cellular entry of lipoplexes are indicated: fusion, lipid-mediated poration, and endocytosis. Endocytosis of lipoplexes is followed by the endosomal escape of (lipid-)DNA into the cytoplasm. In the nucleus, DNA has to be free of lipid to be transcriptionally active. (Reprinted with permission from S.E. Hansen, 1995. That amazing genetic code; unraveling DNA and RNA. Unpublished Lecture notes: National Society for Histotechnology.)

tive charges are relevant for efficient DNA complexation in the first place, for successful in vivo application shielding of those charges from the extracellular milieu appears crucial. In principle, polymers such as polyethylene glycol (PEG) can be used to shield the charge of lipoplexes and to prevent serum protein attachment. However, transfection activity with PEG-stabilized lipoplexes depends on the exchange of PEG, once the complex has reached its target site, in order to 'restore' the transfection activity (Bally et al., 1999; Woodle & Scaria, 2001; Song et al., 2002). Next to exchangeable PEG devices, targeting devices are also needed for appropriate homing of the lipoplexes to the therapeutic site (Pouton & Seymour, 2001). These should be as specific as possible and may include monoclonal antibodies, sugars, and lectins (Barratt, 2000). Ligands for which receptors are ubiquitously present such as, e.g., transferrin or folic acid, are less preferable. Alternatively, problems with systemic delivery of lipoplexes can be circumvented by local administration — if possible — to the site of disease, e.g., delivery of genes encoding antigens or cytokines for immunotherapy by intratumoral injection. Localization techniques like endoscopy expand the possibilities of local administration of genes to virtually any organ in the body (Pouton & Seymour, 2001).

Unlike the in vivo application of lipoplex-mediated gene transfer, ex vivo gene therapy is not met with the above-mentioned problems concerning biodistribution and lipoplex stability in systemic delivery. Thus, where possible, ex vivo gene therapy represents an attractive alternative in the application of gene therapy, a concept that may gain sooner practical application than the 'direct' application of in vivo gene therapy. Thus it would appear that particularly in this context, a further expansion and intensification of optimizing in vitro gene delivery appears fully justified.

Intracellular Barriers

Binding and Internalization of Lipoplexes

A net positive charge of the lipoplexes ensures their efficient binding to the cell surface. However, by increasing the positive charge of the lipoplex, also its

cytotoxic effect is enhanced (Lasic & Templeton, 1996). Apparently, cellular damage is an important event accompanying transfection. Such damage may arise when cationic lipids intercalate into cellular membranes. Indeed, it has been proposed, given the membranous nature of lipoplexes, that their cargo would gain intracellular access via fusion with the plasma membrane. However, lipid mixing assays did not reveal a correlation between fusion of lipoplexes with the plasma membrane and their transfection efficiency (Stegmann & Legendre, 1997). Rather, current data support the view that following binding of the complexes to the cell surface, internalization is required for productive transfection to occur, and that nucleic-acid release takes place at the level of endosomal compartments.

Recently, we were able to show such a correlation by employing a number of approaches relying on both biochemical and cell biological techniques. Inhibition of clathrin-mediated endocytosis by means of cholesterol removal following cyclodextrin-treatment of cells, and its reactivation upon cholesterol resupply showed that endocytosis via clathrin-coated vesicles is required in order to achieve cellular transfection (Zuhorn, Kalicharan & Hoekstra, 2002a). These experiments also provided evidence that entry of nucleic acids at the cell surface can be excluded, and, if occurring, that such a pathway does not lead to significant transfection. As a typical endocytic process is generally believed to have a size restriction of 100–200 nm, it is a priori unexpected that efficient lipofection is often related with colloidal instability of lipoplexes, which results in extensive aggregation and in particle sizes of up to several μm. However, the determination of particle size typically relies on dynamic light scattering (DLS) measurements of highly concentrated lipoplex formulations, which clearly affects lipoplex aggregation behavior. In addition, lipoplex formulations exhibit a heterogeneous size distribution and it could well be that the lipoplexes that are responsible for productive transfection are those with a relatively smaller size than the mean size of the total population. As the colloidal instability could indicate the presence of a hexagonal conformation of the lipoplexes, it is probably more accurate to propose that lipoplexes that have adopted the hexagonal phase, and hence display a strong tendency to aggregate, rather than large-sized lipoplexes are necessary for obtaining efficient transfection. In this context, it is of interest to note that, although PEG-coated lipoplexes are as effectively internalized by cultured cells in vitro as PEG lipid-devoid (control) complexes, effective transfection is not observed, as the presence of PEGylated lipids prevent the complexes from adopting the hexagonal phase. When dissociated from the complex in the endosomal compartments, release of nucleic acids occurs as effectively as for control lipoplexes, and the removal of the PEGylated lipid can be correlated with the ability of the complex to adopt the hexagonal phase (Shi et al., 2002). These observations indicate that, although internalization is a necessary step for transfection to occur, size neither determines the efficiency of endocytic internalization nor does the efficiency of internalization necessarily determine eventual transfection efficiency (Song et al., 2002; Zuhorn et al., 2002c; Shi et al., 2002).

Although the lipoplexes enter the cells along the pathway of receptor-mediated endocytosis, the nature of the receptor(s) remains to be established. Membrane-associated glucosaminoglycans (GAGs) have been suggested as potential binding sites for lipoplexes (Mislick & Baldeschwieler, 1996). Recently, extracellular GAGs were shown not to significantly affect lipoplex uptake but to affect their intracellular trafficking, thereby affecting gene expression. In fact, interaction with glycosaminoglycans can induce the release of DNA from lipoplexes, a necessary and crucial step in the overall transfection process (Wiethoff et al., 2001; Ruponen et al., 2001). If so, the question remains whether interaction with GAGs as such suffices or whether additional conditions have to be met, given that release at the cell surface does not lead to productive transfection.

Transfection in the presence of an acid pH nuclease inhibitor enhances reporter gene expression mediated by cationic peptides and cationic lipids, suggesting that nuclease action in the endosomes/lysosomes could be detrimental for transfection (Ross et al., 1998a). To prevent degradation of the lipoplex in lysosomes, triggered-release mechanisms have been developed. Incorporation of fusogenic devices (adenovirus capsid, HA) in lipoplexes greatly enhance gene expression in vitro. However, incorporation of virus-derived proteins or peptides also enhances the immunogenicity of the formulation, a phenomenon not encountered when including a lipid with membrane-perturbing activity (Hope et al., 1998). Inclusion of unsaturated PE increases the ability of lipoplex to disrupt the endosomal membrane. The appreciation of the importance of H_{II} (Hexagonal) phase formation of the lipid phase of the lipoplexes for endosomal release led to the synthesis of acid-sensitive lipids that similarly undergo a lamellar to H_{II} phase transition in an acidic environment, providing a tool for site-specific (e.g., within endosomes or in ischemic tissue) contents release (Boomer & Thompson, 1999; Li & Schick, 2001).

Instead of facilitating the escape of lipoplexes from the endosome by conveying additional membrane destabilizing properties, the chances of escape could also be improved by inhibiting the transport of lipoplexes towards lysosomes, thereby increasing the time-span for membrane interaction between lipoplex and endosomal membrane, necessary for the release

of lipoplex into the cytoplasm. Downregulation of dynein activity has been proposed to slow the kinetics of membrane traffic from endosomes to lysosomes, thereby increasing the chance for plasmids to escape into the cytosol prior to reaching the lysosomal degradation compartment (Hamm-Alvarez, 1999). Consistent with such a scenario is the recent observation that rupture of microtubules enhances transfection efficiency (Hasegawa, Hirashima & Nakanishi, 2001). Since microtubules are required for transport between early and late endosomes, these data thus indicate that the complexes, likely being excluded from recycling, remain trapped in early endosomal compartments, thus improving the options for nucleic-acid release prior to degradation.

Endosomal Escape and Disassembly of Lipoplexes

Interactions between cationic amphiphiles and anionic (phospho-)lipids are driven by ion-pairing and hydrophobic forces, which are stronger than interactions relying on electrostatic forces involved in cationic lipid-DNA complex formation and stabilization. Consequently, the interaction of acidic (phospho-)lipids with lipoplexes may cause the release of DNA, as proposed in the hypothesis put forward by Zelphati and Szoka (1996) (Bhattacharya & Mandal, 1998). This proposal relies on the notion that following arrival of the lipoplex in the endosomal compartment, translocation of phosphatidylserine into the complex may occur, which would cause the dissociation of DNA from the complex and its subsequent release into the cytosol. The exact mechanism of the latter step is obscure, as is its relation with the PS translocation step as such. Recently, it was claimed that the release of DNA from lipoplexes coincides with their fusion with the endosomal membrane (Nakanishi & Noguchi, 2001). It is evident, though, that a tight interaction between lipoplex surface and endosomal membrane is essential to preclude that DNA dissociates from the complex prematurely, which may result in its processing into the degradation pathway with the lysosomes as the endpoint. Accordingly, it would thus be advantageous that the dissociation step and subsequent release kinetically coincide. Recent evidence strongly suggests that the lipid structural phase is instrumental in these steps, implying a correlation between the motional properties of endogenous lipids and transfection potential. Particularly relevant in this regard is the presence of nonbilayer structures, which may promote membrane destabilization and/or disrupt membrane integrity (Mok & Cullis, 1997; Koltover et al., 1998; Sternberg et al., 1998; Mui et al., 2000; Smisterova et al., 2001). Presumably, the formation of a (transient) pore or membrane perturbation as such is crucial for the DNA to escape from the endosome, as will be discussed in the next paragraph. A convenient procedure for screening the capacity of lipoplexes to release DNA is to follow the accumulation of FITC-labeled oligonucleotides (ODN) in the nuclei of cells incubated with cationic lipid-ODN complex.

Adaptation of a hexagonal (H_{II}) phase of lipoplexes, as can be determined by small-angle X-ray scattering, has been correlated with an efficient in vitro transfection capacity of these lipoplexes (Sternberg et al., 1998; Koltover et al., 1998; Smisterova et al., 2001). Whereas lamellar lipoplexes (L_α) bind stably to anionic phospholipid vesicles, the more transfective, i.e., hexagonal lipoplexes were unstable and readily fused with anionic phospholipid vesicles, thereby releasing their DNA (Koltover et al., 1998). Also with cells the different structures interacted differently, showing a correlation between structure and transfection efficiency (Lin et al., 2000). Similarly, a close correlation was found between the shape of the amphiphile and the presence of helper lipids like DOPE in promoting the hexagonal phase transition, and thereby transfection (Smisterova et al., 2001). By systematic chemical synthesis it was concluded that amphiphiles with a small head group area relative to hydrophobic tail region strongly prefer the hexagonal phase and displayed the highest transfection efficiency.

In conjunction with the likelihood of a role of the hexagonal phase of lipoplexes in bringing about efficient transfection, some recent work described by Lewis & McElhaney (2000) is of particular interest in this context. These authors noted that mixtures of cationic and anionic lipids (e.g., PS, PG, cardiolipin, phosphatidic acid), could form a hexagonal phase when approaching charge neutrality, whereas neither of the lipids in isolation exhibits a nonlamellar phase-forming propensity (Lewis & McElhaney, 2000). This finding further gains in significance when taking into account the concept of potential flip-flop of PS at the level of the endosomal membrane, as noted above. If cationic lipids in the lipoplex are able to form a hexagonal phase upon intercalation of PS into the lipoplex, for example when the surface boundary of the lipoplex is interacting with the inner endosomal leaflet, this could induce the formation of (transient) pores or sites of membrane destabilization, which would create a way for (part of) the lipoplex and/or DNA to escape from the endosomal compartment. Cationic lipids that undergo an H_{II} phase transition in mixtures with anionic, bilayer-forming phospholipids should be considered potent bilayer-destabilizing agents. These cationic lipids often are active without helper lipid activity (Hafez & Cullis, 2001).

According to this scenario it might thus be possible that at least part of the lipoplex as such may gain access into the cytosol. In fact, when endosomes loaded with lipoplexes are osmotically ruptured, re-

leased complexes (into the cytosol) still display transfection capacity (Zuhorn et al., 2002c), suggesting that release of plasmid may continue into the cytosol. Indeed, the presence of RNA in the cytosol and/or interactions of lipoplexes with cytoskeletal elements may trigger release of DNA from the complexes (Scherman et al., 1998; Barenholz, 2001).

Do these considerations imply that the mechanism of lipofection has been clarified? On the contrary, thus far only a few representative amphiphiles have been examined and direct evidence for PS translocation during cellular transfection has yet to be presented. In addition, thus far not all parameters have been defined, as examplified by the observation that DOTAP/DOPE-DNA, which forms hexagonal structures, transfects poorly, and a series of cationic phospholipids did not show enhanced transfection efficiencies when a complex was generated that displayed hexagonal phase properties (Ross et al., 1998b; Rakhmanova, McIntosh & MacDonald, 2000).

Nuclear Translocation and Mitosis

DNA that has been released from its carrier should finally reach the nucleus and be transcribed. How the DNA transfers across the nuclear membrane is unknown. However, it has been established that for nuclear import of large nucleic acids (i.e., viral genomes, mRNA, snRNA), an association of nucleic acids with proteins is required (Escriou et al., 2001). In this context it is of relevance to note that viral nucleocapsids with diameters up to 36 nm are able to cross the nuclear pore without disassembly, likely relying on a signal-mediated (NLS) mechanism (Panté & Kann, 2002). Support for a protein-mediated mechanism in plasmid nuclear import could also be derived from a study using microinjection (Wilson et al., 1999). Possibly, in analogy with the transport of adenoviral DNA (Trotman et al., 2001), the plasmid may bind to endogenous histones and thus piggyback into the nucleus.

Alternatively, it has also been proposed that mitosis is required for plasmids to gain access into the nucleus upon its reassembly. During mitosis the nuclear envelope is fragmented, which would facilitate the transfer of the (large) DNA into the nucleus. The observation of a close correlation between the onset of transgene expression and mitosis (in synchronized cell cultures) suggests that event(s) during mitosis augment transfection (Brunner et al., 2000; Escriou et al., 2001). If nuclear envelope fragmentation would represent an absolute requirement for plasmid entry, the relatively low transfection efficiency obtained with lipoplexes, compared to viral particles, could thus be explained. Within a typical cell cycle of 24 hours, mitotic breakdown of the nuclear membrane is limited to 1 hour, thus providing a very narrow time window for the plasmid to merge with the nuclear machinery (Mortimer et al., 1999). More likely, while 'waiting' for this opportunity, the DNA will be degraded in the cytoplasm (Pollard et al., 2001), since its half-time in the cytoplasm is approximately 90 minutes (Lechardeur et al., 1999). Still, it has been shown that non-dividing cells are also transfectable. Accordingly, cell division is not an absolute prerequisite for transfection, although it likely facilitates the nuclear transfer of DNA.

At this time, it cannot be excluded that plasmids arriving at the nuclear membrane are still (partly) coated with lipid. Therefore, such coated plasmids could be more condensed and thus smaller than free DNA, facilitating their entry into the nucleus and implying that eventual transfection might be particularly attributed to such a fraction. In analogy with the cationic nature of nuclear-homing sequences (NLS), the cation polylysine was suggested to exert a nuclear-localization activity, and similarly, cationic lipids could facilitate nuclear transport. However, it was noted that the DNA compaction rather than the ionic charge of cationic polymer-DNA complexes improved nuclear targeting (Pollard et al., 1998). This would be consistent with restrictions in particle size, enabling the intact transfer across the nuclear pore complex (Panté & Kann, 2002). Uncoating of DNA in the nucleus might be a result of a competitive distribution of the cationic lipid between DNA from exogenous plasmids and that from a large pool of chromatin (Behr, 1994; Scherman et al., 1998). However, microinjection of entire lipoplexes into the nucleus prevents transgene expression (Zabner et al., 1995; Pollard et al., 1998), which suggests that the uncoating of DNA should take place before the plasmid acquires access into the nucleus. Alternatively, if DNA were still coated with lipid as it reaches the nucleus, the negatively charged nuclear membrane could play a role in the final uncoating of the plasmid, needed for making it available to the transcription machinery. We observed an efficient delivery of ODN into the nucleus when ODN complexed to cationic lipid was incubated with isolated nuclei, which means that at the nuclear membrane uncoating of nucleotides can occur (*unpublished observations*). Finally, it is possible that the cationic lipid itself could play an active role in the translocation of DNA across the nuclear membrane. Friend, Papahadjopoulos & Debs (1996) described an interaction between cationic lipids and the nuclear envelope in CV-1 and U937 cells, resulting in vesicular and reticular intranuclear membranes. However, further studies are needed, using isolated nuclei, microinjection techniques, and digitonin-permeabilized cells, to extend our knowledge about such a direct role of cationic lipid in the nuclear translocation of DNA.

Compaction of DNA by association with peptides like human histone or protamine prior to complexation with lipids offers better protection against

enzymatic digestion, and increases the in vivo transfection efficiency when compared to conventional cationic lipid-DNA complex (Li & Huang, 1997; Schwartz et al., 1999). Alternatively, the NLS in protamine might facilitate nuclear uptake of the lipid-protamine-DNA (LPD) complex, while protamine might also be more readily dissociated from the DNA than cationic lipids. However, cationic lipids seem necessary to induce endosomal escape of the LPD complex (Gao & Huang, 1996). Similarly, condensation of DNA with oligolysine-RGD peptide enhances the nuclear transfer of DNA through the nuclear pore complex (NPC). The combination of RGD peptide and cationic lipids enhances the transfection efficiency of plasmid compared to transfection with solely cationic lipids (Colin et al., 2001). Degradation of DNA in the cytoplasm may be prevented by inhibition of cytosolic nuclease activity or cotransfection with noncoding DNA, saturating the enzyme (Pollard et al., 2001).

Attaching NLSs to synthetic gene delivery systems is thought to enhance the nuclear delivery of DNA, particularly in nondividing cells (Ziemienowicz et al., 1999). The NLS can be linked to both the carrier and the DNA (Bremner, Seymour & Pouton, 2001), although only heavily substituted DNA seems to significantly enhance DNA nuclear import (Pouton, 1998; Ludtke et al., 1999). Unfortunately, the covalent binding of many NLS to DNA may be detrimental for DNA functioning (Wolff & Sebestyén, 2001). Recently, Zanta et al. (1999) found that the coupling of a single NLS to DNA enhanced gene expression in several cell lines, irrespective of the cationic vector that was used. By hybridizing a bifunctional peptide nucleic acid (PNA), consisting of a nucleic acid binding moiety and an NLS, it is possible to link protein functions directly to nucleic acids (PNA-NLS/DNA). It is likely that the conformation of the NLS and its binding affinity for its cytoplasmic receptors will be affected by the method used to couple the NLS. These issues have to be clarified before nuclear delivery of DNA can be optimized (Pouton, 1998). The variation of NLS receptor species in different tissues might also be exploited to achieve a higher degree of selectivity in transfection of targeted organs, potentially in combination with tissue-specific promoters (Branden, Christensson & Smith, 2001). Alternatively, steroid-mediated gene delivery has been reported to enhance gene transfer, by targeting the DNA to the glucocorticoid receptor, inducing translocation to the nucleus (Rebuffat et al., 2001).

Also, newly designed plasmids could be evaluated in cell-free systems, since there is no consistency between the nuclear accumulation of plasmids and gene expression (Tachibana et al., 2001; Zanta, Belguise-Valladier & Behr, 1999). The use of single-molecule fluorescence spectroscopy would make it possible to follow in real time the complete route of lipoplex internalization, from passing the plasma membrane to entering the nucleus. This would allow the further development of advanced strategies in gene therapy research (Seisenberger et al., 2001).

Perspective

The properties of synthetic amphiphiles, relevant to their ability to function as an effective substitute for virus-based particles in gene delivery, are gradually emerging. In particular, dynamic lamellar-to-non lamellar transitions, well-established in research concerning phospholipid membranes, appear to be a key factor in the mechanism of lipoplex-mediated transfection. This property sets requirements as to the shape and hence the structure of the amphiphile, implying that the synthesis of novel compounds may take such features into account. It is apparent that the nonlamellar hexagonal (H_{II}) phase is not required for cellular internalization of the complex, but rather appears crucial for the cytosolic delivery, which appears to proceed via endosomal compartments. The intriguing possibility exists that the insertion of cellular lipids, in particular anionic lipid, into the lipoplex may in fact trigger the lamellar to hexagonal phase transition. Thus, such lipids may fulfill a dual function in that they compete with DNA for the cationic lipid, causing DNA dissociation from the complex and simultaneously promoting the nonlamellar transition as an important step that eventually results in plasmid expulsion into the cytosol. It is thus relevant to bear in mind that cationic lipids that display lamellar properties need not necessarily be useless in lipoplex assembly and delivery, as subsequent insertion of anionic lipids, following cellular uptake of the complex, may still trigger the hexagonal phase. Interestingly, it has been shown that poly(ethyleneglycol)-coated lipolexes, although being internalized by cells, and hence being capable of binding electrostatically to membranes, including the endosomal membrane, fail to deliver genes or oligonucleotides, unless the PEGylated lipid dissociates from the (internalized) complex (Song et al., 2002). These observations suggest that the lipoplex membrane is stabilized as long as the PEG lipid prevents the complex from intimately interacting with the endosomal target membrane; such an interaction likely is necessary for engaging in a lamellar-to-hexagonal phase transition. Whether such a translocation actually occurs in situ and what the role of cellular lipids is in this process remain to be determined. Furthermore, it also is unclear by which mechanism lipoplexes, being organized in the hexagonal phase, perturb the endosomal membrane such that plasmids can be released. Does fusion take place or is the hexagonal phase relevant to translocation of

cationic lipids into the target membrane, thereby causing a destabilization, often reflected by release of contents from liposomes or upon hemolysis of erythrocytes? These and other issues, of interest to a multidisciplinary field of research, ranging from organic chemistry, biophysics to cell biology, will warrant continued interest in this area, both at the level of fundamental and of applied membrane research.

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