CHAPTER 2

Cationic lipids, lipoplexes and intracellular delivery of genes

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Abstract

As a consequence of several setbacks encountered by viral technology in achieving efficient and safe gene therapy in clinical trials, non-viral gene delivery vectors are considered to date as a valuable alternative and to hold promise for future therapeutic applications. Nevertheless, the transfection efficiency mediated by these non-viral gene delivery vectors has to be improved, especially in vivo, to benefit fully from their advantages. Cationic lipid/nucleic acid complexes or lipoplexes have been the subject of intensive investigations in recent years to understand the parameters governing the efficiency of transfection. Specifically, the comprehension of such mechanisms, from the formation of the complexes to their intracellular delivery, will lead to the design of better adapted non-viral vectors for gene therapy applications. Here, we will discuss some recent developments in the field on the structure/function relationship of cationic lipids in the mechanism of transfection, and where appropriate, we will make a comparison with mechanisms of viral and polyplex-mediated gene delivery. Cationic lipids are often used in combination with helper lipids such as DOPE or cholesterol. The effect of DOPE on lipoplex assembly and the relevance of the structural properties of the lipoplexes in destabilizing endosomal membranes and mediating endosomal escape of DNA will be discussed.
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Introduction

Over the last decades, the principle of gene delivery for therapeutic purposes has been well established. However, several shortcomings in the development of devices employed for effective delivery have frustrated ground breaking progress in this area [1]. Thus, although viral vectors, compared to non-viral vectors such as lipoplexes and polyplexes, are far more efficient in bringing about cellular transfection, innate immunity issues compromise their productive use in vivo [2]. Additional hazards include mutational insertion risks, potentially leading to oncogenicity [3,4], while large scale production of viral vectors as such might also constitute an obstacle. Although being less efficient, particularly in vivo [5], both lipoplexes, consisting of a complex of nucleic acids and cationic lipids, and polyplexes, composed of cationic polymers and nucleic acids, are thought to be immunologically inert, and potentially more safe than viral vectors for in vivo use. Since they are also relatively easy to produce and to modulate chemically for improvement of transfection efficiency, research efforts in this particular area have drastically increased in recent years [6]. Thus, numerous cationic devices have been synthesized and for rational development structurally modified in a systematic manner in order to correlate structure with transfection activity. In addition, non-viral vector performance might also be optimized by their targeting to particular cell types and into a distinct cellular internalization pathway, taking into account the possibility that not every pathway may be equally effective in releasing DNA into the cytosol, an important step in the eventual expression of the genes [7-10]. Indeed, a number of potentially rate limiting steps in the process of non-viral-mediated gene delivery have been identified, which include the efficiency of cell surface association, internalization, release of genes from intracellular compartments such as endosomes, transfer via the cytosol to and translocation into the nucleus and transcription efficiency. Insight into molecular features of each of these steps is essential in order to determine their effectiveness as a barrier and to identify means of overcoming these hurdles. From such studies it is also becoming apparent that it is important to obtain quantitative insight into these processes such as, for example, concerning the amount of DNA present in endosomes, following internalization of non-viral vectors by endocytosis, the efficiency of actual escape of the genes and the number of copies that arrive and are expressed in the nucleus. Only such knowledge will provide an exact appreciation of the relevance of each of the supposed (intra)cellular barriers. Thus one of the reasons for a low gene transfer efficiency by cationic lipids has been suggested to relate to insufficient
DNA protection against intracellular nucleases [11]. However, by using a novel strategy based on a combined approach of PCR and confocal-image-assisted three dimensionally integrated quantification (CIDIQ), Hama et al. [12], were able to obtain quantitative insight into the amount of DNA present in the endosomal track relative to that in the nucleus, and their study suggested that transcription rather than delivery might be a crucial rate limiting step in non-viral gene delivery.

Size and charge of the non-viral vector are important parameters but their precise role remains unclear. Often, larger particles show a higher transfection efficiency than smaller particles, which has been attributed to an enhanced sedimentation of the former onto the cells [13]. However, others [14] have shown that lipopolyplexes (consisting of a mixture of cationic lipid and polymers) of medium size particles with diameters of 140-220 nm may transfect cells as effectively as 1 µm large aggregated lipopolyplexes. Whether this difference reflects differences in complex stability and/or size-driven differences in cellular processing remains to be determined, but better insight into such parameters is essential for the rational development of non-viral vectors and improvement of their transfection efficiency. Here we will briefly review some (recent) work in the field of lipoplex-mediated gene delivery to accomplish that goal. In particular we will focus on in vitro studies and limit this overview to lipoplex assembly and structure, and highlight some aspects of their mechanism of cellular internalization and the intracellular dissociation of nucleic acid from the lipoplex, a prerequisite for eventual delivery to the nucleus for transcription. For in vivo application, the reader is referred to some recent excellent reviews in this area, as published elsewhere [15,16].

**Cationic lipids - properties and structure**

Cationic lipids are amphiphilic molecules, implying that they consist of a hydrophilic and a hydrophobic region, i.e., a (charged) cationic (amine) headgroup, attached via a linker (for example glycerol) to a usually double hydrocarbon chain or a
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cholesterol derivative (Fig. 1). An important property of the amphiphile, with regard to its application as a vector, is its geometry. Like any amphiphile, when suspended in an aqueous environment, cationic lipids can adopt various structural phases, including the micellar, lamellar, cubic and inverted hexagonal phase (Fig. 2). The type of structure can be predicted by a factor known as the packing parameter, P. This packing parameter, $P = \frac{v}{a l_c}$, is defined as the ratio of the hydrocarbon volume, $v$, and the product of the effective head group area, $a$, and the critical length of the lipid tail, $l_c$ [17]. In short, the correlation emphasizes the

Figure 1. Chemical structures typical of some cationic lipids used in transfection: DOTAP, SAINT-2, DC-Chol and GS1. DOTAP contains a quaternary ammonium polar head group, a glycerol linker, and two oleyl chains as hydrocarbon tail. SAINT-2 consists of a pyridinium base with a quaternary amine whose cationic charge is delocalized over the pyridinium ring; it has two oleyl chains as hydrophobic tail. DC-Chol is typified by a charged head group attached to cholesterol as hydrophobic tail. GS1 is a sugar-based Gemini surfactant, which consists of two single-tail surfactants with a reduced glucose and a pH dependent amino moiety in the headgroup and an oleyl chain as hydrocarbon tail linked via an ethylene oxide spacer.
relevance of the ratio of the area occupied by the hydrophobic region versus that of the hydrophilic region (Fig. 2). Thus, when \( P \) exceeds the value of 1, i.e. the area occupied by the hydrocarbon chains is much larger than that of the head group, the lipid tends to adopt the inverted hexagonal phase, which in essence is a bilayer destabilizing structure, and as will be discussed below, adoption of this phase appears an important step in the mechanism by which genes or oligonucleotides (ODNs) are delivered into the cytosol, following lipoplex internalization. For transfection application, cationic lipids are often mixed with so-called helper lipids, like dioleoylphosphatidylethanolamine (DOPE) or cholesterol, both lipids potentially promoting conversion of the lamellar lipoplex phase into a non-lamellar structure, which presumably rationalizes their ability to often improve cationic lipid mediated transfection efficiency.

The structure of cationic lipids is readily amenable to chemical modification [18] allowing attachment of other functional groups like polyethylene glycol (PEG; [19-21]), thus conveying so-called stealth properties to cationic vectors, which precludes their rapid

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**Figure 2. Schematic representation of the phase structure of cationic lipids as a function of their packing parameter.** The geometry of the amphiphile, defined by the packing parameter \( P \), determines the organization of self-assembled cationic lipids. For \( P < \frac{1}{2} \) it is predicted that a cone-like shaped monomer will assemble into structures with positive curvature corresponding to a micellar phase (spherical micelles, wormlike or cylindrical micelles or non-inverted hexagonal \( H_I \) phase). When \( P \) is between \( \frac{1}{2} \) and 1 the curvature of the self-assembled amphiphiles is close to 0 leading to the lamellar phase (bilayers). For \( P > 1 \) the structures formed display a negative curvature leading to inverted phases (inverted micelles or inverted hexagonal \( H_{II} \) phases). For further details, see text.
elimination from the blood circulation by macrophages, upon injection in vivo [22]. By attachment of sugar residues, sugar-linked biosurfactants have been prepared, thereby providing targeting properties to particular cellular receptors, thus promoting cell type-dependent vector specificity and a potential enhancement of its cellular internalization. Thus inclusion of a sugar-linked biosurfactant into dimethylaminoethane carbamoyl cholesterol (DC-Chol)/DOPE based lipoplexes improved the transfection efficiency of the lipoplex due to an enhancement of cell attachment and subsequent internalization [23]. It also follows that an enhanced internalization in this case presumably also led to an increase in gene delivery to nucleus, but it should be stressed that both events are not necessarily directly related [24]. Importantly, the presence of such functional groups may preclude extensive interactions between lipoplexes, thus avoiding particle clustering. As a result, the average diameter of the ensuing lipoplex particles will be smaller and may be differently processed by cells than larger complexes, as will be discussed below.

When knowledge advanced that endocytosis played a major role in the mechanism of lipoplex-mediated gene delivery, acidification of the endosomal compartment has been exploited to design specific cationic lipid systems that will only be destabilized at acidic pH, thus providing pH-sensitive drug delivery vectors facilitating endosomal escape. These systems are assembled from pH sensitive cationic lipids like DC-Chol or sugar-based gemini surfactants [25-29]. Gemini surfactants are obtained by connecting two single-tailed surfactants via a spacer at the level of the headgroups (Fig. 1). They usually posses a lower critical micellar concentration and a higher ability to decrease the surface tension of water, when compared to the parent surfactant.

The mildly acidic properties of endosomes have also been exploited to eliminate the interference of PEG-linked lipid analogues, intercalated into lipoplexes for the purpose of conveying stealth properties, with lipoplex-endosomal membrane interaction. Thus PEGylated derivatives have been synthesized in which the PEG moiety is cleaved off when exposed to mildly acid pH. Accordingly, at neutral pH these compounds will stabilize lipoplexes by precluding particle clustering, which would be detrimental for in vivo application, and/or interaction with serum proteins which precludes rapid elimination by macrophages. However, once in the endosomal compartment, the analogue will be cleaved off and released when exposed to the mildly acidic environment, thus allowing destabilization of the lipoplexes, necessary for disruption of the endosomal membrane and concomitant dissociation and release of the plasmid (see below and [30]).
With the aim of exploiting the mechanism of polyplex-mediated gene delivery, more recently a polycationic sphingolipid analog, containing a spermine as its headgroup has been synthesized. Interestingly, it was shown that the capacity of this compound in delivering ODNs into cells, as reflected by an efficient anti-bcl-2 antisense effect, was superior to that of vectors prepared from DOTAP or DC-Chol [31]. However, in comparing amphiphiles per se, it is also relevant to take into account a number of other determining parameters, such as concentration, charge ratio, and size of the liposomes used for lipoplex preparation, and the size of the assembled complex, which are also of importance, emphasizing the complexity of defining proper conditions for optimizing gene and/or ODN delivery.

**Interaction of DNA with cationic lipid vesicles: Lipoplex assembly**

Lipoplexes are self-assembling nanosystems and their formation entails a multistep mechanism as revealed by detailed studies employing monolayers, atomic force microscopy and cryo electronmicroscopy [32-34]. In a millisecond first step electrostatic interactions between phosphate (DNA) and the positively charged amine headgroup of the cationic lipid occur, the one-sided DNA-liposome surface interaction presumably giving rise to packing constraints in the bilayer, the defects triggering extensive interactions between adjacent bilayers as reflected by extensive lipid mixing [33-35] and concomitant release of vesicle contents, indicating the rupture of the membrane structure [32-34,36]. Rupturing presumably causes exposure of hydrophobic edges that may serve as nucleation sites for interaction with similar adjacent structures leading to further membrane merging, lipid mixing and aggregate growth. Compared to addition of DNA to vesicles, the reverse slows down the complex assembly process, indicating that DNA-mediated vesicle-vesicle interaction is instrumental in destabilization rather than DNA-vesicle interaction per se [33,34]. Eventually, the cationic lipids are wrapped entirely around the plasmids and the surfaces of the assembled complex show a smooth appearance implying proper DNA packaging. At that stage, the tendency of adjacent complexes to further undergo extensive lipid mixing largely ceases [32,33], since potential interaction sites are blocked.

Obviously, effective DNA compaction and elasticity of the cationic lipid for packaging is important. In fact, those amphiphiles that are easily hydrated, form fluid aggregates, and undergo a transition to the inverted hexagonal phase in the presence of plasmid DNA at physiological ionic strength are often most favorable for obtaining effective transfection
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[37]. In terms of chemical structure of the cationic lipids, these requirements involve the presence of unsaturation in the alkyl or acyl tails and a chain length of 16-18 carbon atoms. Saturated long tails often display relatively strong attractive intermolecular interactions, accompanied by a low propensity for hydration and mixing with helper lipids like DOPE. The introduction of double bonds leads to less compact crystal packing and consequently to easier dispersal in water, and the increase in the volume of the acyl chains favors the H_{II} phase (c.f. Fig. 2). Thus such parameters should be considered when synthesizing and applying particular amphiphiles. Hydrocarbon tail length and saturation will thus affect lipoplex intradynamics and thereby the packing efficiency of the DNA. Rigid assemblies of cationic lipids preclude efficient recruitment of cationic lipid by the DNA, causing DNA aggregation of larger particles to sizes of several microns, resulting in lower transfection efficiency, presumably caused by a reduced internalization of complexes by the cells.

Counterion release from cationic lipid and DNA, a concomitant decrease in hydration and the resulting increase in entropy is the driving force for assembly of the lipoplex [34,38]. DOPE decreases the surface potential in many cationic lipid systems [39] and causes a more ready release of counter ions from the lipid surface by DNA [40] and its presence diminishes the state of hydration of the lipid surface, properties that are also instrumental in promoting the lamellar to hexagonal phase change [17,41]. An enhanced ionic strength will reduce the extent of counter ion release from DNA and cationic lipid, and thus affect the intimacy of DNA-cationic lipid interaction, which decreases [34,39]. It is therefore anticipated that an increase in ionic strength will have a pronounced effect on the morphology of the aggregates. During the electrostatic interaction between the positively charged vesicles and the negatively charged phosphate groups, the cooperative collapse of the DNA structure occurs, known as condensation or compaction, in such a way that the DNA is effectively shielded by the lipids.

Apart from the physicochemical properties of the amphiphile, there are a number of parameters that determine the final outcome of the assembly process in terms of lipoplex structure and stability, which obviously translate into transfection capacity of a given lipoplex. Thus, the nature of the initial liposome preparation and plasmid size may determine overall transfection efficiency by modulating lipoplex instability defects [31] and lipoplex size [36,42]. Also the role of inclusion of helper lipids in cationic vectors is of importance. DOPE, which in isolation prefers the inverted hexagonal phase, is not required for lipoplex assembly but this lipid may strongly facilitate this process, as opposed to the bilayer structure preferring (saturated) dipalmitoylphosphatidylethanolamine (DPPE), which
negatively interferes with the wrapping of DNA into such complexes [43]. Specifically, plasmid DNA injected into the subphase of mixed cationic lipid/DPPE monolayers remains attached to the monolayer, while lipoplexes form, and dissociate into the subphase when the plasmid interacts with cationic lipid/DOPE monolayers, indicating that in the former case the plasticity of the DPPE-containing monolayer is insufficient to wrap around the plasmid structure. Thus charge and membrane flexibility in terms of structural dynamics of the PE species drive the packaging of DNA into a lipoplex.

**Structural phases of lipoplexes; role of DOPE**

DOPE profoundly affects the polymorphic features of lipoplexes in that it may promote the transition from a lamellar to a hexagonal phase, and its presence causes neutralization of cationic charges by the negatively charged phosphodiester of DOPE [39,44-46]. This feature, in conjunction with the presence of NaCl, presumably brings about a decrease of the surface potential of the complexes, thereby facilitating intermembrane interactions, leading to phase changes. In fact, the presence of DOPE in lipoplexes may lead to a looser binding of the cationic lipid to the DNA, compared to DOPE-devoid complexes, as reflected by ethidium bromide intercalation into the DOPE-containing complexes even upon complete DNA binding [47], and the direct binding of DOPE to DNA [40] likely plays a role in this regard. This molecular parameter will thus be important for DNA dissociation and hence eventual transfection efficiency.

Lamellar phase forming cationic lipids do not necessarily undergo an inverted transition when DOPE is included in such bilayers. However, upon charge neutralization by NaCl or upon ion pairing with anionic phospholipids such as phosphatidylserine (PS), thereby decreasing the mean surface charge of the membrane, a marked enhancement in non-lamellar phase forming propensity is observed, even though both PS and the cationic lipid per se might be lamellar phase forming lipids [41,43,48].

Interestingly, plasmid is usually not needed for the transition of the lipid phase of the vector from a lamellar to a hexagonal phase, i.e., an increase in ionic strength to physiological values often suffices to promote the H$_{II}$ phase. However, recently certain 1,4-dialkylpyridinium amphiphiles [47], which display a very large surface area of the hydrophobic tails giving rise to a $P \gg 1$, have been shown to adopt a cubic phase prior to their transition to a hexagonal H$_{II}$ phase, which only occurs after DNA complexation.
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Binding of DNA generally affects the main phase transition temperature of the carrier mixture and the mixing with the helper lipid.

Cationic complexes are usually prepared at low ionic strength which avoids their clustering seen upon mixing the complexes at physiological conditions. Indeed, in water, SAINT-2/DOPE complexes exist in a lamellar phase, as revealed by SAXS and NMR measurements. However, when suspended in a salt solution of physiological concentration, the system converts quantitatively from a lamellar $L_{\alpha}$ to a thermally highly stable non-lamellar $H_{II}$ phase [37,46], in which there is a homogeneous mixing of the unsaturated cationic lipid and DOPE. Eliminating head group repulsion of the cationic heads by charge screening at physiological ionic strength, will increase the packing parameter $P$, thus resulting in the observed shift to the hexagonal phase. Interestingly, decreasing the mole ratio of DOPE from 50 to 20 mol % leads to the formation of the more strongly curved cubic phase in this cationic lipid system [37], emphasizing that DOPE is instrumental in promoting the transition to the hexagonal phase of the lipoplex, the cubic one being a transition stage from the lamellar to the hexagonal $H_{II}$ phase [49].

As noted above, the rate of lipoplex assembly is affected by the order of addition of DNA and liposomes which may be of consequence for the eventual efficiency of transfection. Thus, the kinetics of complex formation may bring about the coexistence of structures with different sizes, possibly reflecting differences in particle stability which in turn could be a reflection of different structural phases. Different coexisting phases may also arise when cationic lipids and helper lipids poorly mix, which often results in assembly of poorly stabilized complex with low transfection efficiency [34,47].

More importantly and less well addressed is the issue whether the kinetics and efficiency of complex assembly could affect the structural integrity of the plasmid DNA and thereby transfection efficiency. Such effects could be two-fold. Improper packaging may relate to inefficient wrapping of plasmid into the cationic lipid shell, causing protrusions that affect the interaction of lipoplexes with cell surfaces [24]. In addition, it is not unlikely that the transcription quality of the gene has become greatly diminished under such circumstances. In this context, it is becoming apparent that condensation and compaction may well be as yet poorly characterized but decisive factors, particularly in light of a recent report [12] in which viral versus non-viral gene delivery were compared. In this study, a high lipoplex-mediated delivery of genes to the nucleus was accomplished, compared to an adenovirus system, yet a very poor transcription was obtained in the former case. It remains to be determined whether DNA conformational defects could be attributed to the poor
lipoplex performance. However, a variety of structural conformational changes of plasmid DNA have been noted such as a change from native B-DNA into highly condensed ψ-DNA in cationic [50] and Z-DNA in ternary anionic lipoplexes [51]. The precise effects of such changes on DNA dissociation from lipoplexes, their transport into the nucleus and transcription efficiency require further investigations.

Having thus identified a number of parameters that influence lipoplex assembly and structural features of the eventually formed complex, the next issue is how these features may affect initial processing of lipoplexes by the cells, taking into account the various steps that are relevant to ultimate transfection.

**Mechanism of lipoplex-mediated transfection**

*Endocytosis of lipoplexes*

The initial event in the transfection process, i.e. lipoplex - cell surface interaction, is driven by electrostatic interactions, unless the lipoplex contains a specific targeting compound, which is specifically recognized by a cell surface receptor. However, to completely eliminate the dominant charge driven interactions of lipoplexes, it is important to take appropriate precautions that preclude such non-specific cell surface interaction, requiring a diminishment of the charge ratio and the presence of PEG-analogues that convey stealth properties to the complex [21,52-56]. Importantly, the phase of the lipoplexes is not relevant to internalization as lamellar lipoplexes are as efficiently internalized as those adopting the HII phase [43]. Rather, lamellarity would favor the stable protection of DNA and from that point of view might be advantageous.

As revealed by electron and fluorescence microscopy, following an incubation with cultured cells, lipoplexes can be detected in intracellular vesicles beneath the cell membrane [57,58], suggesting that lipoplexes enter cells by endocytosis (Fig. 3). Eukaryotic cells exploit various endocytic pathways involving clathrin-mediated endocytosis via coated pits, or endocytic internalization independent of clathrin, which includes phagocytosis, macropinocytosis and caveolae-mediated endocytosis [59-61]. The relative contribution of either pathway in lipoplex internalization has been poorly defined [62-65]. Except for uptake in specialized cells, like macrophages, monocytes and neutrophils, phagocytosis is likely to be excluded as a significant mechanism of lipoplex uptake in normal cells. Recently, the potential entry of octaarginine modified liposomes via macropinocytosis has been described
Intracellular delivery of genes [66], yet little is known thus far as to the relative importance of this pathway in transfection mediated by cationic lipoplexes. However, clathrin-dependent endocytosis of lipoplexes leading to transfection has been demonstrated for several cationic lipid systems and for different cell types. Thus an inhibition of transfection is seen upon treatment of the cells with specific inhibitors of the clathrin-mediated pathway such as chlorpromazin, [67,68], but more convincingly, also overexpression of a dominant negative mutant of Eps15, a protein necessary for the formation of coated pits, leads to an effective inhibition of internalization of the lipoplexes and hence transfection efficiency [67]. Moreover, filipin III a specific inhibitor of caveolae-mediated endocytosis, only slightly (10-20 %) reduced transfection efficiency [67], suggesting that in this case (transfection of COS7 or HepG2 cells by SAINT-2/DOPE lipoplexes) entry via caveolae-mediated endocytosis is not a major pathway. Interestingly, using cytochalasin B as inhibitor, such a caveolae-mediated pathway was
suggested to represent a prominent pathway of entry for human serum albumin coated
DOTAP/DOPE lipoplexes [69]. This pathway may well have been triggered by an effect of
albumin in targeting the lipoplexes to caveolae, since human serum albumin can trigger
transcytosis across capillary endothelial cells via a caveolar pathway [70]. Importantly, for
all such studies it is very relevant to directly correlate the pathway of entry with transfection
efficiency in order to appreciate the relative contribution of a given pathway to productive
transfection.

Depending on the endocytotic machinery of the cell it can be expected that nature and
efficiency of endocytosis of lipoplexes can be cell type dependent. An important factor in
determining the nature of the entry pathway by endocytosis can also be the size of the
lipoplexes. The effect of different inhibitors and cholesterol depletion on the internalization
of fluorescent beads of various sizes by non phagocytic B16 cells was shown to depend on
the size of the particles [71]. The particles of sizes up to 250 nm were preferably and almost
exclusively endocytosed via clathrin coated pits, while particles of 500 nm were endocytosed
via caveolae. However, while polyplexes of sizes exceeding 500 nm, like latex beads of such
a size, may be internalized by caveolae [68], no data have been reported thus far as to
whether such a pathway is also operating for a size dependent internalization of lipoplexes.
On the other hand, as we discussed elsewhere [65], proper quantitation of different endocytic
pathways for internalization and in particular their relative contribution to eventual
transfection, has been scanty thus far [64,72].

**Release of lipoplex cargo from endosomes**

Once in the endocytic pathway, the plasmid may become degraded when reaching the
lysosomes. Accordingly, for productive transfection the plasmid needs to acquire cytosolic
access at an earlier stage, presumably by escape from (early) endosomal compartments. In
this process, the hexagonal structure of the lipoplex is thought to play a crucial role, and in
this regard a parallel can be drawn with the mechanism by which adenovirus particles
transfect cells. This virus, also a popular tool in gene delivery, enters cells via clathrin-
mediated endocytosis and macropinocytosis [73] and the actual escape into the cytosol
involves lysis of the endosomal membrane structure [74]. Evidently, the lipoplex lacks a
protein machinery to destabilize the endosomal membrane. However, under conditions
where inverted and micellar structures could arise or integrate within a membrane, including
the endosomal membrane, destabilization of that membrane structure likely occurs. Such an
interaction between the bilayer-departed lipoplex structure with the endosomal membrane
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could thus promote gene translocation. Among others, this is dictated by the notion that those lipoplexes adopting such phases in isolation are most effective in transfection [45,46,75]. However, mechanism and molecular details of the process itself are largely lacking because of difficulties in properly simulating such an event in vitro. Yet, an important role of the phospholipid phosphatidylserine (PS), localized in the outer leaflet of the endosomal membrane has been proposed in this process, initially as a means of releasing DNA from the lipoplex, following its flip-flop to the inner leaflet of the endosomal membrane, thus allowing its interaction with the net positively charged lipoplex [11]. More recently a prominent role of PS in (further) promoting hexagonal phase formation by ion pairing with cationic lipids in lipoplexes, even in those that adopt a lamellar phase in isolation, has also become apparent [41,43,48]. PS is not likely promoting the hexagonal phase by undergoing a lamellar to hexagonal transition itself, as this only occurs below pH 4.0, a pH the lipid is not likely to encounter in eukaryotic cells [76]. Rather, mixtures of cationic lipids and anionic phospholipids can adopt an inverted H\textsubscript{II} phase that can be rationalized by molecular shape arguments, electrostatic interactions neutralizing charges and repelling hydration and hence decreasing monolayer curvature [41,48]. Importantly, these data also imply that a lamellar complex may only be converted to a non-lamellar hexagonal phase within cells, following translocation of acidic phospholipids into the complex. This process of phase conversion from a lamellar to a non-lamellar phase, which may include the hexagonal H\textsubscript{II} or H\textsubscript{I} phase as well as the micellar cubic phase, also seems to be instrumental in causing the efficient dissociation of the DNA from the lipoplex, necessary for release into the cytosol [29,43,75,77]. Interestingly, the stability of such phases may possibly depend on the nature of the acidic phospholipid and cationic lipid involved [75]. This was suggested by revealing a stable highly curved micellar cubic phase, induced in lipoplexes consisting of a cationic phospholipid analogue, upon their interaction with phosphatidylglycerol or cardiolipin-containing bilayers. At these conditions, the highest DNA dissociation was obtained as opposed to a lower dissociation upon interaction with PS-containing bilayers, which showed a transient formation of the micellar cubic phase. Similarly, cubic phase adopting cationic lipid systems expressed the highest transfection activity as opposed to mixtures giving rise to less curved phases such as the hexagonal H\textsubscript{II} phase.

A tight intermembrane interaction between lipoplex surface and endosomal membrane is crucial for effective nucleic acid release into the cytosol. Thus PEG-lipid analogues, included in lipoplexes to avoid early capture by macrophages upon in vivo application,
should dissociate from the complex, once localized within the endosome, which is accomplished by including either exchangeable PEG-lipid analogues [20,21,53,56] or cleavable, pH-sensitive PEG analogues, as described above. Presumably, the PEGylated analogues stabilize the lamellar phase by sterically interfering with apposed intermembrane interactions, needed for structural conversion to the hexagonal phase. In this context, it would also appear necessary to design targeting devices on lipoplexes such that they do not interfere with close intermembrane interactions, once the complex reaches the endosomal compartment. By way of example, Igarashi et al., recently accomplished targeting of lipoplexes that contained sugar-derivatized amphiphiles [23]. These lipoplexes were smaller than ligand-devoid lipoplexes (170 vs 270 nm), suggesting less clustering (like PEG-stabilization of lipoplexes) and, accordingly, were internalized more rapidly and to a higher extent than control complexes, consistent with a size-dependent processing. However, like stabilized PEGylated lipoplexes, these sugar-derivatized lipoplexes showed a relatively poor delivery capacity, in spite of improved targeting, as the nucleus was poorly accessed by the ODNs [23], suggesting a poor escape from the endosomal compartment, presumably due to their interference with the membrane destabilization induced by lipoplex-endosomal membrane interaction.

In an interesting study Schwille and colleagues [78] recently monitored the interaction of lipoplexes with giant unilamellar vesicles (GUV), using laser scanning imaging in conjunction with fluorescence cross-correlation analysis. The GUV system, with vesicle diameters that may vary between 5 and 50 µm, provides the unique opportunity to directly visualize its membrane for structural examination during intermembrane interactions, for example in case of membrane fusion with reconstituted membrane vesicles [79], or as occurs during endosome-lipoplex interaction. Interestingly, binding of DOTAP/DOPE lipoplexes to GUVs composed of PS/PE/phosphatidylcholine induced vesiculation at the surface of the GUV, but no rupture of the vesicles was observed. Importantly, vesiculation might be indicative of lipid translocation to the outer leaflet of the bilayer [80], as has been proposed to occur for PS translocating from outer to inner leaflet within the endosomal compartment [11]. During lipoplex-GUV interaction, polynucleotides with a length of 66 base pairs were effectively delivered inside the GUV, delivery being enhanced when the GUV contained increasing levels of PS and DOPE, i.e., lipids which are facilitating release (PS) of nucleotides by charge neutralization of the cationic lipid and destabilize membranes (PE) by promoting hexagonal phases. Moreover, by fluorescence cross correlation spectroscopy diffusion rates of the polynucleotides were obtained consistent with diffusion of
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polynucleotides that were no longer associated with the lipoplex, suggesting that lipoplexes per se were not delivered into the GUV via a fusion event of the outer layer of the lipoplexes, ejecting the remainder into the GUV lumen, thereby paralleling a mechanism that has been proposed to occur in cells [77]. Evidently, this system may be of great value as a mimic in further characterizing and understanding the nature of the interaction of lipoplexes with endosomal target membranes. By employing asymmetrically labeled GUV, exploiting properties of fluorescently tagged PS, direct evidence for PS translocation might be obtained, while labeling of the lipid phase of lipoplexes could provide further insight into the role of membrane fusion in DNA release, DNA translocation across a perturbed bilayer and/or issues of ‘escape’ of cationic lipids/lipoplexes into the cytosol. Also, thus far this work has only been carried out with polynucleotides, rather than with plasmids, thus issues of efficiency of nucleic acid translocation might be similarly mimicked in this system.

In this context it also of interest to note that membrane insertion of a mixture of cationic lipid (SAINT-2) and DOPE followed by a brief pulse of ultrasound allowed, in a time-restricted manner, the passage of an artificial chromosome with a size of approx. 1 µm in diameter across the plasma membrane of cultured cells [81]. Since insertion of the cationic lipid alone was ineffective, these data were taken to suggest that the cationic lipid /DOPE mixture destabilized the plasma membrane of cells, presumably relying on the ability of the mixture to adopt the hexagonal phase, thereby providing a transient pore that allowed the passage of the artificial chromosome, while subsequent lateral diffusion determined the life span of the process. However, whether transient pores are also instrumental in plasmid release from endosomes, or whether the endosome is completely ruptured, remains unclear. Yet, the fact that additional tools that promote destabilization of the endosomal membrane, such as pH-sensitive polymers or peptides [82-84] with a detergent like mechanism of action, or osmotic rupture of endosomes [43] promote transfection would indicate that efficient escape of plasmid from endosomes represents a major hurdle in lipoplex-mediated gene delivery.

Given the relevance of the lipoplex phase structure on overall transfection efficiency, it is finally of interest to briefly consider the potential role of exogenous factors such as serum on such properties. Serum has been shown to diminish or even completely eliminate cation-lipid-mediated gene or ODN delivery [24,36,85,86]. Negatively charged serum components may simply bind to the lipoplex surface and thereby interfere with the efficiency of cell surface interactions. In fact, binding of albumin can induce charge reversal of the complex [87]. Also, it has been proposed that serum may penetrate into lipoplexes, and interfere with
the packaging of DNA causing unfolding of the nucleic acid and its protrusion at the lipoplex surface thus potentially interfering with lipoplex-cell surface interaction [24]. However, given the relevance of the structural phase of lipoplexes, it is also possible that serum could interfere with lipoplex polymorphism. In this regard, it has been recently shown [88] that lipoproteins such as LDL and HDL effectively interact with lipoplexes consisting of vectamidine or DOTAP, as a result of which lipoprotein lipids may mix with the cationic lipids, as revealed by a lipid mixing assay. Moreover, although lysoPC, due to its positive bilayer curvature inducing properties (see Fig. 2, P < 1/2 ) effectively inhibited lipid mixing, lipoplexes containing this lipid transfect cells as efficiently as lysoPC-devoid lipoplexes. In case non-bilayer phases would be important for release of DNA from endosomes, this observation is somewhat puzzling, as cone-shaped lysoPC would stabilize the bilayer structure, unless the lyso-lipid dissociates from the complex, once localized in the endosomal compartment. Whether this occurs, will require further work and is of obvious relevance in the context of optimization of lipoplex formulations.

**Concluding remarks and perspective**

In recent years considerable progress has been made in defining conditions that favor or promote lipoplex-mediated transfection, particularly with regard to the structural features of the lipoplex/cationic lipid per se. It is thus becoming apparent that such structural features, i.e., non-lamellar phases including cubic and hexagonal H_{II} phases, in conjunction with cellular lipids such as PS, and perhaps PG, play a major role in destabilizing intracellular membranes, which allow the escape of nucleic acids into the cytosol. Whether endosomes are the sole intracellular organelles involved remains to be determined, as well as whether lipoplexes can be targeted into other internalization pathways such as via caveolae, which would avoid their capture into lysosomal compartments and thus possibly improve chances for and efficiency of nucleic acid escape into the cytosol. With regard to advancing our insight into the molecular mechanisms involved in membrane destabilization, nucleic acid dissociation and release of DNA into the cytosol, further progress might benefit from novel model systems such as the use of giant unilamellar vesicles, but other more biologically relevant model membranes (erythrocyte ghosts) could be equally relevant. These systems should also be helpful in clarifying as to whether or not part of the lipoplex itself is released into the cytosol, accomplished via initial fusion of its outer membrane boundary. This aspect will be relevant to a better understanding of intracellular dissociation of the nucleic acid
cargo, an important aspect of overall transfection. Indeed, once in the cytosol, the plasmid or ODN requires transfer to and translocation into the nucleus for transcription or blockage of mRNA processing, respectively. Whereas oligonucleotides acquire readily and efficiently access to the nucleus (see e.g. [62]), which given the nuclear pores and ODN size is not unexpected, the efficiency of plasmid translocation is generally thought to be extremely poor (see for example [72]) and efforts to improve this include coupling to nuclear localization signals. Indeed, in this context it is generally assumed that the efficiency of release of DNA from endosomes represents a major hurdle in gene delivery. Accordingly, alternative entry pathways via targeting or an improved efficiency of endosomal release, aided by pH sensitive devices such as polymers or peptides linked to lipoplexes, would be directions for optimizing delivery. However, very recently Hama et al. [12] made a comparison between the tranfection efficiency of adenoviral and lipoplex systems and determined by a novel quantitative fluorescence microscopical approach in conjunction with PCR, that DNA release via endosomes into the cytosol occurred as efficiently via lipoplexes as it did via adenoviral particles. Remarkably, about 3 orders of magnitude more intracellular gene copies per cell were needed when delivered by lipoplexes (Lipofectamine plus) in order to accomplish a comparable level of gene expression when compared to the number of copies delivered by adenovirus. Similarly, the efficiency of transcription for polyplex delivered DNA has been shown to be much less efficient than that obtained via viral particle-mediated gene delivery [89]. Thus, the superior transfection efficiency of viral genes, in spite of an orders of magnitude lower delivery efficiency, suggests that viral factors (proteins) may positively affect transcription by modulating efficiency and/or (nuclear) stability. Obviously, these kinds of factors are lacking in case of cationic lipid delivered plasmid DNA. Thus, these studies not only emphasize the need for better quantitation of delivery, but also that transcription efficiency and not necessarily differences in intracellular trafficking could be a major hurdle in lipoplex-mediated gene delivery. This issue thus raises questions of the relevance of employing nuclear localization signals per se to promote nucleic acid delivery to the nucleus. Rather, issues related to the effect of cationic lipids on compaction and condensation of genes, their stability in the cytosol and ensuing effects on transcription efficiency, which include effects of DNA condensation on intra-nuclear transcription [90] need to be included in future studies that aim at improvement of cationic lipid-mediated gene delivery.
Chapter 2

Abbreviations

DNA, deoxyribonucleic acid; ODN, oligonucleotides; PEG, poly(ethylene glycol); PEI, polyethylenimine; lysoPC, lysophosphatidylcholine; DPPE, 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine; DOPE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; DOTAP, N-[1-(2,3-dioloyl)propyl]-N,N,N-trimethylammonium chloride; SAINT-2, N-methyl-4-(dioleyl)methylpyridinium; PS, 1,2-dioleoyl-sn-glycero-3-[phospho-L-serine]; DC-Chol, 3β-[N-(N''-dimethylaminoethane)-carbamoyl] cholesterol; GUV, giant unilamellar vesicles; P-NMR, phosphorus nuclear magnetic resonance; P, packing parameter; L_{α}, lamellar phase; H_{II}, inverted hexagonal phase; H_{I}, non-inverted hexagonal phase.

References

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