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REVIEW

Gene delivery by cationic lipid vectors: overcoming cellular barriers

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Abstract Non-viral vectors such as cationic lipids are capable of delivering nucleic acids, including genes, siRNA or antisense RNA into cells, thus potentially resulting in their functional expression. These vectors are considered as an attractive alternative for virus-based delivery systems, which may suffer from immunological and mutational hazards. However, the efficiency of cationic-mediated gene delivery, although often sufficient for cell biological purposes, runs seriously short from a therapeutics point of view, as realizing this objective requires a higher level of transfection than attained thus far. To develop strategies for improvement, there is not so much a need for novel delivery systems. Rather, better insight is needed into the mechanism of delivery, including lipoplex–cell surface interaction, route of internalization and concomitant escape of DNA/RNA into the cytosol, and transport into the nucleus. Current work indicates that a major obstacle involves the relative inefficient destabilization of membrane-bounded compartments in which lipoplexes reside after their internalization by the cell. Such an activity requires the capacity of lipoplexes of undergoing polymorphic transitions such as a membrane destabilizing hexagonal phase, while cellular components may aid in this process. A consequence of the latter notion is that for development of a novel generation of delivery devices, entry pathways have to be triggered by specific targeting to select delivery into intracellular compartments which are most susceptible to lipoplex-induced destabilization, thereby allowing the most efficient release of DNA, a minimal requirement for optimizing non-viral vector-mediated transfection.

Keywords Cationic lipid · Phase structure · Cellular transfection · Endocytosis

List of Abbreviations

DOTAP	*N*-(1-(2,3-dioleoyloxy)propyl,*N*,*N*,*N*-trimethylammonium chloride)
DOPE	1,2-dioleoyl-sn-glycerol-3-phosphatidylethanolamine
DOTMA	*N*-(1-(2,3-dioleyloxy)propyl)-*N*,*N*,*N*-trimethylammonium chloride
HSPG	Heparin sulfate proteoglycans
PEI	Polyethylenimine
SAXS	Small angle X-ray scattering
NMR	Nuclear magnetic resonance
SAINT-2	*N*-methyl-4(dioleyl) methylpyridiniumchloride

Introduction

Cationic lipids (Niculescu-Duvaz et al. 2003), such as DOTMA (Felgner et al. 1994) and its ester analogue DOTAP (Simberg et al. 2004), or SAINT-2 (van der Woude et al. 1997), are amphipatic structures that in several respects display physicochemical properties, which are also commonly observed for natural phospholipids.

Dedicated to Prof. K. Arnold on the occasion of his 65th birthday.

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Thus when dispersed in an aqueous solution they spontaneously form bilayers, the core structure of membranes. Polymorphic lamellar-to-non-lamellar transitions of cationic lipid-containing membranes can occur, depending on composition and environmental factors, such as salt and temperature, and these features are instrumental in biologically relevant processes like membrane fusion or other (transient) membrane-perturbing processes that often depend on a dynamic interplay between lipids and proteins. Evidently, cationic lipids, usually bearing a charged amine group, differ in charge from natural phospholipids which are either zwitterionic or (net) negatively charged. As such, liposomes prepared from cationic lipids readily interact with negatively charged compounds. Thus when mixed with nucleic acids, i.e., entire genes or oligonucleotides [including antisense oligonucleotides or small interference RNA (siRNA)], so-called lipoplexes are formed instantaneously (ms scale), driven by electrostatic interaction forces. Importantly, in contrast to such complexes formed with liposomes prepared from phospholipids, which relies on entrapment rather than that effective electrostatic interactions between DNA and bilayers occur, lipoplexes display the capacity to relatively efficiently deliver genes or nucleotides into cells, a feature that likely relates to the aforementioned polymorphic properties. Hence, from both a fundamental perspective, i.e., as a model for understanding membrane dynamics, and the application point of view, i.e., in the case of gene delivery for therapeutic or cell biological reasons, vectors based upon cationic lipids are of interest. Here, we will focus on recent progress made in understanding mechanisms relevant to their capacity as delivery vehicle, a feature that may also inherently improve our insight into the dynamics of biological membranes, acting as lipoplex targets. Where relevant for advancing insight into cationic lipid-mediated gene transport, a comparison will be made with current views on the mechanisms by which gene complexes prepared from polymers, the so-called polyplexes, enter cells. Progress discussed here is largely based on in vitro studies. However, from a therapeutic point of view such in vitro studies have their own merit for ex vivo gene therapy, in which patient-derived cells are transfected in vitro followed by their re-implantation. Furthermore, in vitro experiments are indispensable in optimizing lipoplex formulations for in vivo application relying on systemic administration, for example in terms of targeting, lipoplex stability and delivery efficiency, but evidently not all parameters can be appropriately simulated, including e.g. effects of serum. For more detailed accounts of in vivo application, the reader is referred to reviews published elsewhere (Audouy and Hoekstra 2001; Conwell and Huang 2005; Dass and Choong 2006; Huynh et al. 2006; Evans et al. 2006).

Interaction of lipoplexes with the cell surface and initial processing

Since extensive mixing of lipids occurs when cationic lipid vesicles are mixed with DNA, and since this process was taken as a reflection of the occurrence of membrane fusion, it was initially thought that lipoplexes readily delivered their DNA into cells by a fusion event between lipoplexes and the plasma membrane (for a review and references: Zuhorn and Hoekstra 2002). However, more recently it has been firmly established that endocytosis acts as the major path of entry, as revealed and inferred from data that include evidence from electron microscopy (Friend et al. 1996; El Ouahabi et al. 1997), the use of mutant cell lines defective in endocytosis (Zuhorn et al. 2002a), the role of temperature dependence (endocytosis being effectively inhibited at temperatures below approximately 15°C), and the application of a host of inhibitors (although one should be aware of toxic side effects in this case, often leading to a contradictory outcome) and, much more reliable, co-localization experiments with markers for labeling-specific endocytic compartments (for recent reviews: Medina-Kauwe et al. 2005; El Ouahabi and Ruysschaert 2005; Khalil et al. 2006). From a mechanistic point of view, endocytosis, rather than fusion at the plasma membrane is of obvious advantage for lipoplex-mediated transfer of the gene since it carries the plasmid nearer to the site where its transcription and replication occurs, i.e., the nucleus, thereby also shortening the plasmid's residence time in the cytosol thus avoiding inactivation by cytosolic nucleases or otherwise. Importantly, this mechanism also allows easy passage of barriers like cortical actin filaments, localized just underneath the plasma membrane.

The extent to which cellular parameters affect the efficiency of interaction of lipoplexes with cell surfaces, as the first step in entry, is of obvious relevance for optimizing target properties of lipoplexes and/or exploiting cellular properties of a given cell type. Indeed, it has been well established that cell type-dependent differences in transfection efficiency are obtained, which, in part, have been attributed to effects of cell cycle and cell division frequency, but also to endocytic capacity (Audouy and Hoekstra 2001;

Simberg et al. 2004). However, whether specific cellular molecules are involved in the processing of lipoplexes in terms of trafficking is still largely obscure. Clearly, consistent with a requirement for a net positive charge of a lipoplex for productive transfection, electrostatic interactions are involved in the initial binding of lipoplexes to the cell surface, such interactions (and hence, eventual transfection) being essentially negligible for "neutral" lipoplexes (Lenssen et al. 2002; Wasungu et al. 2006b).

The potential involvement of "specific" cell surface receptors in lipoplex–cell interaction has been poorly addressed thus far, although it is apparent that cell surface molecules like (negatively charged) heparin sulfate proteoglycans (HSPG), which mediate the entry of pathogens (Sawitzky 1996; Finlay and Cossart 1997; Dehio et al. 1998), can also facilitate internalization of cationic lipo- and polyplexes (Mislick and Baldeschwieler 1996; Kopatz et al. 2004; Simberg et al. 2004). Interestingly, these membrane-anchored proteins, which function as specific growth factor receptors, also physically interact with cytoskeletal components, thereby conveying cytoskeletal reorganization properties to HSPGs that are of potential relevance for endocytic internalization of pathogens (Freissler et al. 2000; Pelkmans and Helenius 2003), and a similar mechanism was recently proposed for cellular entry of polyplexes (Kopatz et al. 2004).

Negatively charged proteoglycans are likely, but presumably not exclusively, involved in the binding of lipoplexes (and polyplexes). This may be inferred from the notion that it is not a single, but rather a variety of endocytic pathways that have been claimed to be involved in lipoplex internalization, including phagocytosis (which is, however, a rare event in cells other than professional phagocytes such as macrophages), macropinocytosis, clathrin-mediated and non-clathrin-mediated endocytosis, and entry via caveolae (for a recent review see Khalil et al. 2006). Importantly, these pathways need not necessarily be mutually exclusive and in that regard it will be important to precisely define which entry pathway is actually involved in mediating productive transfection, i.e., gene expression. Thus (the extent of) internalization per se does not necessarily correlate with transfection efficiency (Zuhorn et al. 2002b), and not every pathway may necessarily lead to productive transfection, as recently suggested for polyplex-mediated transfection (Rejman et al. 2005).

Once bound to the cell surface, lipoplex internalization is not instantaneous, as is commonly observed for endocytosis of single, molecular ligands. This suggests that additional events may be required such as receptor-driven clustering, reflected by surface aggregation of polyplexes (Godbey et al. 1999) and lipoplexes (Rejman et al. 2004a), into particular lateral domains of the plasma membrane, capable of invagination, like clathrin coats or caveolae, or at domains enabling membrane ruffling to engulf particles for internalization via macropinocytosis. These "additional events" should also be considered in the context of the often relatively large size of the lipoplexes, which may vary between 150 and 1,000 nm that needs to be internalized. In that sense it is quite remarkable that the clathrin-mediated pathway is capable of internalizing lipoplexes with an average size of 150–200 nm (Zuhorn et al. 2002a) or polyplexes of similar size (Grosse et al. 2005), taking into account the restricted size of "regular" clathrin-coated vesicles, which is around 100 nm, imposed by the likely relative rigidity of the clathrin basket. Similarly, it is puzzling how to reconcile that polyplexes with a size as large as 700–800 nm can be internalized via caveolae, thus reaching caveosomes (Rejman et al. 2005), whereas the size of the omega-shaped invaginations seen on the cell surface are often about an order of magnitude less in diameter. Indeed, uptake of smaller polyplexes (<100 nm) via this pathway (Grosse et al. 2005) would be more consistent with caveolar vesicle size. Nevertheless, the cell surface seems to display a considerable degree of flexibility, and thus appears to be capable to adjust and extend existing machineries, such as caveolae- and clathrin-mediated endocytosis, for particle size-dependent engulfment and internalization (Rejman et al. 2004b). Possibly, this may involve a merging of several microdomains, harboring the required machinery and normally operating for processing of smaller, molecular entities. Indeed, although caveolae are often visualized on the cell surface as flask-shaped invaginations with a diameter of 50–100 nm, flattened caveolin domains may act as precursor domains for such invaginations (Gumbleton et al. 2003). In fact, analogies may exist with clathrin domains where substrates bound to distinct receptors are clustered in such domains, as observed for caveolae-mediated internalization of Simian Virus 40 (SV40; Pelkmans et al. 2001; Empig and Goldsmith 2002), followed by signal-driven invagination. Thus, the internalization of caveolae is a triggered event, in which a substrate like a lipoplex or polyplex could interact with a lipid raft component, moving laterally along the plasma membrane until trapped in caveolae domains in a size determining manner. In the case of SV40 internalization, actin is recruited to the virus-loaded caveolae (Pelkmans et al. 2001) from where it radiates tail-like wise, which seems important for the mechanism of invagination. In this regard, a gradual electrostatic zippering of the plasma membrane onto lipoplexes or polyplexes, as recently

proposed (Kopatz et al. 2004), mediated by a lateral diffusion and clustering of particular HSPGs in the lateral plain of the plasma membrane, is reminiscent of such a mechanism. As noted, clustering of several of such domains may thus be required for processing of large lipoplexes or polyplexes, which is not unprecedented as suggested by the entry of (opsonized) bacteria and viruses along the same pathways (Shin et al. 2000; Shin and Abraham 2001). In fact, within cells cavernous structures of interconnected caveolae, which may bud as such from the plasma membrane, have been noted (McIntosh et al. 2002).

Evidently, to further clarify the internalization mechanism, it will also be necessary to take into account the extent to which the cationic charges themselves may govern invagination, for example by modulating plasma membrane curvature, a parameter that obviously will facilitate internalization (Hagerstrand et al. 1999), and in which cholesterol, its depletion eliminating invagination, may play a role as a (co-)regulator of membrane curvature (Rodal et al. 1999; Huttner and Zimmerberg 2001; Zuhorn et al. 2002a), i.e., different from its role as raft stabilizer (Kopatz et al. 2004).

Targeting of lipoplexes, involving coupling of specific antibodies or specific ligands directed toward cell surface receptors potentially provides options to improve delivery (Medina-Kauwe et al. 2005 and references therein). However, as noted, delivery per se needs not necessarily improve transfection implying that targeting devices directing lipoplexes into a distinct and in terms of transfection, most susceptible endocytic pathway may be more beneficial. Furthermore, lipoplex charge often obscures the specificity of such interactions, requiring additional precautions to accomplish receptor specificity, for example by charge diminishment in conjunction with inclusion of PEGylated lipid derivatives that may convey stealth properties to such complexes (Harvie et al. 2000; Song et al. 2002; Shi et al. 2002; Rejman et al. 2004a). In addition, it is not always apparent that receptor-mediated endocytosis of a given ligand (e.g. transferrin) will also lead to effective endocytosis of the same ligand when coupled to a particle, orders of magnitude larger in size, which as such may be handled differently by cells (cf. Simoes et al. 1999).

Lipoplex entry versus transfection efficiency

Although binding of lipoplexes to the cell surface is an obvious prerequisite for subsequent internalization, the process as such is not predictive for transfection efficiency, neither needs this to be the case for the efficiency of lipoplex internalization per se. Intriguingly, more recently evidence has become available that might suggest that the nature of the internalization pathway may determine transfection efficiency, implying distinct properties of intracellular compartments that allow faster or slower release of plasmid across their membranes, which furthermore seems to depend on the physicochemical properties and possibly the size of the delivery vehicle, although the correlation as such, being of great potential interest (Rejman et al. 2004b), is still obscure (cf. Fig. 1). For example, Rejman et al. (2005) have reported that PEI polyplexes can be internalized by both clathrin and caveolae-mediated mechanisms. Intriguingly, inhibitor studies revealed that only the caveolae-mediated pathway led to productive transfection. The particle size in this study varied between 200 and 800 nm and the smaller particles may have entered the cells by clathrin-mediated endocytosis, although Grosse et al. (2005) noted that particularly ligand-derivatized PEI, in contrast to unsubstituted complexes, enter via this pathway. The separate effectiveness of transfection of the PEI particles along this pathway was not established, although a diminished nuclear gene delivery via this route was suggested. However, particles with sizes exceeding 200 nm transfected cells following internalization via macropinocytosis, rather than via a caveolae-mediated pathway (Grosse et al. 2005). Whether derivatization of the polymer or cell type differences may underlie the differences in internalization pathway in either study remains to be determined, but evidently, a pathway-dependent transfection susceptibility is highlighted, and the efficiency of particularly larger PEI particles in bringing about transfection would be in agreement with previous observations (Ogris et al. 2001b).

Careful analyses of DOTAP lipoplex data have revealed that in fact size is more critical to transfection efficiency than lipoplex charge or the zeta potential, and in analogy to size-dependent effects of polyplex transfection efficiency, for DOTAP lipoplexes 500 nm particles were shown to be more effective in transfecting cells in culture than 100 nm lipoplexes (Fig. 1; Kawaura et al. 1998; Trubetskoy et al. 2003; Simberg et al. 2004). Interestingly, by preparing DOTAP lipoplexes at different cationic lipid/DNA ratios, Sakurai et al. (2000) obtained particles of different sizes displaying differences in transfection efficiency, the highest activity occurring with lipoplex particles of the largest diameter (approximately 1,000 nm) in caveolae-enriched HUVEC cells, when compared with the other cell lines. However, no evidence was provided in these

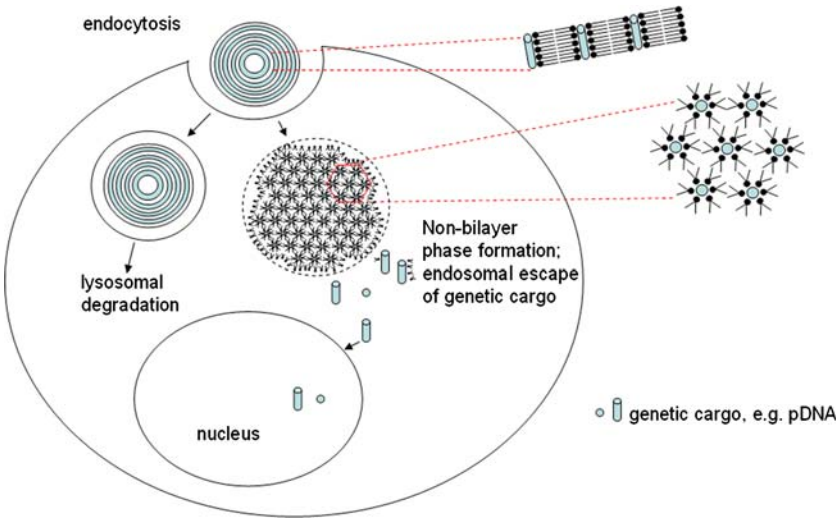

Fig. 1 Cellular processing of lipoplexes by endocytosis. Following attachment to the cell surface, lipoplexes are internalized by endocytosis, the efficiency of which is not necessarily dependent on the structural phase of the lipoplex (Zuhorn et al. 2002b), which can be lamellar, with DNA sandwiched between the lamellae, or hexagonal (H_{II}), the latter phase being required for efficient release of the DNA from an (early) endosomal compartment. Note that given the average size (approximately 200 nm) of lipoplexes, only a few of such complexes likely tightly fit within an endosomal compartment. Intermingling of the lipid phase of the lipoplex with (cellular) acidic phospholipids within this compartment can (still) trigger or further promote a non-lamellar transition within the lipoplex, allowing endosomal membrane destabilization and concomitant release of the genetic cargo. Smaller complexes and those of which the lamellar phase is stabilized are transfection-wise less productive, as the majority of these complexes will reach the lysosomes, prior to the effective release of DNA. Following release into the cytosol, DNA is transferred to and into the nucleus, needed for eventual transcription and translation. For further details see text

studies as to the exact intracellular localization of these complexes. Nevertheless, these data would argue in favor of better defining the potential role of size in governing the route of cellular entry of lipoplexes, i.e., the nature of the intracellular compartment for nucleic acid cargo escape and thereby potential transfection efficiency.

With increasing charge ratio (+/−), the size of the lipoplexes decreases, and the particles are more stable (less contents are released from initial liposomes) (Simberg et al. 2004). For instance, for lipoplexes such as DOTAP/DOPE and SAINT-2/DOPE the highest instability is expected at a charge ratio of 2:1, just above neutrality, where the highest transfection efficiency of these complexes is seen (Simberg et al. 2004; van der Woude et al. 1997). Instability is reflected by particle size, usually growing with increasing instability, which, in turn, is often a reflection of the polymorphic dynamics of the lipid phase (Shi et al. 2002), and, as will be discussed below, likely plays an important role in destabilizing the endosomal membrane. Thus size, known as an important parameter in transfection (Kircheis et al. 2001) but not properly defined thus far, may trigger uptake via a distinct transfection-efficient pathway, while size may also be a reflection of structural instability, needed for perturbing intracellular membrane barriers to allow cytosolic escape of DNA.

However, it is apparent that no consensus has been reached yet with regard to the size-dependent entry of DNA complexes, but the appreciation of its role is often complicated by subtle, yet apparently highly relevant changes in the chemical nature of a given compound (Grosse et al. 2005; Mennesson et al. 2005). Thus, whereas PEI, substituted with a lactosyl group primarily enters the clathrin-coated pathway, unsubstituted PEI does not (Grosse et al. 2005). Using linear PEI, Mennesson et al. (2005) produced particles with a size of 250–300 nm in salt-free media, the uptake of which was partly inhibited upon blocking clathrin-mediated endocytosis, the efficiency being, moreover, dependent on cell type. Since no direct correlation was established between the pathway of entry and transfection efficiency, it is difficult to precisely determine, whether the clathrin-dependent pathway was also transfection effective in this case.

Taken together, these studies underscore that if further insight is to be obtained into the mechanism of transfection by non-viral vectors with the aim of improving therapeutic impact, it is essential to better define the correlation between size and internalization mechanism. This insight requires a careful *quantitative*

analysis of the mechanism of entry, defining the relative contribution of each pathway as such, rather than conclusions that rely on the involvement of "a major pathway" in lipoplex (or polyplex) uptake. In addition, a given pathway of entry and ensuing transfection efficiency by non-viral vectors requires verification in several cell types in order to appreciate the general significance. Finally, instructive in this context could also be some recent observations on the intracellular uptake of differently sized and shaped colloidal gold particles, the kinetics of which were shown to be highly dependent on the physical dimension of the nanoparticles (Chithrani et al. 2006), spherical particles being internalized better than rod-shaped particles. This further suggests that a careful control of particle shape and size of poly- and lipoplexes, allowing correlations to be drawn between size and shape, pathway of entry and the efficiency of transfection accomplished via a given pathway, will be highly desirable in the rational development of gene delivery devices.

Lipid shape and lamellar-hexagonal transitions

To clarify the mechanistic role of cationic lipids in gene delivery, apart from their role in serving as an efficient template for lipoplex assembly, an understanding of their properties in terms of the so-called packing parameter P has proven highly useful. P is defined as the ratio of the cross-sectional areas of the hydrophobic hydrocarbon chains and the polar head group (Israelachvili et al. 1980). Cationic lipids with a relatively small head group and unsaturated alkyl- or acyl hydrocarbon chains, occupying an extended area in a wedge-like manner, have a tendency to adopt the inverted hexagonal H_{II} phase. A similar cone-shaped structure is preferred by the phospholipid DOPE ($P > 1$), as opposed to the lamellar L_α structure, adopted by most other phospholipids species, which show a cylindrical shape ($P \approx 1$). In contrast, micellar (non-inverted) structures are preferred by lipids with a head group that occupies a relative large surface area as compared to the cross-sectional area taken by the hydrophobic chains ($P < 1$), such as for example in the case of lysophospholipids.

DOPE is often included in cationic lipid mixtures as a so-called "helper lipid" because in the case of many but not all cationic lipid systems, inclusion of DOPE has been shown to promote transfection efficiency. Above pH 9.0, DOPE is negatively charged and adopts a lamellar phase, but when lowering the pH to near neutral or acidic pH (Siegel and Epand 1997) the lipid becomes zwitterionic. Thus, around neutral pH, when $P > 1$, DOPE in isolation undergoes a lamellar to H_{II} phase transition, which occurs at temperatures above 10–15°C.

Cationic lipids like DOTAP and SAINT-2 prefer a bilayer organization. However, mixing with DOPE will affect the packing parameter and when this additive average packing parameter becomes sufficiently large (>1), the system will revert to an hexagonal phase as observed for DOTMA (Mok and Cullis 1997), DOTAP (Koltover et al. 1998; Simberg et al. 2001) and SAINT-2 (Smisterova et al. 2001) when mixed with an equimolar amount of DOPE. Within the hexagonal phase adopting lipoplexes, lipid-coated DNA strands are arranged on a hexagonal lattice, contrasting the sandwich organization of DNA between lipid bilayers for lipoplexes in a lamellar organization (see Fig. 1; Koltover et al. 1998).

The efficiency of DOPE to promote the hexagonal phase in cationic lipid containing systems may depend on conditions. Thus for the cationic lipid SAINT-2, which is in the lamellar phase at pH 7.4 in water, addition of DOPE facilitates the conversion to an H_{II} phase, which is further promoted when the system is suspended in a physiological salt solution (Smisterova et al. 2001). In this context, it is also relevant to note that inclusion of other lipids, such as PEGylated lipid analogues, in cationic lipid/DOPE mixtures may strongly affect lamellar to hexagonal phase transitions. Thus with increasing head group size, relative low amounts of PEG-lipids (2–3 mol %) already suffice to stabilize the bilayer structure (Johnsson and Edwards 2001), presumably by short-range interference of bilayer–bilayer interactions, necessary for triggering the transition from the lamellar to hexagonal phase. This approach is often taken to "stabilize" lipoplexes, providing stealth properties that preclude (extensive) clustering of the complexes, which is obviously undesirable for in vivo application of these devices.

Intra-endosomal events necessary for nucleic acid release into the cytosol

Once lipoplexes have been internalized and thus arrive in an endosomal (or caveolar) compartment (Fig. 1), two essential processes have to occur: (i) the dissociation of the nucleic acid from the complex and (ii) the destabilization of the endosomal (caveolar) membrane to allow the release of the nucleic acid into the cytosol, an essential step in making the plasmid accessible for transport to the nucleus for transcription. Which cellular components are involved in these steps? And which molecular properties of lipoplexes could facilitate

these events? Clearly, several studies indicate that inclusion of the helper lipid DOPE or cholesterol triggers or promotes the transfection potency of lipoplexes (Farhood et al. 1995; Hirsch-Lerner and Barenholz 1999; Hafez and Cullis 2001; Smisterova et al. 2001; Zuhorn et al. 2005), an effect which appears inherently related to their ability to facilitate a conversion of the lipoplex structure to adopt an hexagonal H_{II} phase. Moreover, ion pairing of negatively charged cellular lipids, like PS, with cationic lipids in lipoplexes also strongly promotes the formation of the hexagonal phase of lipoplexes (Lewis and McElhaney 2000; Hafez et al. 2001; Zuhorn et al. 2002c). Of interest, the presence of the lamellar phase preferring DOPC instead of DOPE in cationic lipid/PS mixtures inhibits transition to the H_{II} phase, i.e., apparently prevents effective ion pairing of the cationic lipid and PS (Hafez et al. 2001). However, simultaneous inclusion of DOPE in such mixtures triggers again the H_{II} phase, emphasizing the strong facilitating role of DOPE in H_{II} phase formation. Similarly, when lipoplexes are mixed with PS/PE/PC-containing liposomes an H_{II} conversion can be detected by both SAXS (Zuhorn et al. 2002b) and NMR (Hafez et al. 2001). In this context it should be noted that although PS itself can undergo an L_{α} (lamellar) to H_{II} (hexagonal) phase transition, the required pH at which this event occurs is too low (<4.0; Hope and Cullis 1980) to be of significance in the case of lipoplex-mediated transfection. Another important role for PS stems from in vitro studies, which revealed that interaction of PS-containing lipid vesicles with lipoplexes causes the efficient dissociation of nucleic acid from the complexes, presumably due to competition between PS and nucleic acid for binding with the cationic lipid (Xu and Szoka 1996); see further below). To envision a role of PS in endosomal escape of lipoplex-delivered DNA, it is furthermore relevant to bear in mind that at the plasma membrane, aminophospholipids, including PS and PE, are restricted to the cytoplasmic leaflet of the bilayer, the asymmetry being maintained by an ATP-dependent aminophospholipid translocase activity. Whether and how PS acquires access to the luminal lealflet of the endosomal membrane has not been experimentally resolved thus far. Nevertheless, its potential translocation into and within the lipoplex may readily occur when cationic lipid/DOPE domains that have adopted the hexagonal phase interact with endosomal membranes (cf. Hoekstra and Martin 1982), while at the same time the reverse process, i.e., cationic lipid and DOPE intermingling with endosomal membrane domains may similarly take place (Fig. 1). The relevance of this intermingling for obtaining transfection is suggested by observations on the interaction of lipoplexes, labeled with the fluorescent lipid analogue N-Rh-PE. Thus, laterally diffuse Rh-fluorescence is only present in endosomal membranes of cells that were also efficiently transfected, whereas Rh-fluorescence appeared as patches, in association with the endosomal membrane, in cells where uncondensed DNA protrudes from the lipoplex surface, preventing intimate contact between lipoplexes and endosomal membranes, emphasizing the need for such interactions between lipoplex and endosomal membrane in bringing about translocation of the plasmid across a perturbed or disrupted endosomal membrane (Zuhorn and Hoekstra 2002).

Although the role of DOPE in controlling lipid/DNA phase behavior by virtue of its ability to promote/facilitate non-lamellar phase formation is highly relevant for eventual transfection, it is also evident that the degree of phase conversion is not necessarily related to that measured of the complexes per se. Thus, such a transition may also be triggered upon interaction at the level of endosomes following mixing with cellular lipids such as PS. Hence lipoplexes, displaying after preparation a predominant lamellar phase, may adopt (partially) a hexagonal phase following their interaction with PS-containing lipid vesicles (Hafez et al. 2001; Zuhorn et al. 2002c, 2005), thus fulfilling criteria, minimally required for DNA release/translocation.

Besides in complex assembly with nucleic acids and association with the cell surface, the charge of the cationic lipids also plays a role in the destabilization of the endosomal membrane. It has been calculated that a lipoplex of 90 nm contains sufficient cationic charges to form ion pairs with all the anionic lipids in the endosomal membrane and to induce non-bilayer structures (Lewis and McElhaney 2000). Lipoplexes with a size between 200 and 400 nm, and a cationic lipid to DNA charge ratio ≥ 2, are generally most effective in mediating transfection (Zhdanov et al. 2002; Rakhmanova et al. 2004; Tarahovsky et al. 2004). Lipoplexes with this size, stretching the limit of clathrin-mediated endocytosis (Rejman et al. 2004b) totally fill up an endocytic vesicle (Zuhorn et al. 2002a). This occupation of space will obviously promote intimate intermembrane contact, thereby facilitating lipid mixing and exchange (Fig. 1). An additional advantage of this "tight fitting" of lipoplexes within endosomal compartments could be that it may slow the kinetics of processing along the endocytic pathway, thus potentially expanding the time span for accomplishing endosomal destabilization and/or rupture, necessary to translocate DNA into the cytosol. Likewise, transferrin-PEI complexes with

a size of 500 nm were shown to efficiently mediate transfection (Ogris et al. 2001b), while relatively small lipoplexes (Zuhorn et al. 2002b) and polyplexes (Rejman et al. 2004a; Grosse et al. 2005; Mennesson et al. 2005) are relatively rapidly transported to lysosomes. Possibly, the tight electrostatic interactions of lipoplexes or polyplexes with endosomal membranes precludes membrane folding necessary for pinching off vesicles from the filled endosomal compartments thereby impeding rapid processing into a lysosome-directed pathway toward the perinuclear region of the cell (Fig. 1).

In addition to the cationic lipid/DNA charge ratio, the membrane charge density of the lipid component (i.e., the percentage of cationic lipid relative to total lipid) can also be a critical parameter in lipoplex-mediated transfection (Lin et al. 2003, Ahmad et al. 2005). Using newly multivalent lipids, an optimal membrane surface charge in lamellar lipoplexes, prepared from these lipids, was shown to result in transfection efficiencies that were compatible to those obtained for H_{II} lipoplexes (DOTAP). Thus, at low membrane charge density, a low transfection efficiency was observed and the authors (Ahmad et al. 2005) suggested that lipoplexes remained trapped in the endosome, while at an intermediate charge density efficient endosomal escape and release of DNA occurs. However, at high membrane charge density, endosomal escape is efficient, yet DNA is not released from the complex, resulting in suboptimal transfection efficiencies. These data were taken to suggest (Ahmad et al. 2005) that following their first encounter with the endosomal membrane, the lamellar lipoplexes will release only one layer of DNA, the remaining lamellae of the lipoplexes (at optimal membrane surface charge density) being slowly dismantled by sequential peeling upon their interaction with (macro)molecules in the cytoplasm, thereby releasing their DNA layer by layer. However, such a mechanism ignores the fact that by ion pairing of cationic and (cell-derived) anionic lipids, non-bilayer phases are readily formed (Lewis and McElhaney 2000; Hafez et al. 2001). In fact, it has been shown that a lamellar lipoplex, as determined in isolation, may revert to a hexagonal phase upon interaction with anionic lipids, and mediates the translocation and subsequent nuclear accumulation of oligonucleotides as efficiently as lipoplexes, which adopt a hexagonal phase upon (initial) lipoplex assembly (Zuhorn et al. 2002b). Accordingly, these data strongly suggest that no predictions can be made on the structural phase of a lipoplex, solely based upon its structure observed immediately following assembly, i.e., prior to its interaction with (endosomal) target membranes. Thus once internalized in cells, it is not apparent that a lamellar complex will maintain its lamellar phase. Nevertheless, these data also revive the issue as to the extent to which cationic lipids per se may become exposed to cytosolic structures, as it is generally thought that in contrast to polyplexes, the lipidic part of lipoplexes does not cross endosomal membranes.

Fusion, endosomal membrane destabilization or endosomal rupture?

An intriguing issue is whether during or upon H_{II} phase formation, membrane fusion is of relevance in the process of DNA translocation across the endosomal membrane. In this context, it has been sown that when the membrane charge density of oppositely charged vesicles is sufficiently high, such that it results in high adhesion strength and flattening of the vesicles, the vesicles will fuse (Lei and MacDonald 2003). Also, PS exposure on the cell surface of erythrocytes (by inhibition of flippase activity), or HeLa cells and Jurkat T cells (by treatment of the cells with pro-apoptotic agents, causing the flop of PS) was shown to induce lipid mixing between lipoplexes and cell surface, leading to transfection in the latter case (Stebelska et al. 2005, 2006). The latter observation implies that the lipid asymmetry in unperturbed, natural membranes apparently prevents intimate interactions between lipoplexes and plasma membrane, necessary for destabilization and DNA translocation, although as mentioned before, a priori direct translocation of plasmid at the plasma membrane into the cytosol might not be the most effective path of DNA entry due to the presence of a cytoskeletal barrier. Yet, these experiments support the notion of a potential role of PS in membrane destabilization (as reflected by lipid mixing) and plasmid translocation (as reflected by ultimate transfection). Possibly, loss of ATP-ase activity at the endosomal level may similarly result in randomization of anionic lipids, facilitating membrane charge fluctuations, and consequently promote lipid mixing. However, to claim fusogenic properties upon H_{II} conversion solely based on lipid mixing is unjustified. As noted, H_{II} phases readily and effectively transfer lipids between bilayers without the occurrence of genuine fusion (Hoekstra and Martin 1982). Thus, membrane destabilization need not necessarily be the equivalent of membrane fusion.

A priori, in cell-free systems membrane fusion, defined as the concomitant mixing of lipids and entrapped contents, of lipoplexes is difficult to establish because of a lack of a well-defined membrane-bounded

aqueous space (Hafez et al. 2001; Simberg et al. 2004), let alone to reveal fusion at the level of cells. As others and we have argued and summarized elsewhere, there is no apparent correlation between the extent of fusion, based on lipid mixing, and transfection efficiency (Zuhorn and Hoekstra 2002; Simberg et al. 2004). Moreover, membrane fusion is very much dependent on the physical properties of the contacting membranes and has been proposed to proceed via a stalk as an intermediate structure (Zimmerberg and Chernomordik 1999). According to this concept, the two apposing monolayers of fusing membranes form a highly bent semi-toroidal lipid structure and the geometry of this structure favors inclusion of lipids which exhibit negative bilayer curvature such as DOPE, thereby promoting membrane fusion. Consistently, fusion is strongly inhibited by lipids like lysoPC ($P < 1$), adopting a micellar phase and exhibiting positive membrane curvature, which is not compatible with the configuration of the stalk structure. Accordingly, one would anticipate that micellar phase preferring amphiphiles would be inhibitory to transfection. Intriguingly, when such systems are mixed with DOPE, a molecular shape compatible with a cylindrical shape is obtained and it would thus be anticipated that transfection is inhibited, which is indeed observed (Wasungu et al. 2006b). One would therefore also anticipate that micellar H_I phase adopting amphiphiles in the absence of DOPE, like pH-sensitive gemini surfactants (Bell et al. 2003; Wasungu L. et al. 2006a) by virtue of an inherent lack of fusion susceptibility, should be highly inefficient transfectants. This appears not to be the case and they transfect cells as efficiently as H_{II} phase adopting complexes (Wasungu L. et al. 2006a). Thus, their capacity to adopt a non-bilayer phase, thereby destabilizing and perturbing a lamellar phase seems more relevant than so-called "fusion-promoting" H_{II} conditions. Similarly, Ewert et al. (2006) recently reported the ability of lipoplexes consisting of newly multivalent lipids and DOPC to adopt a cubic (H_I) phase, capable of bringing about efficient cellular transfection.

Whether entire lipoplexes escape from endosomes is unclear, although some authors have reported the cytosolic localization of such complexes by electron microscopy (Zhou and Huang 1994) or fluorescence microscopy of tagged lipoplexes (El Ouahabi et al. 1999); yet, the resolution is often too low to firmly establish the appearance of lipoplexes per se, free in the cytosol. A fusogenic mechanism could, however, lead to a potential role of intracellular structures other than endosomal compartments in releasing plasmid from lipoplexes, such as cytoskeletal elements. But also in this case, it will be difficult to establish whether this "release" actually resulted from a fusion event or that it was due to complete or partial endosomal rupture.

Endosomal rupture by osmotic shock enhances transfection efficiency of cationic formulations with relatively poor non-bilayer phase forming capacity (Zuhorn et al. 2005). Less rigorous approaches like the application of molecular devices that promote destabilization of the endosomal membrane are also beneficial, such as pH-sensitive polymers or peptides (Plank et al. 1994; Simoes et al. 1999; Wagner 1999; Ogris et al. 2001a; Li et al. 2004; Kakudo et al. 2004). These data would suggest that complete rupture of endosomes is likely not occurring and/or necessary for plasmid release as such, although obviously, complete rupture would improve the potential extent of cytosolic delivery. In fact, the ability of a plasma membrane inserted mixture of the cationic lipid SAINT-2 and DOPE, capable of maintaining the opening of an ultrasound-induced pore for a restricted period of time to allow passage of an artificial chromosome, would be compatible with a "channel" function rather than a lytic activity (Oberle et al. 2004).

Acidic pH and plasmid release from intracellular compartments

Acidification of lipoplexes within the endosomal compartment is not an obvious prerequisite in a model of hexagonal phase-mediated membrane destabilization, which may involve charge neutralization via ion pairing of cationic and cellular acidic lipids. Thus, except for pH sensitive amphiphiles, synthesized and employed for that particular purpose, most cationic lipid formulations, with or without helper lipids, do not require a mild acidic pH, neither for interacting with the endosomal target membrane nor for actual release of DNA. Accordingly, endosomal escape of genetic cargo does not depend on a drop in pH, implying that gene delivery as such does not necessarily depend on lipoplex transport through a compartment of mildly acidic pH. Indeed, observations on transfection, mediated after complex internalization by caveolae, must occur in a pH-independent manner, given that the pH in caveosomes is thought to be (near) neutral (Parton and Richards 2003; Pelkmans and Helenius 2003). If polyplexes of certain sizes follow this pathway and moreover show a transfection dependency of such a pathway of entry (Rejman et al. 2005), this would thus raise intriguing questions on their mechanism of membrane destabilization, which in caveosomes must then underlie a mechanism that differs from that of

acidification in conjunction with an osmotic sensitive step. This latter mechanism is know as "the proton sponge effect" and considers polymers like PEI to express endosomolytic activity (Boussif et al. 1995) upon lowering of the pH in endocytic compartments. The mildly acidic pH will cause an enhanced protonation of the nitrogen atoms in the polymer, thus creating a charge gradient, which induces Cl^- influx. The increased Cl^- concentration triggers water influx, presumably leading to endosomal swelling and rupture. However, the effectiveness of the buffering capacity of PEI in the context of its ability to efficiently rupture endosomal membranes has been questioned (Sonawane et al. 2003; Forrest and Pack 2002); yet, the following considerations may be of interest in this regard. As noted above, glycosaminoglycans (GAGs) are potential binding sites for lipoplexes and/or influence lipoplex trafficking (Mislick and Baldeschwieler 1996; Wiethoff et al. 2001; Ruponen et al. 2001). It is therefore possible that following internalization, GAGs could affect lipoplex properties and thereby cationic lipid-mediated transfection. Ruponen et al. (2001) showed that cationic lipid/DOPE systems are relatively unaffected by exogenously added GAGs. However, carriers with buffering capacity, such as the cationic polymer PEI are sensitive to their presence. Cationic polymers, unlike cationic lipids, do not contain a hydrophobic moiety, and endosomal escape is not mediated by lipid mixing underlying mechanisms as in the case of cationic lipoplexes. Possibly, intimate contact between the polymer and the glycocalix might displace an amount of bound water, sufficient to cause a certain degree of local dehydration leading to a membrane instability, which could eventually allow complex release. In fact, Bieber et al. (2002) have demonstrated that local membrane damages can be induced at the plasma membrane by large polyplexes, without a need for their internalization by cells. Alternatively, it cannot be excluded that cationic polymers per se induce the H_{II} phase structure, for example when interacting with anionic lipids like PS (cf., de Kruijff and Cullis 1980). These alternative mechanisms are thus becoming particularly relevant when envisioning mechanisms of release, such as those via caveosomes, that cannot rely on a mild acidic pH drop, relevant to the proposed "proton sponge" mechanism for polyplex entry.

Dissociation of plasmid from lipoplexes

The occurrence of hexagonal phase formation is not only of relevance in the context of endosomal membrane destabilization, as discussed above. Rather, data also suggest its role in promoting release of DNA from lipoplexes, if not a prerequisite for efficient endosomal escape into the cytosol (Shi et al. 2002; Zuhorn et al. 2005). This can be inferred from observations in which it was shown that incorporation of only a few mol % of a PEGylated lipid analogue into lipoplexes suffices to convey stealth properties to such particles, implying that their stability in vivo is enhanced by precluding interaction with serum proteins, thereby prolonging their blood circulation time and precluding scavenging by macrophages. Also in vitro, their colloidal stability is greatly improved. However, quite surprisingly, for many of such lipoplexes, prepared at a charge ratio of 2–3 (+/−), the particles still interact electrostatically with the cell surface (Harvie et al. 2000; Shi et al. 2002) and are efficiently internalized in the endosomal compartment. Interestingly, however, the inclusion of 2–3 mol % PEGylated lipids into lipoplexes that otherwise rapidly adopt the hexagonal H_{II} phase suffices to stabilize their lamellar phase (Johnsson and Edwards 2001; Shi et al. 2002), and only after dissociation of the (exchangeable) PEGylated lipids by monomeric transfer from the lipoplex, conversion of the lamellar to the H_{II} phase occurs, which is accompanied by release and subsequent nuclear delivery of nucleic acid. In a more direct manner, it has similarly been shown that plasmid is only released from lipoplexes displaying a hexagonal phase structure when incubated with PS-containing vesicles, but not from lamellar complexes at similar conditions (Zuhorn et al. 2005). It is highly likely that for lipoplexes that efficiently transfect cells, both events, i.e, hexagonal phase-mediated destabilization of the endosomal membrane and dissociation of plasmid happens simultaneously although the "threshold" for either event to effectively occur, may differ.

A need for the interaction of cellular anionic lipids with cationic lipids, thereby displacing plasmids from their binding sites, is thought to be instrumental in this event. Consistent with this concept, originally proposed by Xu and Szoka (1996), it was recently shown that the DNA-releasing capacity of a given anionic lipid depends on the nature of the cationic component of the lipoplex (Tarahovsky et al. 2004). In essence, the binding affinity of a negatively charged lipid may differ for different cationic lipids, thus affecting the efficiency of DNA dissociation. By the same token, different negatively charged cellular lipids often display differences in lateral distribution and may similarly display differences in affinity for cationic lipids (cf. Xu and Szoka 1996). Accordingly, apart from endocytic internalization of lipoplexes per se, the molecular composition of a given endocytic compartment may

possibly (co-)determine the anionic (phospho)lipid-induced dissociation of the gene from a lipoplex, and hence the efficiency of cytosolic release.

Concluding remarks and perspectives

It becomes apparent that lipoplexes can be internalized into eukaryotic cells by a variety of endocytic processes. Quantitative knowledge of the relative contribution of each of these internalization pathways is crucial for designing strategies to direct these complexes into the pathway that leads to the highest transfection efficiency. To this end, insight is needed into parameters that (i) direct lipoplexes into a given pathway and (ii) mediate the effective release of DNA from the endocytic compartment in which the complexes localize. Lipoplex size is obviously a relevant parameter, and it is tempting to extrapolate data on the size-dependent internalization and processing by endocytosis of well-defined latex beads (Rejman et al. 2004b). However, apart from the fact that size is not likely the only parameter involved in endocytic processing of lipoplexes, the size of these particles is also very difficult to control and it is unclear what the relevance is of size determinations of the particles per se, given that lipoplexes may cluster to various degrees when incubated with cells or when present at the cell surface. On the other hand, the dynamics of their phase structure is important for the eventual release of DNA from intracellular compartments. Clearly, the efficiency of release seems to depend on the ability of lipoplexes to undergo a transition to a non-lamellar phase, the H_{II} phase being most frequently detected but clearly not necessarily the only phase capable of destabilizing membranes, since systems displaying H_I transitions may also effectively transfect cells (Ewert et al. 2006; Wasungu L. et al. 2006a). Since the efficiency of transfection is often enhanced when endocytic compartments are completely disrupted by osmotic shock (irrespective of whether lipoplexes are employed that underwent a complete non-lamellar transition), it is apparent that part of the problem of transfection inefficiency resides in the efficiency of DNA release from the complex as well as the endosomal compartment. Thus, the transition from a lamellar to a hexagonal phase as such seems insufficient in fully disrupting endosomal membranes. On the other hand, it cannot be excluded that the kinetics of processing along the endocytic pathway may also interfere with this process, implying that delivery to lysosomes represents a dead-end trap, the accessible DNA being destroyed in this compartment. Accordingly, a search for a more efficient and powerful disrupting capacity is needed, and in this context it will be of interest to investigate the possibility that different intracellular compartments could display differences in lipid phase dependent fragility; for example, caveosomes could be more susceptible to lipoplex-induced fragmentation than macropinosomes or early endosomes. Exclusive targeting into a defined pathway will then be a major challenge.

Evidently, nuclear delivery represents a final and equally important step in DNA/RNA delivery. Mechanisms involved in this aspect of overall transfection have been poorly addressed thus far and a detailed account is considered beyond the scope of this overview. Both translocation via nuclear pores as well as access of plasmid after cell division, thereby accomplishing integration into the nuclear translation and transcription machinery (Shi et al. 2003), have been proposed, and the attachment of nuclear localization signals to nucleic acids has been claimed to improve transfection (for recent reviews see Medina-Kauwe et al. 2005; Khalil et al. 2006; and references therein). However, in a study (Hama et al. 2006) in which DNA delivery and transfection was compared between a viral delivery system and lipoplexes, quantitative data revealed that rather than nuclear delivery per se, the poor transcription efficiency of the lipoplex-mediated reporter gene should also be taken into account in efforts aimed at improving lipoplex-mediated gene delivery. Thus, the current status of the field suggests that several issues, as discussed here, are worth to be explored further as they may provide clear options for improving the efficiency of gene delivery by non-viral vectors.

References

Ahmad A, Evans HM, Ewert K, George CX, Samuel CE, Safinya CR (2005) New multivalent cationic lipids reveal bell curve for transfection efficiency versus membrane charge density: lipid-DNA complexes for gene delivery. J Gene Med 7:739–748

Audouy S, Hoekstra D (2001) Cationic lipid-mediated transfection in vitro and in vivo (review). Mol Membr Biol 18:129–143

Bell PC, Bergsma M, Dolbynya IP, Bras W, Stuart MCA, Rowan AE, Feiters MC, Engberts JBFN (2003) Transfection mediated by Gemini surfactants: engineered escape from the endosomal compartment. J Am Chem Soc 125:1551–1558

Bieber T, Meissner W, Kostin S, Niemann A, Elsasser HP (2002) Intracellular route and transcriptional competence of polyethylenimine-DNA complexes. J Control Release 82:441–454

Boussif O, Lezoualc'h F, Zanta MA, Mergny MD, Scherman D, Demeneix B, Behr JP (1995) A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. Proc Natl Acad Sci USA 92:7297–7301

Chithrani BD, Ghazani AA, Chan WC (2006) Determining the size and shape dependence of gold nanoparticle uptake into mammalian cells. Nano Lett 6:662–668

Conwell CG, Huang L (2005) Recent advances in non-viral gene delivery. Adv Genet 53:1–18

Dass CR, Choong PF (2006) Selective gene delivery for cancer therapy using cationic liposomes: in vivo proof of applicability. J Control Release 113:155–63

Dehio C, Freissler E, Lanz C, Gomez-Duarte OG, David G, Meyer TF (1998) Ligation of cell surface heparan sulfate proteoglycans by antibody-coated beads stimulates phagocytic uptake into epithelial cells: a model for cellular invasion by Neisseria gonorrhoeae. Exp Cell Res 242:528–539

de Kruijff B, Cullis PR (1980) The influence of poly(L-lysine) on phospholipid polymorphism. Evidence that electrostatic polypeptide-phospholipid interactions can modulate bilayer/non-bilayer transitions. Biochim Biophys Acta 601:235–240

El Ouahabi A, Ruysschaert JM (2005) Formation and intracellular trafficking of lipoplexes and polyplexes. Mol Ther 11:336–347

El Ouahabi A, Thiry M, Pector V, Fuks R, Ruysschaert JM, Vandenbranden M (1997) The role of endosome destabilizing activity in the gene transfer process mediated by cationic lipids. FEBS Lett 414:187–192

El Ouahabi A, Thiry M, Schiffmann S, Fuks R, Nguyen-Tran H, Ruysschaert JM, Vandenbranden M (1999) Intracellular visualization of BrdU-labeled plasmid DNA/cationic liposome complexes. J Histochem Cytochem 47:1159–1166

Empig CJ, Goldsmith MA (2002) Association of the caveola vesicular system with cellular entry by filoviruses. J Virol 76:5266–5270

Evans CH, Gouze E, Gouze JN, Robbins PD, Ghivizzan SC (2006) Gene therapeutic approaches-transfer in vivo. Adv Drug Deliv Rev 58:243–258

Ewert KK, Evans HM, Zidovska A, Bouxsein NF, Ashmad A, Safinya CR (2006) A columnar phase of dendritic lipid-based cationic liposome-DNA complexes for gene delivery: hexagonally ordered cylindrical micelles embedded in a DNA honeycomb lattice. J Am Chem Soc 128:3998

Farhood H, Serbina N, Huang L (1995) The role of dioleoyl phosphatidylethanolamine in cationic liposome mediated gene transfer. Biochim Biophys Acta 1235:289–295

Felgner JH, Kumar R, Sridhar CN, Wheeler CJ, Tsai YJ, Border R, Ramsey P, Martin M, Felgner PL (1994) Enhanced gene delivery and mechanism studies with a novel series of cationic lipid formulations. J Biol Chem 269:2550–2561

Finlay BB, Cossart P (1997) Exploitation of mammalian host cell functions by bacterial pathogens. Science 276:718–725

Forrest ML, Pack DW (2002) On the kinetics of polyplex endocytic trafficking: implications for gene delivery vector design. Mol Ther 6:57–66

Freissler E, Meyer auf der Heyde A, David G, Meyer TF, Dehio C (2000) Syndecan-1 and syndecan-4 can mediate the invasion of OpaHSPG-expressing *Neisseria gonorrhoeae* into epithelial cells. Cell Microbiol 2:69–82

Friend DS, Papahadjopoulos D, Debs RJ (1996) Endocytosis and intracellular processing accompaning transfection mediated by cationic liposomes. Biochim Biophys Acta 1278:41–50

Godbey WT, Wu KK, Mikos AG (1999) Tracking the intracellular path of poly(ethylenimine)/DNA complexes for gene delivery. Proc Natl Acad Sci USA 96:5177–5181

Grosse S, Aron Y, Thevenot G, Francois D, Monsigny M, Fajac I (2005) Potocytosis and cellular exit of complexes as cellular pathways for gene delivery by polycations. J Gene Med 7:1275–1286

Gumbleton M, Hollins AJ, Omidi Y, Campbell L, Taylor G (2003) Targeting caveolae for vesicular drug transport. J Control Release 87:139–151

Hafez IM, Cullis PR (2001) Roles of lipid polymorphism in intracellular delivery. Adv Drug Deliv Rev 47:139–148

Hafez IM, Maurer N, Cullis PR (2001) On the mechanism whereby cationic lipids promote intracellular delivery of polynucleic acids. Gene Ther 8:1188–1196

Hagerstrand H, Danieluk M, Bobrowska-Hagerstrand M, Pector V, Ruysschaert J, Kralj-Iglic V, Iglic A (1999) Liposomes composed of a double-chain cationic amphiphile (vectamidine) induce their own encapsulation into human erythrocytes. Biochim Biophys Acta 1421:125–130

Hama S, Akita H, Ito R, Mizuguchi H, Hayakawa T, Harashima H (2006) Quantitative comparison of intracellular trafficking and nuclear transcription between adenoviral and lipoplex systems. Mol Ther 13:786–794

Harvie P, Wong FM, Bally MB (2000) Use of poly(ethylene glycol)-lipid conjugates to regulate the surface attributes and transfection activity of lipid-DNA particles. J Pharm Sci 89:652–663

Hirsch-Lerner D, Barenholz Y (1999) Hydration of lipoplexes commonly used in gene delivery: follow-up by laurdan fluorescence changes and quantification by differential scanning calorimetry. Biochim Biophys Acta 1461:47–57

Hoekstra D, Martin OC (1982) Transbilayer redistribution of phosphatidylethanolamine during fusion of phospholipid vesicles. Dependence on fusion rate, lipid phase separation and formation of non-bilayer structures. Biochemistry 21:6097–6103

Hope MJ, Cullis PR (1980) Effects of divalent cations and pH on phosphatidylserine model membranes: a 31P NMR study. Biochem Biophys Res Commun 92:846–852

Huttner WB, Zimmerberg J (2001) Implications of lipid microdomains for membrane curvature, budding and fission. Curr Opin Cell Biol 13:478–484

Huynh GH, Deen DF, Szoka FC Jr (2006) Barriers to carrier mediated drug and gene delivery to brain tumors. J Control Release 110:236–59

Israelachvili JN, Marcelja S, Horn RG (1980) Physical principles of membrane organization. Q Rev Biophys 13:121–200

Johnsson M, Edwards K (2001) Phase behavior and aggregate structure in mixtures of dioleoylphosphatidylethanolamine and poly(ethylene glycol)-lipids. Biophys J 80:313–323

Kakudo T, Chaki S, Futaki S, Nakase I, Akaji K, Kawakami T, Maruyama K, Kamiya H, Harashima H (2004) Transferrin-modified liposomes equipped with a pH-sensitive fusogenic peptide: an artificial viral-like delivery system. Biochemistry 43:5618–5628

Kawaura C, Noguchi A, Furuno T, Nakanishi M (1998) Atomic force microscopy for studying gene transfection mediated by cationic liposomes with a cationic cholesterol derivative. FEBS Lett 421:69–72

Khalil IA, Kogure K, Akita H, Harashima H (2006) Uptake pathways and subsequent intracellular trafficking in nonviral gene delivery. Pharmacol Rev 58:32–45

Kircheis R, Wightman L, Wagner E (2001) Design and gene delivery activity of modified polyethylenimines. Adv Drug Deliv Rev 53:341–358

Koltover I, Salditt T, Radler JO, Safinya CR (1998) An inverted hexagonal phase of cationic liposome-DNA complexes related to DNA release and delivery. Science 281:78–81

Kopatz I, Remy JS, Behr JP (2004) A model for non-viral gene delivery: through syndecan adhesion molecules and powered by actin. J Gene Med 6:769–776

Lei G, MacDonald RC (2003) Lipid bilayer vesicle fusion: intermediates captured by high-speed microfluorescence spectroscopy. Biophys J 85:1585–1599

Lenssen K, Jantscheff P, von Kiedrowski G, Massing U (2002) Combinatorial synthesis of new cationic lipids and high-throughput screening of their transfection properties. ChemBioChem 3:852–858

Lewis RN, McElhaney RN (2000) Surface charge markedly attenuates the nonlamellar phase-forming propensities of lipid bilayer membranes: calorimetric and 31P-nuclear magnetic resonance studies of mixtures of cationic, anionic, and zwitterionic lipids. Biophys J 79:1455–1464

Li W, Nicol F, Szoka FC Jr (2004) GALA: a designed synthetic pH-responsive amphipathic peptide with applications in drug and gene delivery. Adv Drug Deliv Rev 56:967–985

Lin AJ, Slack NL, Ahmad A, George CX, Samuel CE, Safinya CR (2003) Three-dimensional imaging of lipid gene-carriers: membrane charge density controls universal transfection behavior in lamellar cationic liposome-DNA complexes. Biophys J 84:3307–3316

McIntosh DP, Tan X-Y, Oh O, Schnitzer JE (2002) Targeting endothelium and its dynamic caveolae for tissue-specific transcytosis in vivo: A pathway to overcome cell barriers to drug and gene delivery. Proc Natl Acad Sci USA 99:1996–2001

Medina-Kauwe LK, Xie J, Hamm-Alvarez S (2005) Intracellular trafficking of nonviral vectors. Gene Ther 12:1734–1751

Mennesson E, Erbacher P, Piller V, Kieda C, Midoux P, Pichon C (2005) Transfection efficiency and uptake process of polyplexes in human lung endothelial cells: a comparative study in non-polarized and polarized cells. J Gene Med 7:729–738

Mislick KA, Baldeschwieler JD (1996) Evidence for the role of proteoglycans in cation-mediated gene transfer. Proc Natl Acad Sci USA 93:12349–12354

Mok KW, Cullis PR (1997) Structural and fusogenic properties of cationic liposomes in the presence of plasmid DNA. Biophys J 73:2534–2545

Niculescu-Duvaz D, Heyes J, Springer CJ (2003) Structure-activity relationship in cationic lipid mediated gene transfection. Curr Med Chem 10:1233–1261

Oberle V, De Jong G, Drayer JI, Hoekstra D (2004) Efficient transfer of chromosome-based DNA constructs into mammalian cells. Biochim Biophys Acta 1676:223–230

Ogris M, Carlisle RC, Bettinger T, Seymour LW (2001a) Melittin enables efficient vesicular escape and enhanced nuclear access of nonviral gene delivery vectors. J Biol Chem 276:47550–47555

Ogris M, Steinlein P, Carotta S, Brunner S, Wagner E (2001b) DNA/polyethylenimine transfection particles: influence of ligands, polymer size, and PEGylation on internalization and gene expression. AAPS PharmSci 3:E21

Parton RG, Richards AA (2003) Lipid rafts and caveolae as portals for endocytosis: new insights and common mechanisms. Traffic 4:724–738

Pelkmans L, Helenius A (2003) Insider information: what viruses tell us about endocytosis. Curr Opin Cell Biol 15:414–422

Pelkmans L, Kartenbeck J, Helenius A (2001) Caveolar endocytosis of simian virus 40 reveals a new two-step vesicular-transport pathway to the ER. Nat Cell Biol 3:473–483

Plank C, Oberhauser B, Mechtler K, Koch C, Wagner E (1994) The influence of endosome-disruptive peptides on gene transfer using synthetic virus-like gene transfer systems. J Biol Chem 269:12918–12924

Rakhmanova VA, Pozharski EV, MacDonald RC (2004) Mechanisms of lipoplex formation: dependence of the biological properties of transfection complexes on formulation procedures. J Membr Biol 200:35–45

Rejman J, Wagenaar A, Engberts JBFN, Hoekstra D (2004a) Characterization and transfection properties of lipoplexes stabilized with novel exchangeable polyethylene glycol-lipid conjugates. Biochim Biophys Acta 1660:35–42

Rejman J, Oberle V, Zuhorn IS, Hoekstra D (2004b) Size-dependent internalization of particles via the pathways of clathrin- and caveolae-mediated endocytosis. Biochem J 377:159–169

Rejman J, Bragonzi A, Conese M (2005) Role of clathrin- and caveolae-mediated endocytosis in gene transfer mediated by lipo- and polyplexes. Mol Ther 12:468–474

Rodal SK, Skretting G, Garred O, Vilhardt F, van Deurs B, Sandvig K (1999) Extraction of cholesterol with methyl-beta-cyclodextrin perturbs formation of clathrin-coated endocytic vesicles. Mol Biol Cell 10:961–974

Ruponen M, Ronkko S, Honkakoski P, Pelkonen J, Tammi M, Urtti A (2001) Extracellular glycosaminoglycans modify cellular trafficking of lipoplexes and polyplexes. J Biol Chem 276:33875–33880

Sakurai F, Inoue R, Nishino Y, Okuda A, Matsumoto O, Taga T, Yamashita F, Takakura Y, Hashida M (2000) Effect of DNA/liposome mixing ratio on the physicochemical characteristics, cellular uptake and intracellular trafficking of plasmid DNA/cationic liposome complexes and subsequent gene expression. J Control Release 66:255–269

Sawitzky D (1996) Protein–glycosaminoglycan interactions: infectiological aspects. Med Microbiol Immunol (Berl) 184:155–161

Shi F, Wasungu L, Nomden A, Stuart MCA, Polushkin E, Engberts JBFN, Hoekstra D (2002) Interference of poly(ethylene glycol)-lipid analogues with cationic lipid-mediated delivery of oligonucleotides; role of lipid exchangeability and non-lamellar transitions. Biochem J 366:333–341

Shi F, Visser WH, de Jong NM, Liem RS, Ronken E, Hoekstra D (2003) Antisense oligonucleotides reach mRNA targets via the RNA matrix: downregulation of the 5- HT1A receptor. Exp Cell Res 291:313–325

Shin JS, Abraham SN (2001) Caveolae—not just craters in the cellular landscape. Science 293:1447–1448

Shin JS, Gao Z, Abraham SN (2000) Involvement of cellular caveolae in bacterial entry into mast cells. Science 289:785–788

Siegel DP, Epand RM (1997) The mechanism of lamellar-to-inverted hexagonal phase transitions in phosphatidylethanolamine: implications for membrane fusion mechanisms. Biophys J 73:3089–3111

Simberg D, Danino D, Talmon Y, Minsky A, Ferrari ME, Wheeler CJ, Barenholz Y (2001) Phase behavior, DNA ordering, and size instability of cationic lipoplexes. Relevance to optimal transfection activity. J Biol Chem 276:47453–47459

Simberg D, Weisman S, Talmon Y, Barenholz Y (2004) DOTAP (and other cationic lipids): chemistry, biophysics, and transfection. Crit Rev Ther Drug Carrier Syst 21:257–317

Simoes S, Slepushkin V, Pires P, Gaspar R, de Lima MP, Duzgunes N (1999) Mechanisms of gene transfer mediated by lipoplexes associated with targeting ligands or pH-sensitive peptides. Gene Ther 6:1798–1807

Smisterova J, Wagenaar A, Stuart MCA, Polushkin E, ten Brinke G, Hulst R, Engberts JBFN, Hoekstra D (2001) Molecular shape of the cationic lipid controls the structure of cationic lipid/dioleylphosphatidylethanolamine-DNA complexes and the efficiency of gene delivery. J Biol Chem 276:47615–47622

Sonawane ND, Szoka FC Jr, Verkman AS (2003) Chloride accumulation and swelling in endosomes enhances DNA transfer by polyamine-DNA polyplexes. J Biol Chem 278:44826–44831

Song LY, Ahkong QF, Rong Q, Wang Z, Ansell S, Hope MJ, Mui B (2002) Characterization of the inhibitory effect of PEG-lipid

conjugates on the intracellular delivery of plasmid and antisense DNA mediated by cationic lipid liposomes. Biochim Biophys Acta 1558:1–13

Stebelska K, Dubielecka PM, Sikorski AF (2005) The effect of PS content on the ability of natural membranes to fuse with positively charged liposomes and lipoplexes. J Membr Biol 206:203–214

Stebelska K, Wyrozumska P, Sikorski AF (2006) PS exposure increases the susceptibility of cells to fusion with DOTAP liposomes. Chem Biol Interact 160:165–174

Tarahovsky YS, Koynova R, MacDonald RC (2004) DNA release from lipoplexes by anionic lipids: correlation with lipid mesomorphism, interfacial curvature, and membrane fusion. Biophys J 87:1054–1064

Trubetskoy VS, Wong SC, Subbotin V, Budker VG, Loomis A, Hagstrom JE, Wolff JA (2003) Recharging cationic DNA complexes with highly charged polyanions for in vitro and in vivo gene delivery. Gene Ther 10:261–271

van der Woude I, Wagenaar A, Meekel AAP, ter Beest MBA, Ruiters MHJ, Engberts JBFN, Hoekstra D (1997) Novel pyridinium surfactants for efficient, nontoxic in vitro gene delivery. Proc Natl Acad Sci USA 94:1160–1165

Wagner E (1999) Application of membrane-active peptides for nonviral gene delivery. Adv Drug Deliv Rev 38:279–289

Wasungu L, Stuart MCA, Scrazello M, Engberts JBFN, Hoekstra D (2006a) Lipoplexes formed from sugar-based gemini surfactants undergo a lamellar-to-micellar phase transition at acidic pH. Evidence for a non-inverted membrane-destabilizing hexagonal phase of lipoplexes. Biochem Biophys Acta (in press). DOI 10.1016/j.bbamem.2006.06.019

Wasungu L, Scarzello M, van Dam G, Molema G, Wagenaar A, Engberts JBFN, Hoekstra D (2006b) Transfection mediated by pH-sensitive sugar-based gemini surfactants; potential for in vivo gene therapy applications. J Mol Med 84:774–784

Wiethoff CM, Smith JG, Koe GS, Middaugh CR (2001) The potential role of proteoglycans in cationic lipid-mediated gene delivery. Studies of the interaction of cationic lipid-DNA complexes with model glycosaminoglycans. J Biol Chem 276:32806–32813

Xu Y, Szoka FC Jr (1996) Mechanism of DNA release from cationic liposome/DNA complexes used in cell transfection. Biochemistry 35:5616–5623

Zhdanov RI, Podobed OV, Vlassov VV (2002) Cationic lipid-DNA complexes-lipoplexes-for gene transfer and therapy. Bioelectrochemistry 58:53–64

Zhou X, Huang L (1994) DNA transfection mediated by cationic liposomes containing lipopolylysine: characterization and mechanism of action. Biochim Biophys Acta 1189:195–203

Zimmerberg J, Chernomordik LV (1999) Membrane fusion. Adv Drug Deliv Rev 38:197–205

Zuhorn IS, Hoekstra D (2002) On the mechanism of cationic amphiphile-mediated transfection. To fuse or not to fuse: is that the question? J Membr Biol 189:167–179

Zuhorn IS, Kalicharan R, Hoekstra D (2002a) Lipoplex-mediated transfection of mammalian cells occurs through the cholesterol-dependent clathrin-mediated pathway of endocytosis. J Biol Chem 277:18021–18028

Zuhorn IS, Visser WH, Bakowsky U, Engberts JBFN, Hoekstra D (2002b) Interference of serum with lipoplex–cell interaction: modulation of intracellular processing. Biochim Biophys Acta 1560:25–36

Zuhorn IS, Oberle V, Visser WH, Engberts JBFN, Bakowsky U, Polushkin E, Hoekstra D (2002c) Phase behavior of cationic amphiphiles and their mixtures with helper lipid influences lipoplex shape, DNA translocation, and transfection efficiency. Biophys J 83:2096–2108

Zuhorn IS, Bakowsky U, Polushkin E, Visser WH, Stuart MCA, Engberts JBFN, Hoekstra D (2005) Nonbilayer phase of lipoplex-membrane mixture determines endosomal escape of genetic cargo and transfection efficiency. Mol Therapy 11:801–810