Nonbilayer Phase of Lipoplex–Membrane Mixture Determines Endosomal Escape of Genetic Cargo and Transfection Efficiency

Inge S. Zuhorn,1,2 Udo Bakowsky,3 Evgeny Polushkin,4 Willy H. Visser,1 Marc C.A. Stuart,5 Jan B.F.N. Engberts,5 and Dick Hoekstra1,*

1Department of Membrane Cell Biology, University of Groningen, A. Deusinglaan 1, 9713 AV Groningen, The Netherlands
2Biomade Technology Foundation, Nijenborgh 4, 9747 AG Groningen, The Netherlands
3Department of Pharmaceutical Technology and Biopharmacy, Philipps University, Ketzerbach 63, 35037 Marburg, Germany
4Department of Polymer Chemistry, University of Groningen, and Laboratory of Polymer Chemistry, Dutch Polymer Institute, Materials Science Center, Nijenborgh 4, 9747 AG Groningen, The Netherlands
5Physical Organic Chemistry Unit, Stratingh Institute, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands

*To whom correspondence and reprint requests should be addressed. Fax: +31 50 3632728. E-mail: d.hoekstra@med.rug.nl.

Available online 18 January 2005

Cationic lipids are widely used for gene delivery, and inclusion of dioleoylphosphatidylethanolamine (DOPE) as a helper lipid in cationic lipid–DNA formulations often promotes transfection efficacy. To investigate the significance of DOPE’s preference to adopt a hexagonal phase in the mechanism of transfection, the properties and transfection efficiencies of SAINT-2/DOPE lipoplexes were compared to those of lipoplexes containing lamellar-phase-forming dipalmitoylphosphatidylethanolamine (DPPE). After interaction with anionic vesicles, to simulate lipoplex–endosomal membrane interaction, SAINT-2/DOPE lipoplexes show a perfect hexagonal phase, whereas SAINT-2/DPPE lipoplexes form a mixed lamellar–hexagonal phase. The transition to the hexagonal phase is crucial for dissociation of DNA or oligonucleotides (ODN) from the lipoplexes. However, while the efficiencies of nucleic acid release from either complex were similar, SAINT-2/DOPE lipoplexes displayed a two- to threefold higher transfection efficiency or nuclear ODN delivery. Interestingly, rupture of endosomes following a cellular incubation with ODN-containing SAINT-2/DPPE complexes dramatically improved nuclear ODN delivery to a level that was similar to that observed for SAINT-2/DOPE complexes. Our data demonstrate that although hexagonal phase formation in lipoplexes is a prerequisite for nucleic acid release from the complex, it appears highly critical for accomplishing efficient translocation of nucleic acids across the endosomal membrane into the cytosol for transport to the nucleus.

Key Words: cationic lipid, DNA, hexagonal, endosomes, transfection

INTRODUCTION

It has been well documented that the inclusion of dioleoylphosphatidylethanolamine (DOPE) in cationic lipid–DNA formulations may significantly improve the transfection activity of these formulations, whereas incorporation of dioleoylphosphatidylcholine (DOPC) usually diminishes this activity [1–3]. Accordingly, DOPE is often defined as a “helper lipid” in such formulations. The augmentation in transfection efficiency of DOPE-containing formulations compared to formulations without DOPE has been attributed to the propensity of DOPE to adopt nonbilayer structures. These nonbilayer structures are known to play a role in membrane fusion and—in the entry mechanism of lipoplexes into cells—could serve to destabilize the endosomal membrane, thereby allowing the release of the genetic cargo into the cytosol [3–6]. However, the extent of lipid mixing of lipoplexes with cellular membranes does not correlate with their transfection activity [5]. This lack of correlation has been attributed to the fact that lipid mixing of lipoplexes with cellular membranes does not necessarily result in the concomitant release of DNA from the lipoplex [7,8], a prerequisite for DNA to become eventually transcriptionally active [9–11].

More recently, it was hypothesized that DOPE has a function in weakening the binding between the cationic lipid and DNA, resulting in a facilitated release of DNA from lipoplexes. Interaction of the phosphatidylethanol-
amine (PE) phosphate with the amine group of the cationic lipid [12–15], as well as interaction of the PE amine with the DNA phosphate [8], has been proposed as a possible site of interference in the cationic lipid–DNA binding reaction. For pH-sensitive liposomes, neutralization of the (negatively charged) acidic lipids at acidic pH weakens intermolecular interactions in the lateral plane of the bilayer, causing local phase separation into DOPE-enriched domains. This allows the formation of a non-bilayer phase of DOPE at low pH, which could facilitate lipoplex destabilization [16]. However, for cationic lipids the ionization state is not expected to change at acidic pH. Thus, lowering of the pH in endosomal compartments is not expected to affect the stability of lipoplexes.

To define better the role of DOPE in facilitating the transfection activity of cationic lipid–DNA complexes, we compared its helper lipid function with that of dipalmitoylphosphatidylethanolamine (DPPE). DPPE does not display the intrinsic propensity to form non-bilayer structures (at cell culture temperature and physiological pH) and remains in the lamellar phase. Here, we investigated the influence of either lipid, assembled with the cationic lipid N-methyl-4(dioleyl)methylpyridiniumchloride (SAINT-2), on the interaction of the thus-formed lipoplexes with (cellular) membranes and on the biological activity of the lipoplex in terms of nuclear delivery of oligonucleotides and cellular transfection. Our data demonstrate that the formation of a hexagonal conformation of the lipoplex is critical for an efficient endosomal escape of its genetic cargo in terms of both its dissociation from the lipoplex and its translocation across the endosomal membrane and hence for efficient transfection.

Results

Transfection Efficiency of COS-7 Cells with SAINT-2 Lipoplexes Is Dependent on the Type of Helper Lipid

To determine whether DPPE-containing SAINT-2 lipoplexes were able to transfect cells, we incubated COS-7 cells with SAINT-2 lipoplexes, which contained equimolar amounts of either DPPE or DOPE, using pEGFP-N1 as reporter gene. Both complexes interacted with equal efficiency with the cells, as established by the cellular association of N-Rh-PE (incorporated in the lipoplexes at 0.5 mol%, final concentration) as tracer for the extent of lipoplex–cell interaction (not shown). However, the transfection activity of SAINT-2 lipoplexes containing DPPE as a helper lipid was significantly reduced compared to that of lipoplexes containing DOPE. While transfection with SAINT-2/DOPE lipoplexes resulted in 73.1 ± 4.8% reporter gene-positive cells, SAINT-2/DPPE lipoplexes transfected 25.3 ± 2.5% of the cells, as determined by FACS analysis. Moreover, reporter gene expression per cell was significantly enhanced following transfection with DOPE-containing lipoplexes compared to DPPE-containing lipoplexes. Following transfection with SAINT-2/DOPE lipoplexes 27% of the transfected cells showed a reporter gene expression level higher than 10,000 (AU) compared to 8% in the case of transfection with SAINT-2/DPPE lipoplexes. To clarify the underlying cause for this difference, we investigated complex assembly and structural features known to govern transfection efficiency.

DPPE in an Equimolar Mixture with SAINT-2 Is Present in a Fluid Phase

We first investigated the lipid phase properties of DPPE in a mixture with SAINT-2, which we compared to those of the SAINT-2/DOPE mixture. To this end we studied monolayers of pure SAINT-2, DOPE, or DPPE, as well as equimolar mixtures of SAINT-2 with DOPE and DPPE, at room temperature. All monolayers showed a liquid-expanded film and fluid phase behavior, as determined by measuring molecular area–pressure curves (isotherms), except for the pure DPPE monolayer, which exhibited a constant pressure plateau (Π = 10 mN/m) phase coexistence and transition region between the liquid-expanded and the liquid-condensed (gel) phase (Fig. 1). This implies that at room temperature the DPPE lipid film shows a mixed phase behavior, involving the coexistence of both the fluid and the gel phase of the lipid. At surface pressures below 10 mN/m the molecular

FIG. 1. Isotherms of SAINT-2, DOPE, DPPE, and their equimolar mixtures. Monolayers composed of pure SAINT-2 (●), DOPE (○), and DPPE (●) and the equimolar mixtures of SAINT-2 and DOPE (◆) and SAINT-2 and DPPE (▲) were compressed at a constant temperature, while the molecular area occupied by a single lipid molecule was recorded. The isotherm of SAINT-2 shows a liquid-expanded film behavior, indicating the presence of SAINT-2 in a fluid phase. DPPE shows a phase-transition region, which implies that the lipid is present in a mixed liquid-condensed (gel) and liquid-expanded (fluid) phase. Addition of an equimolar amount of SAINT-2 to DPPE results in a monolayer with a fluid phase behavior. The inset shows isotherms of mixed monolayers upon addition of DNA.
packing density within the film is low and DPPE is in the fluid state. At higher pressures DPPE molecules tend to cluster in small circular domains. These domains are characteristic of a gel-phase film behavior and increase in size with increasing surface pressure. The highest molecular surface density, i.e., when all domains fuse to form a homogeneous film, was reached at a (collapse) pressure of 52 nM/m and a molecular area of 0.42 nm². We visualized the lateral morphology of the lipid film by epifluorescence microscopy (Fig. 2). The fluorescent dye 1-hexadecanoyl-2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) aminohexanoyl)-sn-glycero-3-phosphocholine (NBD-PC), which was included in the lipid film (1 mol%), is preferentially dissolved in the liquid-expanded phase, resulting in the visualization of DPPE domains as circular black areas (Fig. 2A). When DPPE was mixed in a 1:1 molar ratio with SAINT-2 the mixture displays liquid-expanded behavior. The expansion of the molecular area and the lack of a constant pressure region imply that—during compression—the film is in a fluid phase. Examination by epifluorescence microscopy shows a homogeneously bright image without any condensed domains (Fig. 2B). Similar images were obtained for the monolayer films of pure DOPE and pure SAINT-2 and their equimolar mixture (not shown). To investigate lipoplex formation carefully using SAINT-2, mixed (1:1) with either DOPE or DPPE, we next employed the film balance technique as previously described [17], which allows monitoring of complex assembly under equilibrium-like conditions.

Distinct Interaction of Plasmids with SAINT-2/DOPE versus SAINT-2/DPPE Monolayers

We prepared monolayers consisting of SAINT-2/DOPE or SAINT-2/DPPE as described under Materials and Methods and initiated lipoplex assembly at the air–water interface by injection of DNA underneath the monolayer. The onset of complex assembly could be inferred from the observation that bright domains were formed in monolayers, in which a fluorescent analog of SAINT-2 (diphenylhexatriene (DPH)–SAINT-2) had been included (Fig. 2C). These domains presumably represent the sites where cationic lipids are recruited at a relative high density, reflected by an enhancement in local fluorescence, due to their interaction with the negatively charged DNA. We confirmed this by examination of the film by atomic force microscopy (AFM). Thus, after lipoplex formation at the air–water interface of the lipid film, we transferred the film to a silicon wafer for examination by AFM. The structures that were detected by AFM were of the same dimension as the bright domains found in the monolayer after injection of DNA into the subphase (not shown), thus providing support for the notion above that the bright fluorescent spots reflected the sites of lipoplex assembly in the monolayer system.

Following injection of DNA into the subphase, the surface pressure of SAINT-2/DOPE decreased, whereas the pressure of SAINT-2/DPPE remained constant (Fig. 3). In time, SAINT-2/DOPE lipoplexes detached from the interface, which was accompanied by a transition from a patchy fluorescence into a more cloudy appearance of the monolayer (cf. Fig. 3 in Ref. [19]), indicative of its collapse into three-dimensional structures that diffused into the subphase, i.e., lipid recruitment into lipoplexes and their dissociation from the interface will cause collapse of the monolayer when the pressure is kept constant, consistent with previous observations (see Ref.
Interestingly, SAINT-2/DPPE lipoplexes did not dissociate from the monolayer and remained located at the air–water interface, as reflected by the persistent presence of the fluorescent complexes at the interface. Since both SAINT-2/DOPE and pure SAINT-2 lipoplexes dissociate from the subphase [17], the presence of DPPE apparently frustrates the detachment process, as also indicated by the constancy of the surface pressure following DNA addition to the aqueous subphase (Fig. 3), possibly reflecting an interference of DPPE in the final lipoplex assembly steps.

Since lipoplex formation in practice is a highly dynamic event, involving the relatively uncontrolled interaction between liposomes and plasmids, we also visualized lipoplex formation in bulk phase. As shown in Fig. 4, SAINT-2/DPPE liposomes engaged in lipoplex formation leading to formation of particles with a size distribution of 150–250 nm, which is indistinguishable from the size distribution obtained for complexes assembled with SAINT-2/DOPE. However, the relative refractoriness of SAINT-2/DPPE to complex assembly as noted in the monolayer experiments was also apparent in bulk phase. As shown in Fig. 4B, numerous merged vesicles (original size of the vesicles was approx 60 nm), devoid of plasmid, were present in the sample. In contrast to a typical hexagonal phase, observed by electron microscopy when SAINT-2/DOPE–DNA complexes are suspended in 150 mM NaCl/10 mM Hepes, pH 7.4 [18], we observed stacks of bilayers for SAINT-2/DPPE–DNA complexes that were slightly curved (Fig. 4C).

**FIG. 3.** Comparison of monolayer pressure versus time of SAINT-2/DOPE and SAINT-2/DPPE mixtures, following DNA injection into the aqueous subphase. Upon DNA injection underneath the lipid monolayer, the pressure of the SAINT-2/DOPE monolayer decreases in time, reflecting the dissociation of lipid–DNA complexes from the air–water interface into the subphase. The pressure of the SAINT-2/DPPE monolayer remains constant upon injection of DNA, reflecting the refractoriness of the lipoplex assembly and/or its subsequent dissociation into the subphase.

**FIG. 4.** AFM and EM images of SAINT-2/DOPE and SAINT-2/DPPE lipoplexes. Lipoplexes were prepared in bulk phase and examined with AFM. (A) SAINT-2/DOPE lipoplexes are round-shaped and have a size of 150–200 nm (total image 1.2 by 1.2 μm). (B) SAINT-2/DPPE lipoplexes show irregular morphology and have a size of 150–250 nm (total image 1.2 by 1.2 μm). In addition, vesicles that are devoid of plasmid can be seen. (C) SAINT-2/DPPE lipoplexes were rapidly frozen in liquid nitrogen and examined with EM. Stacks of bilayers can be distinguished (total image 0.57 by 0.57 μm).
Because of the natural preference of pure DPPE to form a bilayer, a priori we would predict that SAINT-2/DPPE–DNA would display a lamellar organization. This is consistent with the electron microscopy (EM) observations, shown in Fig. 4C. To obtain further support for this notion, we carried out small-angle X-ray measurements. As shown in Fig. 5, analysis of SAINT-2/DPPE lipoplexes revealed four X-ray diffraction maxima with regular spacing according to the ratio 1:2:3:4 and a q₁ value of 0.109, typical of a lamellar structure. The periodicity of the phase is 57.7 Å (Fig. 5). Elsewhere we demonstrated that inclusion of acidic phospholipids into SAINT-containing lipoplexes promotes a transition from a lamellar to a hexagonal phase [19]. The hexagonal topology strongly facilitates transfection efficiency [18,20], and recruitment of acidic phospholipids like phosphatidylserine (PS) into the complex [18,19,21], when tightly interacting with the endosomal membrane, may be instrumental in this process. Such a recruitment of PS and its consequences for the lipid phase of the lipoplex can be adequately simulated by incubating the lipoplexes with PS-containing liposomes [19]. Interestingly, as shown in Fig. 5B, after interaction of SAINT-2/DPPE lipoplexes with anionic PS:PC:PE (1:1:2) vesicles a mixed lamellar–hexagonal phase was obtained. Hence, given that the hexagonal phase strongly facilitates transfection efficiency, this only partial transition from a lamellar to a hexagonal phase would be consistent with the less efficient transfection efficiency observed for the DPPE-containing complex. To investigate further the potential efficiency of interaction of the DOPE versus DPPE SAINT-2 lipoplexes with target membranes, simulated by the PS-containing lipid vesicles, we next determined how such an interaction affected DNA release, i.e., a major factor that codetermines eventual transfection efficiency.

**DPPE Affects (Nuclear) Delivery Rather Than Nucleic Acid Dissociation from Lipoplexes**

The release of DNA from lipoplexes is strongly dependent on the presence of salt in the incubation medium. If lipoplexes were prepared in water and subsequently incubated with PS:PC:PE (1:1:2) target vesicles, we could observe no release of DNA, irrespective of the nature of the helper lipid (Fig. 6, lanes 3, 5, 7, and 9). Moreover, small-angle X-ray diffraction (SAXS) analysis revealed that the lamellar phase is maintained under these conditions, i.e., a shift to the hexagonal phase does not occur (not shown, cf. Ref. [18]). In the presence of salt, we could observe differences in the ability of the helper lipids to facilitate DNA release from lipoplexes (Fig. 6). Without helper lipid, DNA was only marginally released from SAINT-2 lipoplexes upon incubation with PS:PC:PE vesicles (lane 4), while inclusion of DOPC in the lipoplex formulation exerted no effect on the amount of dissociated DNA (lane 10). However, inclusion of DOPE in the SAINT-2 complex significantly enhanced the extent of DNA that was released, when incubated with the anionic lipid-containing vesicles (lane 6). Interestingly, a similar effect on the release of DNA was apparent when the formulation contained DPPE (lane 8) instead of DOPE, resulting in a release of 39.9 ± 3.4% in the latter case and...
52.1 ± 8.4% in the former. Thus, membrane destabilization of lipoplexes, reflected by DNA release induced by anionic lipid-containing vesicles, seems to require complexes that intrinsically can display the propensity to adopt the hexagonal phase (i.e., SAINT-2/DOPE in the presence of salt) and/or incubation conditions that promote the adoption of such a phase, as occurs (i.e., for SAINT-2/DPPE, Fig. 5, but not SAINT-2/DOPC, not shown) in the presence of anionic vesicles. Note, however, that comparing the DOPE versus DPPE lipoplexes, the slight differences in DNA release do not match the threefold higher transfection efficiency, as seen for the DOPE-containing SAINT-2 complexes. Apart from dissociation of the DNA from the lipoplex, (concomitant) translocation across the endosomal membrane is imperative for the plasmid to reach eventually the nucleus for transcription. Accordingly, the data could imply that the efficiency of plasmid delivery into the cytosol might have been hampered in the case of the DPPE-containing SAINT-2 lipoplexes. Apart from dissociation of the DNA from the lipoplex, translocation across the endosomal membrane is imperative for the plasmid to reach eventually the nucleus for transcription. Accordingly, the data could imply that the efficiency of plasmid delivery into the cytosol might have been hampered in the case of the DPPE-containing SAINT-2 lipoplexes. To investigate this issue, we monitored the nuclear delivery of fluorescently tagged ODNs, which can be more conveniently determined and quantified than that of plasmid. Moreover, as electrophoresis is not the most sensitive assay to determine DNA release from lipoplexes, the in situ nuclear accumulation of ODNs will more accurately reflect the efficiency of DNA release and endosomal escape. We incubated COS-7 cells for 1 h with SAINT-2/DOPE and SAINT-2/DPPE lipoplexes, complexed with FITC-labeled ODNs, as described under Materials and Methods. As shown in Fig. 7, fluorescently tagged ODNs localized to the nucleus in essentially all cells in the case of delivery with SAINT-2/DOPE lipoplexes. However, delivery with SAINT-2/DPPE lipoplexes resulted in a more scattered intracellular distribution of ODNs. Specifically, the nuclear staining obtained after incubation of cells with SAINT-2/DPPE lipoplexes was less abundant and substantially less bright than that observed after an incubation with SAINT-2/DOPE lipoplexes. Indeed, quantification by the computer software Scionimage revealed a reduction of approximately 60% in the extent of nuclear accumulation in the case of incubation of cells with SAINT-2/DPPE complexes, which is compatible with the differences observed in transfection. In addition, cells incubated with SAINT-2/DOPE lipoplexes appeared almost devoid of cytoplasmic ODNs, whereas cells incubated with SAINT-2/DPPE lipoplexes showed numerous ODN-positive cytoplasmic structures, presumably representing endosomes. Hence, these data suggest that SAINT-2/DPPE lipoplexes are defective in facilitating endosomal escape of nucleic acids, resulting in entrapment of ODNs in endosomes. If so, endosomal rupture by artificial means should result in an improved nuclear delivery. We examined this issue next.

**Endosomal Rupture by Osmotic Shock Treatment Facilitates ODN Delivery with SAINT-2/DPPE Lipoplexes**

To investigate whether the reduced transfection efficiency and diminished nuclear delivery of ODNs, mediated via SAINT-2/DPPE complexes compared to those consisting of SAINT-2/DOPE, was related to a limited capacity of the DPPE-containing system to rupture endosomal membranes, we applied osmotic shock to cells following an incubation with SAINT-2/DOPE or SAINT-2/DPPE lipoplexes. Osmotic shock treatment will result in rupture of endosomal membranes, allowing escape of their contents, as previously described [22]. As a control, we observed no destabilization or release of ODNs or plasmid when the lipoplexes as such were suspended and incubated in the shock medium (not shown). However, as can be seen in Fig. 8, osmotic shock treatment

![FIG. 6. Release of DNA from lipoplexes induced by anionic vesicles: influence of presence of salt and type of helper lipid. Lane 1, DNA. Lanes 3, 5, 7, and 9 show DNA released from SAINT-2, SAINT-2/DOPE, SAINT-2/DPPE, and SAINT-2/DOPC lipoplexes in water, respectively. Lanes 4, 6, 8, and 10 show DNA released from similar lipoplexes, prepared in the presence of salt.](image)

![FIG. 7. Nuclear accumulation of FITC–ODN in COS-7 cells incubated with SAINT-2/DOPE and SAINT-2/DPPE lipoplexes. COS-7 cells were incubated with SAINT-2/DOPE and SAINT-2/DPPE lipoplexes containing FITC–ODN for 1 h (37°C). Subsequently, the cells were examined by fluorescence microscopy. (A) All the nuclei of cells incubated with SAINT-2/DOPE lipoplexes are positive for FITC–ODN. (B) In cells incubated with SAINT-2/DPPE not all nuclei are positive for FITC–ODN and many intact endosomes containing FITC–ODN are visible.](image)
following incubation with SAINT-2/DPPE lipoplexes results in a decrease in the amount of ODN-positive endosomes. Concomitantly, we observed a significant increase in the extent of nuclear accumulation of ODNs. Thus, these results are fully in line with the notion that disruption of the endosomal membrane barrier allows ODNs to diffuse readily to and accumulate into the nucleus.

**DISCUSSION**

The present study provides new insight into the relevance and involvement of helper lipids in the mechanism of lipoplex-mediated gene or ODN delivery. Such insight was obtained by comparing the properties of the hexagonal-phase-forming SAINT-2/DOPE lipoplexes versus lamellar-phase-preferring SAINT-2/DPPE complexes. This distinction in phase affected neither the size of the complexes nor their ability to associate with cells as such. However, once internalized the phase properties of the helper lipid did dramatically affect the efficiency of nuclear delivery of plasmids and ODNs. Thus our data reveal that the polymorphic properties of the helper lipid play a prominent role in lipoplex-mediated nucleic acid translocation across the endosomal membrane and, to a lesser extent, in nucleic acid dissociation from the lipoplexes.

As shown previously [19], a distinct degree of membrane flexibility is required for appropriate and efficient wrapping of plasmids by cationic lipids. A relative tightness of the cationic lipid membrane impedes such an efficient interaction, resulting in a relatively slow lipid recruitment causing a partial decondensation of the plasmid upon electrostatic interaction with the interface of (mixed) lipid mono- or bilayers [19,23]. Although plasmid delivery to the nucleus may in this case not be impaired, conformational DNA “defects” (i.e., linear vs supercoiled) may affect the efficiency of transcription and thereby transfection efficiency. Interestingly, the present work demonstrates that lipoplex assembly is also frustrated upon inclusion of the helper lipid DPPE instead of DOPE in the lipid mixture. The inability of the SAINT-2/DPPE lipoplexes to dissociate from the monolayer indicates that the monolayer does not collapse, as occurs upon assembly of SAINT-2/DOPE complexes, thus reflecting differences in the kinetics of lipid recruitment. Therefore, the distinction between the assembly of either complex presumably relates to the differences in polymorphous properties of the helper lipid. However, in SAINT-2/DPPE lipoplexes, the DNA conformation is not affected, as demonstrated by agarose gel electrophoresis, which does not reveal structures other than supercoiled DNA (Fig. 5). This implies that factors other than

**FIG. 8.** Osmotic rupture of endosomes following incubation with SAINT-2/DOPE and SAINT-2/DPPE lipoplexes enhances nuclear accumulation of FITC–ODN. COS-7 cells were incubated with SAINT-2/DOPE and SAINT-2/DPPE lipoplexes containing FITC–ODN for 1 h (37°C). Subsequently the cells were subjected to an osmotic shock to rupture the endosomes artificially. Osmotic shock treatment diminishes the extent of intact ODN-containing endosomes in cells incubated with (B) SAINT-2/DOPE (cf. to A) as well as (D) SAINT-2/DPPE lipoplexes (cf. to C). In cells incubated with SAINT-2/DPPE lipoplexes a significant increase in the amount of ODN-positive nuclei can be seen following osmotic shock (D cf. to C).
plasmid structure determine the diminished transfection efficiency observed for the DPPE-containing SAINT-2 lipoplexes.

The transfection efficiency between SAINT-2/DOPE and SAINT-2/DPPE lipoplexes differs almost threefold, i.e., 70 and 25%, respectively. Yet the dissociation of DNA from either complex, measured in vitro, is similar: approx 40% (DOPE) versus 50% (DPPE). Accordingly, the limiting step in the overall transfection pathway is likely at the level of DNA translocation across the endosomal membrane. Since DNA is nearly as efficiently dissociated from DOPE-containing lipoplexes as from those containing DPPE, but not DOPC (Fig. 5), our data emphasize that the presence of the ethanolamine head group promotes DNA release, when the lipoplexes interact with their (acidic phospholipid-containing) target membrane. This conclusion is consistent with previous suggestions that the PE head group of DOPE facilitates DNA release from lipoplexes by lowering the efficiency of interaction between the cationic lipid and the DNA [13]. However, to accomplish release, adaptation of the hexagonal phase, at least to some extent, appears essential as no significant dissociation is observed from lamellar phase complexes, i.e., for example from SAINT-2/DOPE lipoplexes in the absence of salt (Fig. 5).

Next to a diminished transfection efficiency with SAINT-2/DPPE lipoplexes containing DNA, we observed a reduction in ODN delivery with SAINT-2/DPPE of approx 60%, compared to that obtained for SAINT-2/DOPE lipoplexes. Recently, it has been described that cationic lipids that display a lamellar organization in isolation may assume a hexagonal conformation after interaction with negatively charged lipids, such as PS, that are lamellar in isolation as well [21]. Previously, we showed that SAINT-2/DOPE lipoplexes assume a hexagonal phase both in the absence and in the presence of anionic vesicles. Therefore, we verified whether SAINT-2/DPPE lipoplexes could form a hexagonal phase following an interaction with PS:PC:PE vesicles. As shown by SAXS measurements, SAINT-2/DPPE lipoplexes, which in bulk solution exhibit a lamellar phase, adopted a mixed lamellar–hexagonal phase after interaction with anionic vesicles. This could explain the diminished ability of SAINT-2/DPPE compared to SAINT-2/DOPE lipoplexes to mediate release of either plasmids or ODNs from the endosome, although a partial transformation to a non-lamellar phase apparently suffices for an efficient dissociation of the nucleic acid from the complex as such. It is thus tempting to suggest that the occurrence of only a mixed lamellar–hexagonal phase in case of the DPPE/SAINT-2 lipoplexes is a proper reflection of its reduced ability to intermingle with endosomal target membrane lipids, necessary to destabilize the endosomal membrane, compared to the pure hexagonal phase formed in the case of the DOPE/SAINT-2 system. These data are in agreement with a recent study by Tarahovsky et al. By varying the anionic target liposomes, these authors found a correlation between the efficiency of DNA release and the liquid crystalline phase of the lipoplex–anionic lipid mixture [24].

The efficiency of nuclear accumulation of ODN, as observed in control cells, correlated very well with the relative transfection efficiencies obtained with pEGFP-N1 complexed with SAINT-2/DOPE and SAINT-2/DPPE. Therefore, it seems that the outcome of the in vitro DNA release assay, showing nearly equal efficiencies of DNA dissociation from either complex, does not necessarily correlate with transfection (plasmid) or nuclear delivery (ODN) efficiency, implying an additional barrier in the overall mechanism of DNA/ODN delivery, presumably reflecting passage across the endosomal membrane. The relatively poor nuclear delivery of ODNs as accomplished by SAINT-2/DPPE lipoplexes, which is strongly promoted, following artificial rupture of the endosomal membrane (Fig. 8), is entirely consistent with this notion.

We therefore hypothesize that next to a role in the dissociation of DNA from lipoplexes, the hexagonal phase plays in particular a prominent role in destabilizing the endosomal membrane. The formation of a hexagonal phase at the level of the lipoplex interacting with the endosomal membrane is crucial for bringing about sufficient perturbation and/or destabilization of the endosomal membrane and efficient release of DNA from the lipoplexes, which is then ejected into the cytoplasm.

**Materials and Methods**

*Preparation of vesicles and lipoplexes for transfection, electron microscopy, and X-ray diffraction measurements.* The cationic lipid saint-2 was synthesized as described elsewhere [25]. A methanolic solution of cationic lipid mixed in a 1:1 molar ratio with DOPE or DPPE (Avanti Polar Lipids, Inc., USA) was dried under a stream of nitrogen. The residual solvent was removed under vacuum. The lipid film was dissolved in milliQ water (lipid concentration 1 mm) at room temperature, except in the case of DPPE, for which the sample was heated by a flow of hot air. Subsequently, the lipid formulations were vortexed and sonicated to clarity in a bath sonicator.

For transfection, lipoplexes were prepared in serum-free cell culture medium (DMEM; Gibco). Medium (0.5 ml) containing 1 μg of pEGFP-N1 (Clontech) was added to an equal volume of medium containing the liposome prepared from cationic lipid/helper lipid, as described above. Lipoplexes with a +/- molar charge ratio (cationic lipid/DNA) of 2.5/1 were prepared at 37°C. One day before transfection COS-7 cells were seeded into six-well plates at 3 x 10⁵ cells per well. Cells were incubated with lipoplexes for 4 h at 37°C. Then the transfection medium was replaced by complete cell culture medium. After 24 h the medium was refreshed, while after a total of 48 h cells were screened for reporter gene expression by FACS-analysis (Elite, Coulter, λex 488 nm, λem 530 nm, 5000 events).

For investigation by electron microscopy and X-ray diffraction, SAINT-2/DOPE and SAINT-2/DPPE vesicles were prepared in Millipore water at a 20–50 mM end concentration. The vesicles were diluted in HBS buffer at 37°C (end concentration of 10–15 mM lipid in HBS). Lipoplexes (±/– molar charge ratio 2.5/1) were made at the same temperature by
adding an equal volume of DNA diluted in HBS (end concentration of 0.7–1.0 µg/µl DNA in HBS).

**Monolayer behavior of SAINT-2/DOPE, and SAINT-2/DPPE mixtures.** On a film balance of the Wilhelmy type, lipids solubilized in chloroform at a final concentration of 1 µM were spread over a subphase of borate buffer (pH 7.4) at 25 °C. After spontaneous evaporation of the chloroform phase, the lipid monolayer formed. Subsequently, lipid isotherms were measured to determine the lipid phase, i.e., the gel or liquid crystalline phase. The monolayer was then compressed to a surface tension of 25 mN/m, and 20 µg of DNA (pEGFP-N1; Clontech) was injected into the subphase. The formation of cationic lipid at SAINT-2/DOPE and SAINT-2/DPPE (1:1) monolayers by the negatively charged DNA was visualized. The quality and morphology of the lipid monolayer was characterized by epifluorescence microscopy (Olympus BX2; Planegg, Germany). NBD-PC (Sigma, Germany) was used as a fluorescent dye in the lipid mixture at a concentration of 1 mol%. This dye preferentially dissolves in the liquid-expanded phase.

Inclusion of a fluorescent (DPhP) analog of SAINT-2 in the monolayer resulted in the formation of bright domains. Monolayers with interacting phospholipid were transferred to silicon wafers by the Langmuir–Schaefer technique and examined by atomic force microscopy. Alternatively, lipoplexes were made in bulk phase. Small unilamellar vesicles of SAINT-2/DOPE and SAINT-2/DPPE were solubilized in HBS buffer (150 mM NaCl, 10 mM Hepes, pH 7.4) at a final concentration of 30 µM. An equal volume of buffer, containing 2 µg/ml pDNA, was mixed with the lipid suspension and incubated at room temperature to allow lipoplex formation.

**Atomic force microscopy of lipid vesicles and lipoplexes.** Atomic force microscopy was performed on a Digital Nanoscope Illa Dimension 5000 (Digital Instruments, Santa Barbara, CA, USA). The microscope was vibration-damped. Commercial pyramidal Si34N tips (NCH-W; Digital Instruments) on a cantilever with a length of 125 µm were used in a 1:1 molar ratio with DOPC, DOPE, or DPPE were made in water or 1 mM Hepes, pH 7.4 at a final concentration of 30 µM. An equal volume of buffer, containing 2 µg/ml pDNA, was mixed with the lipid suspension and incubated at room temperature to allow lipoplex formation.

**Transmission EM of SAINT-2/DPPE-DNA.** SAINT-2/DPPE vesicles and lipoplexes, prepared as described above, were transferred to copper grids and frozen by rapid injection into liquid nitrogen. Samples were frozen by rapid injection into liquid nitrogen. Samples were aspirated and the cells were examined by fluorescence microscopy following a 1-h incubation of COS-7 cells with SAINT-2/DOPE and SAINT-2/DPPE lipoplexes, cells were treated with pinocytic cell loading reagent (Molecular Probes) following the manufacturer’s instructions. Briefly, cells were incubated in a 1.5 M sucrose solution for 7 min at 37 °C, followed by an incubation in hypotonic medium (cell culture medium:distilled water 6:4) for 4 min (37 °C). Cells were left to recover for half an hour in complete cell culture medium and subsequently processed for investigation by fluorescence microscopy.

**REFERENCES**


