Phase Behavior of Cationic Amphiphiles and Their Mixtures with Helper Lipid Influences Lipoplex Shape, DNA Translocation, and Transfection Efficiency

Inge S. Zuhorn,*† Volker Oberle,* Willy H. Visser,* Jan B. F. N. Engberts,‡ Udo Bakowsky,* Evgeny Polushkin,§ and Dick Hoekstra*

*Department of Membrane Cell Biology, University of Groningen, 9713 AV Groningen, The Netherlands; and †Biomade Technology Foundation; ‡Physical Organic Chemistry Unit, Stratingh Institute, University of Groningen; and §Department of Polymer Chemistry, University of Groningen and Dutch Polymer Institute, Laboratory of Polymer Chemistry, Materials Science Centre, 9747 AG Groningen, The Netherlands

ABSTRACT Cationic lipids are widely used for gene transfection, but their mechanism of action is still poorly understood. To improve this knowledge, a structure-function study was carried out with two pyridinium-based lipid analogs with identical headgroups but differing in alkyl chain (un)saturation, i.e., SAINT-2 (diC18:1) and SAINT-5 (diC18:0). Although both amphiphiles display transfection activity per se, DOPE strongly promotes SAINT-2-mediated transfection, but not that of SAINT-5, despite the fact that DOPE effectively facilitates plasmid dissociation from either lipoplex. This difference appears to correlate with membrane stiffness, dictated by the cationic lipid packing in the donor liposomes, which governs the kinetics of lipid recruitment by the plasmid upon lipoplex assembly. Because of its interaction with the relatively rigid SAINT-5 membranes, the plasmid becomes inappropriately condensed, which results in formation of structurally deformed lipoplexes. This structural deformation does not affect its cellular uptake but, rather, hampers plasmid translocation across endosomal and/or nuclear membranes. This is inferred from the observation that both lipoplexes effectively translocate much smaller oligonucleotides into cells. In fact, SAINT-5/DOPE-mediated transfection is greatly improved when, before lipoplex assembly, the plasmid is stabilized by condensation with polylysine. The results emphasize a role of the structural shape of the plasmid in gaining cytosolic/nuclear access. Moreover, it has been proposed that such a translocation is promoted when the lipoplex adopts the hexagonal phase, and data are presented that demonstrate that the lamellar SAINT-5/DOPE lipoplex adopts such a phase after its interaction with acidic phospholipid-containing membranes.

INTRODUCTION

To rationally design and to optimize cationic lipid formulations for efficient gene delivery in therapeutics and fundamental cell biological studies alike, many investigations aim at defining a structure-function relationship of cationic amphiphiles. Thus far the outcome of such studies has been unsatisfactory in terms of the ability to predict the functional consequences of a change in chemical structure of the amphiphile on its transfection potential. Clearly, alterations in the hydrophobic region of a particular amphiphile markedly influence its transfection efficacy. Thus, a decrease in acyl chain length or its degree of saturation, which generally results in an increase in membrane fluidity, often leads to an increase in transfection efficiency of the lipoplexes (Felgner et al., 1994; Bennett et al., 1998; Byk et al., 1998). The increase in transfection potential of lipoplexes prepared from fluid lipids as opposed to those assembled with solid lipids has been attributed to an enhanced capability of the fluid amphiphile to condense DNA (Felgner et al., 1994; Reich et al., 1991; Spector and Schnur, 1997). Apparently, besides the electrostatic interaction between the cationic headgroups of the amphiphile and the anionic phosphates of the DNA, packing properties of cationic lipids are important for the condensation of DNA. Although this concept has gained support in several studies (Bennett et al., 1998; Akao et al., 1996), no consensus has been reached yet (Balasubramaniam et al., 1996).

In formulations that contain unsaturated cationic lipids, the inclusion of the helper lipid dioleoylphosphatidylethanolamine (DOPE) often substantially improves the transfection efficiency (Bennett et al., 1998; Meekel et al., 2000). The mechanism by which DOPE exerts its catalytic activity is still poorly defined, but prevailing evidence suggests that its hexagonal-phase-forming propensity likely plays a major role (Smisterova et al., 2001). However, although the negative bilayer curvature resulting from such transitions promotes membrane fusion, there is as yet no compelling evidence that would support direct involvement of a fusion mechanism in lipoplex-mediated gene delivery (Stegmann and Legendre, 1997; Harvie et al., 1998; Scherman et al., 1998).

Because transfection obviously also requires the release of DNA from lipoplexes, a facilitating role of DOPE in this event has also been proposed (Harvie et al., 1998; Bally et al., 1999). The amine group of DOPE may interact with the DNA phosphate groups in a similar way as the cationic lipid does. Such an interaction could weaken the binding between cationic lipids and DNA and therefore make phosphatidylethanolamine (PE)-containing formulations more sus-

Submitted March 13, 2002, and accepted for publication June 11, 2002.

Address reprint requests to Dr. Dick Hoekstra, Department of Membrane Cell Biology, University of Groningen, A. Deusinglaan 1, 9713 AV Groningen, The Netherlands. Tel.: 31-50-363-2741; Fax: 31-50-363-2728; E-mail: d.hoekstra@med.rug.nl.

0006-3495/02/10/2096/13 $2.00

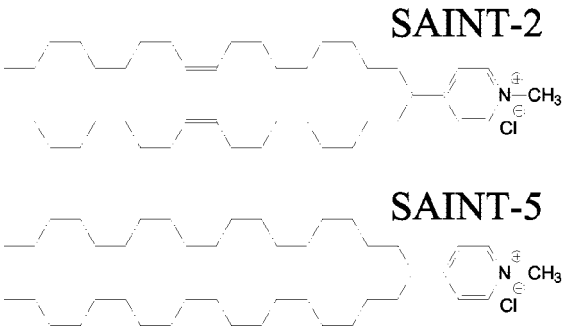

FIGURE 1 The molecular structures of SAINT-2 and SAINT-5 amphiphiles.

ceptible to (cellular) factors that promote disassembly. Because previous work suggested that the beneficial effect of DOPE very much depends on its formulation with unsaturated cationic lipids (van der Woude et al., 1997), the present work was undertaken to obtain further insight into this issue. To this end, we synthesized two pyridinium-based lipid analogs with identical headgroups but differing in alkyl chain unsaturation, i.e., SAINT-5 (C18:0) and SAINT-2 (C18:1) (Fig. 1). Physical properties as well as the transfection potential of the two cationic amphiphiles, with and without the helper lipid DOPE, were compared. The data reveal that neither the fusion-promoting properties of DOPE nor a cationic lipid-dependent facilitation of DNA release can explain the observed differences in transfection efficiency. Although DOPE appears to partly relieve the structural rigidity of cationic lipid membranes, the interaction of plasmid with such poorly deformable cationic lipid membranes leads to structural deformation and decondensation of the DNA instead of efficient lipoplex assembly. We propose that as a result, inefficient plasmid translocation across endosomal and/or nuclear membranes occurs, which thus hampers transfection efficiency.

MATERIALS AND METHODS

Cells and plasmid

COS7 cells were cultured in Costar flasks in Dulbecco's modified Eagle's medium (DMEM; Gibco, The Netherlands), containing 7% fetal calf serum, 2 mM L-glutamine (Gibco), 100 U/ml penicillin (Gist-Brocades, Delft, The Netherlands), and 100 mg/ml streptomycin (Biochemie, Kundl, Austria) at 37°C and 5% CO_2. EGFP-N1 plasmid DNA (Clontech, Palo Alto, CA) was isolated from *Escherichia coli* using a Qiagen plasmid kit (Qiagen, Chatsworth, CA) following the manufacturer's instructions.

Preparation of liposomes and lipoplexes

The cationic lipids *N*-methyl-4(dioleyl)methylpyridiniumchloride (SAINT-2) and *N*-methyl-4(distearyl)methylpyridiniumchloride (SAINT-5) were synthesized as previously described (Meekel et al., 2000; van der Woude et al., 1997). A methanolic solution of pure cationic lipid or cationic lipid mixed in a 1:1 molar ratio with DOPE (Avanti Polar Lipids, Birmingham, AL) was dried under a stream of nitrogen. The residual solvent was removed under vacuum for 2 h. The lipid film was dissolved in Millipore water (final lipid concentration, 1 mM) at room temperature except in the case of SAINT-5 where the sample was heated above its phase transition temperature. (T_m = 43°C) 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): 0.5 ml of medium, containing 1 μg of pEGFP-N1 (Clontech) was added to an equal volume of medium containing cationic lipid. Lipoplexes with a molar charge ratio (cationic lipid/DNA) of 1:1, 2.5:1, and 5:1 were prepared at 37°C and 48°C. One day before transfection, COS7 cells were seeded into six-well plates at 3×10^5 cells per well. Cells were incubated with lipoplex for 4 h at 37°C, and the transfection medium was then replaced by complete cell culture medium. After 24 h, the medium was refreshed, and after another 24 h, the cells were screened for reporter gene expression by FACS analysis (Elite, Coulter, Miami, FL; λ_{ex} = 488 nm, λ_{em} = 530 nm; 5000 events).

Interaction of cationic lipid monolayers with pDNA

On a film balance of the Wilhelmy type, 20 μl of the pure cationic amphiphiles, solubilized in chloroform at a final concentration of 1 mM, was spread over a subphase of ultrapure water. After 60 s, the chloroform was evaporated and a monolayer of the lipids was formed. To prepare equal starting conditions, all monolayers were compressed at 25°C to a surface tension of 25 mN/m. At this surface tension, both SAINT-2 and SAINT-5 and their mixtures with DOPE are in the fluid phase. After injecting 20 μg of plasmid through the amphiphile monolayer into the subphase, the surface tension changed to lower pressures. After ~30 min, the systems reached an equilibrium-like state as described previously (Oberle et al., 2000). To monitor the differences in complex formation, the monolayer with interacting plasmid was transferred by the Langmuir-Schaefer technique (Hagting et al., 1999) to silicon wafers. After cooling for 3 min on air in a box of dry ice, the sample was examined by atomic force microscopy (AFM) in the tapping mode (Digital Instruments, Santa Barbara, CA).

Fluorescence film balance studies

Lipid monolayers containing 0.5 mol% *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-aminophosphatidylethanolamine (*N*-NBD-PE) were prepared as described above (Möhwald, 1990). *N*-NBD-PE partitions exclusively in fluid phases and is therefore excluded from crystalline phases, which appear as black areas in a microscopic image of the monolayer. The distribution of the fluorescent lipid marker was determined by video imaging, using an Olympus fluorescence microscope at ×100 magnification.

AFM of lipoplexes

The AFM studies were carried out as described in detail elsewhere (Oberle et al., 2000). Briefly, complexes were transferred onto silicon wafers, which were used as sample holders, by a dipping procedure. Microscopic images were taken with a Digital Nanoscope IIIa Dimension 5000 (Digital Instruments). The microscope was mounted in an isolated steel box and vibration damped. For the measurements, conventional pyramidal Si3 N4 tips were used, attached to a cantilever with a length of 100 μm. To prevent damage of the sample surface and to allow repeated examination of the same sample region, we used the tapping mode (Oberle et al., 2000; Hansma et al., 1993). A force between 100 and 300 pN at a tapping frequency of ~9 kHz and oscillation amplitude of 20 nm was applied to the sample. The scan speed was proportional to the scan size, and the scan frequency was between 0.5 and 1.5 Hz. Images were obtained by displaying the amplitude signal of the cantilever in the trace direction and the

height signal in the retrace direction, both signals being simultaneously recorded. The results were visualized either in height or in amplitude mode.

Membrane-perturbing activity of cationic lipid and lipoplex

Erythrocytes were freshly isolated from human blood. Erythrocytes (1×10^8) were added to lipid vesicles or lipoplex, containing 4 μg of DNA (volume 0.6 ml), and incubated for 1 h at 37°C or 48°C. At 0, 10, 15, 30, and 60 min, 0.4 ml of precooled Hanks' balanced salt solution (Calbiochem, La Jolla, CA) was added, and the samples were centrifuged in a tabletop centrifuge (4°C for 3 min at 13,000 $\times$ *g*). The supernatants were collected, and absorption at 540 nm was measured in a spectrophotometer to determine the extent of hemoglobin release. The maximal amount of hemoglobin release was determined after complete lysis of the erythrocytes with 1% (v/v) Triton X-100.

Release of DNA from lipoplexes by anionic vesicles monitored by an online fluorometric assay

A picogreen solution was prepared in HBS (150 mM NaCl, 10 mM Hepes, pH 7.4) according to the manufacturer's instructions (Molecular Probes, Eugene, OR; dilution, 1:200). Lipoplexes containing 0.5 μg of pDNA were prepared in HBS in a total volume of 100 μl at 37°C and 48°C and added to the picogreen solution. Subsequently, phosphatidylserine (PS)/phosphatidylcholine (PC)/PE (1:1:2) liposomes were added in a fivefold molar excess. The increase in fluorescence upon addition of the anionic vesicles, indicating the presence of accessible/free DNA, was measured online with a fluorometer (Perkin-Elmer, Norwalk, CT; LS55; λ_{ex} = 502 nm, λ_{em} = 520 nm). Triton X-100 was added at the end of each measurement to obtain the 100% value of fluorescence. To verify the efficiency of detergent-induced DNA release from lipoplexes, the level of fluorescence of the picogreen-labeled free DNA in the presence of PS/PC/PE liposomes and Triton X-100 was measured as well. The level thus obtained was indistinguishable from that obtained for lipoplexes, following addition of detergent after addition of the PS/PC/PE liposomes, indicating a complete release of the DNA from lipoplexes upon addition of Triton X-100.

Release of DNA from lipoplexes by anionic vesicles determined by a quantitative gel retardation assay

Lipoplexes containing 1 μg of pDNA were made in HBS and incubated with a fivefold molar excess of anionic vesicles (PS/PC/PE) for 15 min at 37°C and 48°C. Subsequently, the samples were loaded onto a 0.8% agarose gel containing 1.25 μM ethidium bromide. A voltage of 100 V was applied over the gel, immersed in a 1X TBE buffer (0.045 M tris-borate/0.001 M EDTA, pH 8.3), for 30 min (Zuhorn et al., 2002). The amount of DNA that migrated into the gel was visualized by UV illumination and quantified with computer software (www.totallab.com).

Accumulation of oligonucleotides (ODN) in cell nuclei after incubation of cells with lipid-ODN complexes

COS7 cells were plated 1 day before the experiment in 12-well plates at 1.5×10^5 cells/well. Lipoplexes composed of 15 nmol of SAINT-2/DOPE or SAINT-5/DOPE and 1 μl of fluorescein isothiocyanate (FITC)-ODN were prepared as previously described (Shi et al., 2001). Cells were incubated for 5 h with the lipoplexes and subsequently examined by fluorescence microscopy. Pictures were taken with an Olympus camera.

Small-angle x-ray scattering (SAXS) of SAINT-2/DOPE and SAINT-5/DOPE lipoplexes after interaction with anionic vesicles

Lipoplexes composed of 300 nmol of lipid and 20 μg of DNA were allowed to form in 200 μl of 150 mM NaCl/10 mM Hepes buffer for 10 min at 37°C. Subsequently, PS/PC/PE (1:1:2) anionic vesicles were added in a fivefold molar excess, and the mixture (final volume, 230 μl) was incubated for 15 min at 37°C. Subsequently, the samples were centrifuged at 15,000 rpm for 30 min to pellet the aggregates and stored under argon at 4°C for 2 days. SAXS measurements were performed at 20°C on a Nanostar device (Bruker AXS and Anton Paar), as described in detail elsewhere (Smisterova et al., 2001). Instead of glass capillaries, a metal cell covered with two Kapton windows was used.

Transfection with SAINT-5/DOPE-poly-L-lysine-DNA complexes

Lipoplexes were prepared as described above with the exception that the cation poly-L-lysine was added to the DNA before its addition to the lipid formulation. Lipoplexes were composed of 1 μg of pEGFP-N1 mixed with 0.75 or 1.25 μg of poly-L-lysine (Sigma Chemical Co., St. Louis, MO; molecular weight, 30,000–70,000) and complexed with 15 nmol of SAINT-5/DOPE. As a control, cells were transfected with poly-L-lysine-DNA complexes (0.75 μg of poly-L-lysine, 1 μg DNA). Transfection of COS7 cells was performed and quantified as described above.

RESULTS

DOPE promotes transfection efficiency of fluid-phase cationic lipids

The inclusion of DOPE in SAINT-2 lipoplexes enhances the transfection efficiency at all molar charge ratios tested (1:1, 2.5:1, and 5:1). At a charge ratio of 2.5:1 the enhancing effect of DOPE on transfection efficiency is highest and the transfection efficiency with SAINT-2/DOPE is optimal. For SAINT-5, the liquid-crystalline-phase transition temperature (T_m), which is 43°C, the inclusion of DOPE has no beneficial effect on the transfection efficiency. Interestingly, without the inclusion of DOPE the differences in transfection efficiency with SAINT-2 and SAINT-5 lipoplexes become negligible with increasing charge ratio (5:1), whereas at lower ratios the fluid SAINT-2 reaches efficiencies that are almost an order of magnitude higher (at a charge ratio of 1:1) than those obtained for SAINT-5 (Fig. 2). Finally it should be noted that the extent of lipoplex-cell association and internalization of either complex, determined by measuring the cell-association of *N*-Rh-PE-labeled lipoplexes before (total cell association) and after (internalized fraction) the addition of the *N*-Rh-PE-quencher trypan blue, was very similar (not shown).

To investigate the strongly promoting effect of DOPE on fluid rather than solid SAINT lipoplex-mediated transfection, we next examined the effect of DOPE on the biochem-

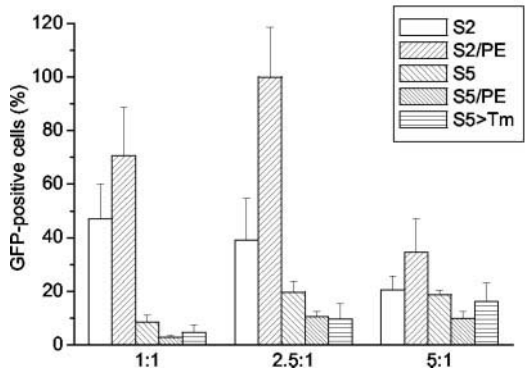

FIGURE 2 The influence of the helper lipid DOPE on the transfection efficiencies obtained with unsaturated SAINT-2 and saturated SAINT-5 cationic amphiphiles. COS7 cells were transfected with lipoplexes, composed of SAINT-2 or SAINT-5 with or without DOPE, at a charge ratio of 1:1, 2.5:1, and 5:1. Two days after transfection the amount of reporter gene (GFP)-positive cells were quantified by FACS analysis. Values represent the means of four individual experiments in duplicate. The transfection efficiency with SAINT-2/DOPE at a charge ratio of 2.5:1 was set as 100%. Error bars indicate the SD.

ical and biophysical characteristics of the cationic lipids SAINT-2 and SAINT-5. First, we characterized the interaction of plasmid with the various cationic lipid formulations in a monolayer system and by AFM.

SAINT-5 is unable to form lipoplexes under equilibrium conditions

After adding plasmid to the subphase of a SAINT-2 monolayer the surface pressure drops slowly and reaches equilibrium after 30 min (Oberle et al., 2000). The ensuing formation of lipoplexes was then visualized by AFM. As shown in Fig. 3 *A*, the SAINT-2/DNA lipoplexes display an ellipsoid-like shape with a length of 250 nm and a width of 50 nm. The surface of these lipoplexes is smooth, and noncomplexed DNA is visible. Single lipoplexes may aggregate. Interestingly, as noted previously (Oberle et al., 2000), the size of the nonaggregated lipoplex very closely resembles that of the pure supercoiled plasmid. By contrast, when similar experiments were carried out with SAINT-5, lipoplex assembly seems to be frustrated. Examination by AFM (Fig. 3 *B*) reveals that the supercoiled DNA structure became unfolded, showing the appearance of a string, which, given its diameter (cf. Oberle et al., 2000), is covered by lipid (see below). The observed structures are remarkably similar to those described previously by Sternberg et al. (1994). Immediately after addition of plasmid DNA to the subphase of the film balance, the surface pressure of the SAINT-5 monolayer sharply drops and the amphiphile monolayer becomes crystalline. This was revealed by the exclusion of the fluorescent lipid analog *N*-NBD-PE from the SAINT-5 monolayer, as reflected by the formation of black domains (Fig. 3, *C* versus *D*).

After injection of plasmid DNA into the water phase, lipoplex formation with SAINT-2 (Fig. 3 *C*) leads immediately to the collapse of the monolayer because the cationic lipid will be recruited on the plasmid surface, driven by electrostatic interactions, and the ensuing complex subsequently enters the aqueous phase. This will diminish the pool of available cationic lipids for maintaining the monolayer (at constant pressure) and hence cause its collapse.

In case of SAINT-5 (Fig. 3 *D*), the interaction with DNA does not result in the formation of complexes that, as in the case of SAINT-2, enter the subphase. Instead, the monolayer crystallizes out with the plasmids merely remaining attached via electrostatic interactions. Thus, in contrast to SAINT-2, SAINT-5 appears unable to dissociate from the lipid monolayer, necessary for recruitment onto the plasmid surface, implying that appropriate lipoplex assembly and effective DNA condensation do not take place. Indeed, whereas free plasmids display a typical supercoiled structure (Oberle et al., 2000), the interaction of plasmid with SAINT-5 results in a loss of the supercoiled conformation, as shown in Fig. 3 *B*.

Lipoplex formation in bulk phase reflects lipid-DNA interactions under equilibrium conditions

Vesicles made from pure SAINT-2 associate rapidly with plasmid DNA, leading to the formation of ellipsoid-shaped particles (Fig. 4A), similarly as observed under equilibrium conditions. However, due to the enhanced

FIGURE 3 Lipoplex formation monitored in a monolayer system. A monolayer of cationic lipids on a surface of ultrapure water was prepared, and plasmid DNA was subsequently injected into the subphase. (*A*) Lipoplexes of SAINT-2 and plasmid are visualized with AFM, after the transfer of the complex from the film balance to a silicon wafer. (*B*) The same experiment (as in *A*) was carried out to monitor the potential assembly of lipoplexes, consisting of SAINT-5 as the cationic lipid. Images *A* and *B* represent an area of 750 nm in length by 750 nm in width. Images *C* and *D* are fluorescence images of the surface of the film balance after lipoplex formation (diagonal, 110 μm). In this case 1 mol % *N*-NBD-PE, as a fluid-phase marker, was mixed with the cationic lipid, before monolayer formation. Bright areas, representing a random distribution of the fluorescent lipid probe, reflect fluid regions, whereas black areas are crystalline, because of phase separation of the probe from these regions. (*C*) The fluid character of SAINT-2 lipoplexes is apparent and the cloudy appearance is indicative of a collapse of the monolayer into a three-dimensional structure. (*D*) The total fraction of the SAINT-5 lipid is in the crystalline phase, causing a phase separation of the fluid marker *N*-NBD-PE, which is visible as intense bright dots. The monolayer collapse is not visible on the film balance. (*E*) The isotherm of SAINT-2 reveals a fluid behavior with a lift-off at 170 Å^2 molecular area and a collapse of the monolayer at 75 Å^2 molecular area. (*F*) By contrast, SAINT-5 behaves entirely crystalline at the same temperature and shows a lift-off at 75 Å^2 molecular area and a collapse of the monolayer at ~35 Å^2 molecular area.

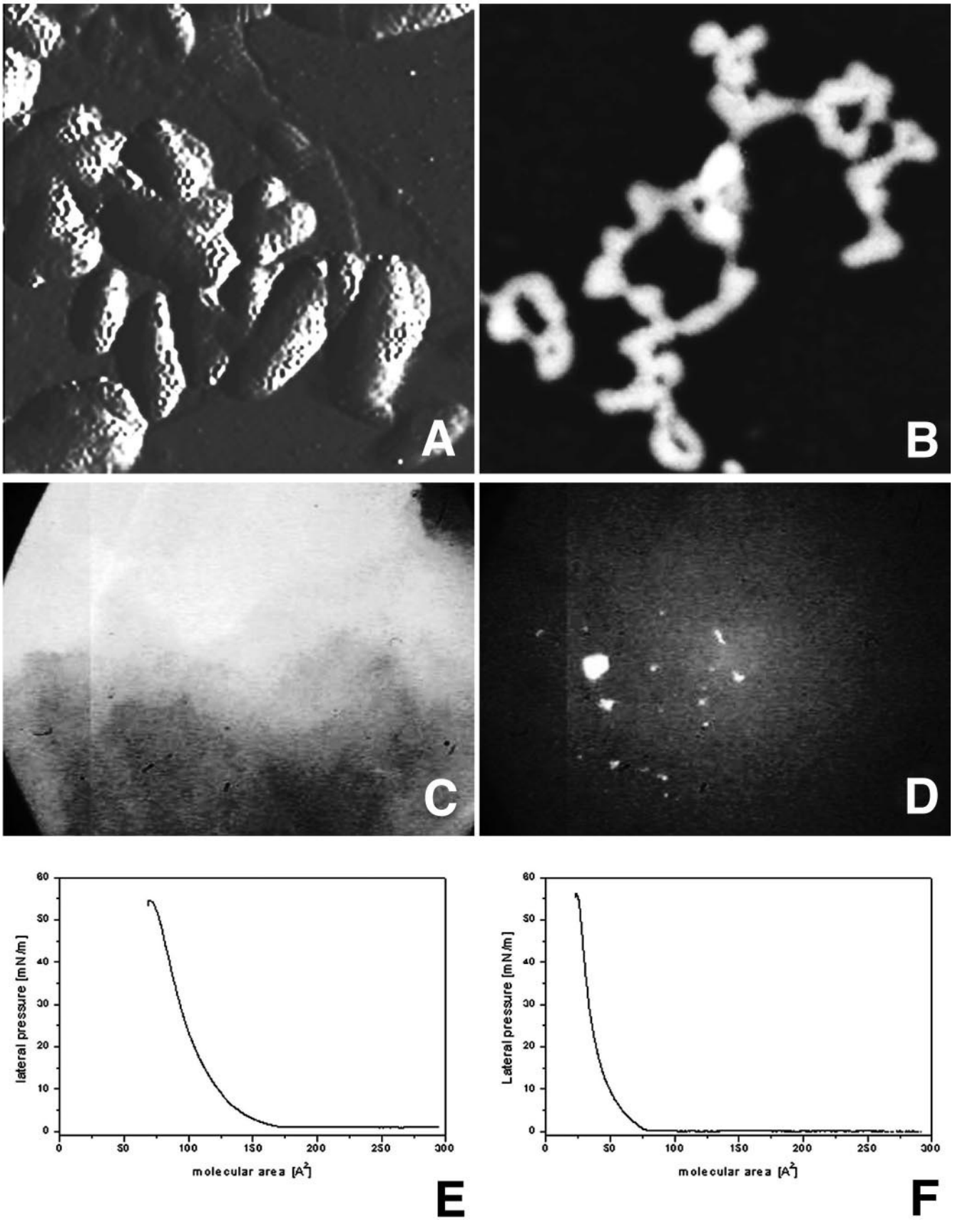
A
B
C
D
lateral pressure [mN/m]
molecular area [A²]
E
Lateral pressure [mN/m]
molecular area [A²]
F

FIGURE 3.

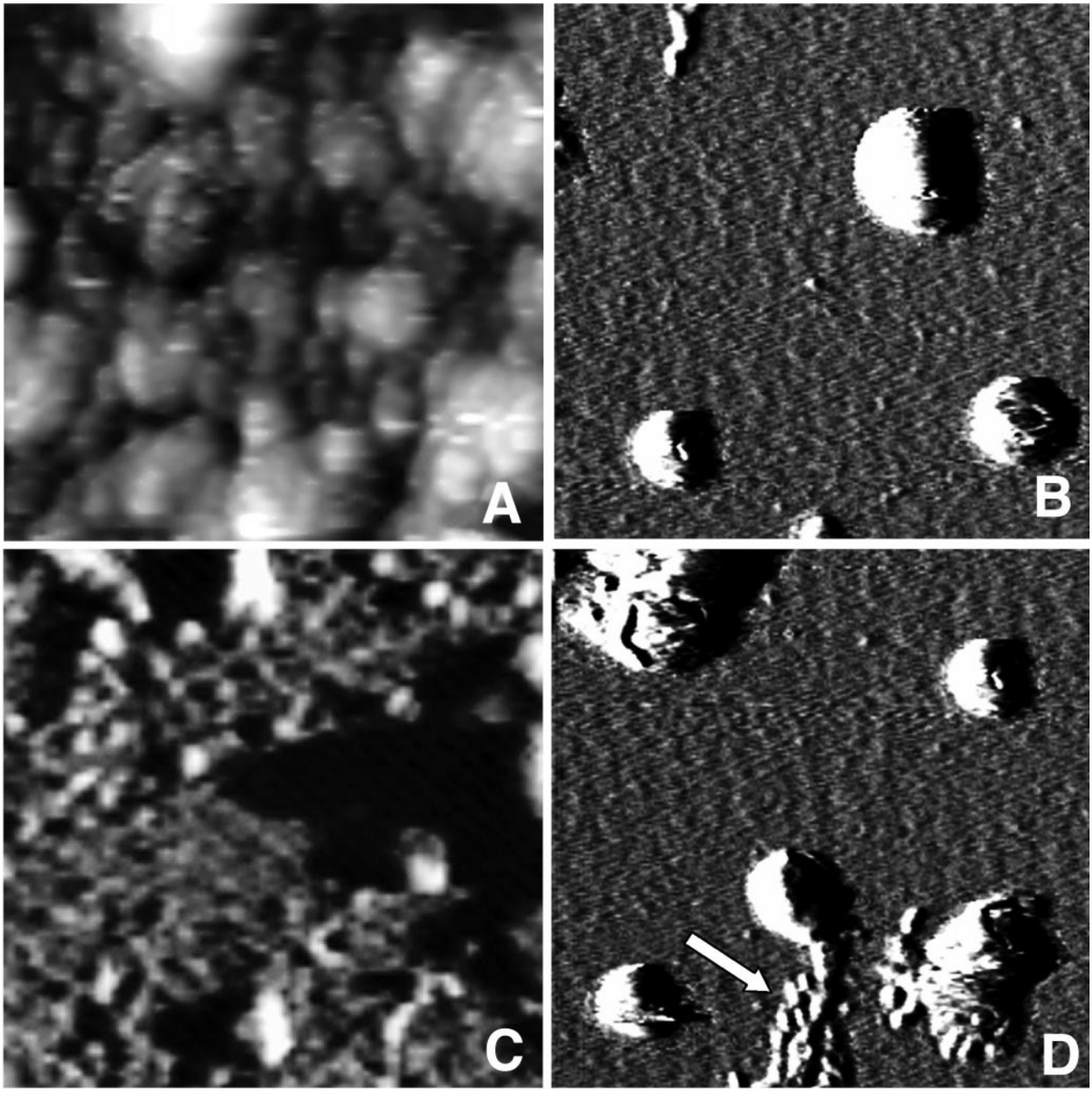

FIGURE 4 Lipoplex formation in bulk phase. Lipoplexes with a 2.5:1 molar charge ratio of the cationic lipid and the plasmid DNA were formed at 25°C in bulk phase. The lipoplexes were subsequently transferred to silicon wafers and examined with AFM. (*A*) Lipoplexes made from pure SAINT-2 and plasmid are shown. They are ellipsoid and exhibit uncovered spots of the plasmid surface. (*B*) DOPE as helper lipid was included in the cationic liposomes (1:1 molar ratio). The lipoplexes are round and have a smooth surface without defects in the lipid layer. (*C*) Lipoplexes are shown, prepared from SAINT-5. Note the presence of uncovered DNA strands. (*D*) DOPE had been mixed with SAINT-5, and in this case a partial coverage of plasmid DNA is apparent, giving rise to a lipoplex-like structure with associated uncovered DNA strands. Images represent areas of 1×1 μm.

rate of lipoplex formation in bulk phase compared with that under equilibrium conditions, the particles are less well defined in their shape, displaying variation in size and showing occasionally the presence of noncomplexed DNA on the surface. In addition, lipoplexes prepared in bulk phase appeared to aggregate faster than the ones prepared under equilibrium conditions. When vesicles consisting of a mixture of SAINT-2 and DOPE (1:1 molar ratio) are incubated with plasmids, round-shaped lipoplexes with a diameter of $\sim$ 250 nm are formed (Fig.

TABLE 1 Percentage recovery of supercoiled plasmid DNA after DNAse exposure of the lipoplexes

	SAINT-2			SAINT-5		
	1:1	2.5:1	5:1	1:1	2.5:1	5:1
− DOPE	82.8 ± 14.4	73.2 ± 17.9	91.6 ± 7.7	83.5 ± 2.7	83.0 ± 13.2	92.2 ± 9.6
+ DOPE	86.1 ± 11.5	90.8 ± 9.1	102.3 ± 1.4	81.2 ± 22.2	80.6 ± 80.6	92.8 ± 4.6

Lipoplexes were composed of SAINT-2 or SAINT-5, with or without DOPE, at three different charge ratios. Values represent the means of three independent experiments (±SD).

4 *B*). In contrast to lipoplexes composed of pure SAINT-2, inclusion of DOPE leads to lipoplexes that are much smoother in appearance, whereas free DNA is not visible.

Addition of plasmid DNA to pure SAINT-5 vesicles causes formation of ill-defined structures (Fig. 4 *C*). Plasmids are visible, which appear coiled or relaxed, but the resolution is insufficient to determine whether or not these plasmids are lipid associated. Nevertheless, a highly distinct assembly pattern is evident when comparing the respective capacities of SAINT-2 and SAINT-5 to complex DNA. The morphology of lipoplexes prepared from a mixture of SAINT-5/DOPE (1:1) resembled that of lipoplexes prepared from SAINT-2 at similar conditions (cf. Fig. 4, *D* and *A*). However, noncomplexed DNA strands are occasionally seen, associated with the lipoplexes (arrow in Fig. 4 *D*), emphasizing the incomplete ability of SAINT-5 to condense and/or package plasmids.

Differences in cationic lipid-mediated condensation of plasmids is not reflected by differences in DNAse sensitivity

As the presence of seemingly uncondensed DNA in SAINT-5 lipoplexes was shown by AFM, we anticipated a diminished protection of DNA from DNAse in these lipoplexes, compared with that in SAINT-2 lipoplexes. However, as can be seen in Table 1, the DNAse sensitivity of SAINT-2 and SAINT-5 lipoplexes is similar. Lipoplexes composed of pure SAINT-2 show an almost complete protection of the DNA at a molar charge ratio of 5:1, whereas inclusion of DOPE at a fixed charge ratio only slightly improves the level of DNA protection compared with that in lipoplexes without DOPE. Quite unexpectedly, for SAINT-5 lipoplexes the level of protection against exogenous DNAse activity is very similar to that observed for SAINT-2, irrespective of the presence of DOPE (Table 1).

For transfection to occur, the lipoplex needs to cross the cellular membrane, either at the level of the plasma membrane or at the level of the endosome. Accordingly, we next examined the membrane-perturbing capacity of SAINT-2- and SAINT-5-containing vesicles and lipoplexes by comparing their ability to interact with red blood cells as a model membrane system, exploiting the release of hemoglobin as a measure of membrane-perturbing capacity.

Differences in membrane-perturbing capacity cannot account for differences in SAINT-2- versus SAINT-5-mediated transfection capacity

In Fig. 5 it can be seen that complexation of cationic lipid by DNA at a charge ratio of 2.5:1 results in a diminishment in membrane-perturbing activity of the lipid (cf. Fig. 5, *A* and *C*). At a charge ratio of 5:1 the difference in membrane-perturbing activity of the pure lipid compared with the lipid-DNA complex is less obvious (cf. Fig. 5, *B* and *D*). Possibly, at this high charge ratio noncomplexed lipid is present in the lipoplex formulation, resulting in an additional membrane-perturbing activity compared with that of the lipid-DNA complex itself.

At a charge ratio of 2.5:1, the membrane-perturbing activity of SAINT-2 and SAINT-5 lipoplexes is similar, irrespective of the presence of DOPE (Fig. 5 *C*). This indicates that the presence of DOPE as such does not enhance the membrane-perturbing activity of the lipoplex. Upon doubling of the charge ratio to 5:1, SAINT-5 lipoplexes show a twofold increase in membrane-perturbing activity compared with a fivefold increase for the other lipoplexes. However, when increasing the temperature above the T_m of SAINT-5 (43°C), a level of hemoglobin release is obtained, similar to that obtained with SAINT-2 lipoplexes. Interestingly, the lower membrane-perturbing capacity of SAINT-5 can also be compensated for by including DOPE (Fig. 5 *D*). This indicates that the remarkable differences in the transfection capacity of SAINT-2/DOPE and SAINT-5/DOPE cannot be accounted for by differences in membrane-perturbing capacity.

The aforementioned data indicate that the differences in transfection efficiency observed between the SAINT-2- versus SAINT-5-containing lipoplexes could not be explained in terms of differences in DNA protection, extent of lipoplex internalization, or membrane-perturbing capacity. Because the release of DNA from the lipoplex and into the cytoplasm of the cell is believed to represent one of the major barriers in the process of lipofection, this parameter was examined next.

Release of DNA from lipoplexes upon addition of anionic target vesicles

Lipoplex destabilization with anionic vesicles may potentially mimic the situation of endosomal escape of DNA (Xu and Szoka, 1996). The amount of DNA in lipoplexes before

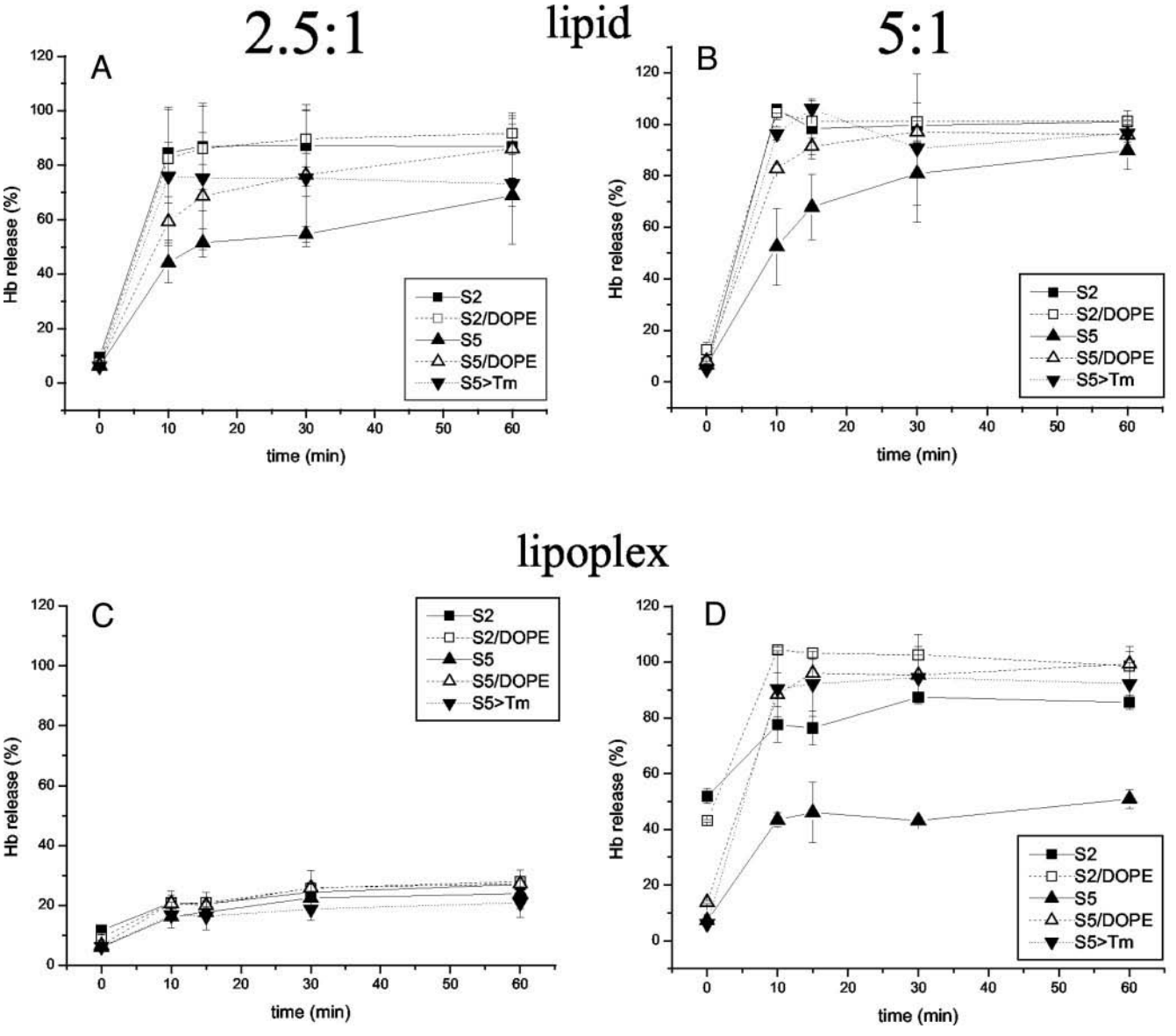

FIGURE 5 The membrane-perturbing activity of SAINT-2 and SAINT-5 lipid and lipoplex as determined with the hemoglobin release assay. Cationic liposomes and lipoplexes were incubated with erythrocytes, and their membrane-perturbing capacity was determined by monitoring the release of hemoglobin. (*A* and *B*) Extent of hemoglobin release induced with liposomes. (*C* and *D*) A similar amount of lipid was used to prepare lipoplexes with a charge ratio of 2.5:1 (*C*) and 5:1 (*D*), respectively. The absorption at 540 nm was measured with a spectrophotometer. Values represent the means of two individual experiments in duplicate. Error bars reflect the SD.

TABLE 2 Accessibility of DNA for picogreen in intact lipoplexes

	SAINT-2	SAINT-5	SAINT-5 (48°C)
− DOPE	9.03 ± 0.86	15.54 ± 1.34	8.23 ± 2.10
+ DOPE	20.33 ± 2.34	51.25 ± 21.41	ND

Results are expressed as percentage relative to the total fluorescence, obtained after addition of Triton X-100. Values represent the means of four independent experiments (±SD). ND = not determined.

(Table 2) and after (Fig. 6) the addition of PS/PC/PE vesicles was therefore measured, using the fluorescent DNA dye picogreen. As shown in Table 2, in the absence of DOPE, plasmid associated with SAINT-5 is only slightly more accessible for picogreen than plasmid associated with SAINT-2. Interestingly, when the accessibility of DNA in SAINT-5 lipoplex was determined at a temperature higher than the phase transition temperature of SAINT-5, the dye-accessible pools of DNA in SAINT-2 and SAINT-5 lipoplexes are essentially indistinguishable.

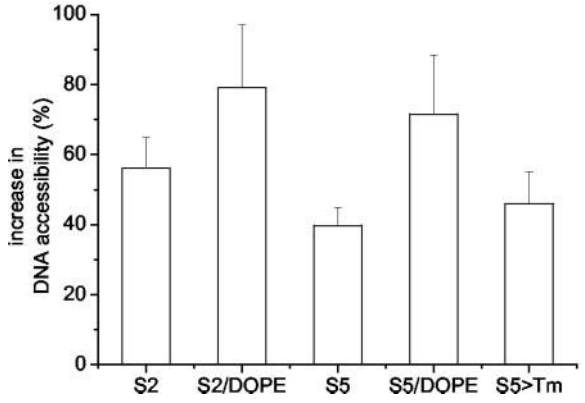

FIGURE 6 The interaction of lipoplexes with anionic phospholipid vesicles increases DNA accessibility. In lipoplexes, the increase in the DNA accessibility for picogreen upon addition of PS/PC/PE vesicles was measured online with a fluorometer. The 100% value represents the level of fluorescence obtained 5 min after the addition of 1% (v/v) Triton X-100. Values represent the means of four independent experiments. Error bars indicate the SD.

When DOPE is included in the lipoplexes, distinct structural changes become clearly apparent, as reflected by an enhancement in the accessibility of the plasmid for labeling with picogreen. The enhancement was highest in the case of SAINT-5, implying a less effective packing of the DNA, which is apparently strongly affected by the presence of DOPE. Note the subtlety of this perturbation as the DNAse sensitivity, as demonstrated above, is not significantly different.

When incubated with anionic vesicles, the increase in DNA accessibility is highest in DOPE-containing lipoplexes, implying an enhanced release of DNA from DOPE-containing lipoplexes (Fig. 6). To be able to distinguish between dye-accessible DNA still bound to lipid and free (released) DNA, we quantified the amount of free DNA in the different PS/PC/PE-treated lipoplex samples by means of agarose gel electrophoresis (Table 3). The data reveal, in good agreement with the data obtained with the fluorometric assay, an enhanced release of DNA from DOPE-containing lipoplexes. Thus, the absolute amount of DNA released from lipoplexes is merely determined by the presence of the helper lipid DOPE. However, the effective release from SAINT-2/DOPE and SAINT-5/DOPE complexes is not significantly different.

TABLE 3 Amount of supercoiled DNA released from lipoplexes upon addition of anionic vesicles

	SAINT-2	SAINT-5	SAINT-5 (48°C)
− DOPE	8.5 ± 4.07	10.01 ± 4.28	6.53 ± 1.40
+ DOPE	33.22 ± 6.82	26.70 ± 4.73	ND

Data are expressed as percentage of the fluorescence of total supercoiled DNA present in the lipoplex. Values represent the mean of three independent experiments (±SD). ND, not determined.

Oligonucleotides are effectively translocated across endosomal membranes by both SAINT-2- and SAINT-5-containing lipoplexes

Despite an equally efficient in vitro release of DNA from SAINT-2/DOPE and SAINT-5/DOPE lipoplexes, only SAINT-2-containing complexes, when incubated with cells, lead to substantial transfection. The issue then arises as to what extent plasmids associated with SAINT-5 complexes were actually released intracellularly. Because for plasmids such a release is a priori low, whereas the event cannot be adequately discerned by fluorescence microcopy, we determined the ability of either complex to deliver fluorescently tagged oligonucleotides (FITC-ODN) into cells. Upon delivery, such compounds rapidly accumulate into the nucleus after release into the cytosol from the endosomal compartment (Shi et al., 2001). As shown in Fig. 7, an incubation of cells with FITC-ODN-containing SAINT-2/DOPE and SAINT-5/DOPE lipoplexes results in a similar nuclear accumulation of ODNs, showing fluorescently labeled nuclei in almost all cells. Accordingly, these data suggest that SAINT-5/DOPE complexes do display a capacity to translocate nucleic acids across the endosomal membrane.

Recently, it was proposed that the hexagonal phase adopted by lipoplexes, possibly promoted when the cationic lipids mix with (cell-derived) anionic lipids (Smisterova et al., 2001; Lewis and McElhaney, 2000; Hafez et al., 2001), is instrumental in the release of DNA from the lipoplex into the cytoplasm. Since SAINT-2/DOPE and SAINT-5/DOPE lipoplexes are equally capable of releasing DNA (and ODN), this could imply the presence of a hexagonal phase after interaction of either lipoplex with anionic target membranes. Therefore, we next examined the lipid phase of SAINT-2/DOPE and SAINT-5/DOPE lipoplexes, and the phase arising upon interaction with PS:PC:PE vesicles, to mimic the potential translocation of anionic lipid into the complexes, following interactions between lipoplex and endosomal membrane.

SAINT-2/DOPE and SAINT-5/DOPE lipoplexes are capable of forming a hexagonal phase

Previously, we demonstrated that SAINT-2/DOPE lipoplexes adopt the hexagonal phase, as measured by SAXS, when incubated in a physiological salt solution (Smisterova et al., 2001). By contrast, under similar conditions the diffraction pattern of SAINT-5/DOPE lipoplexes reflects a lamellar organization as shown in Fig. 8 *A*. Thus, the diffraction maxima at $q = 1.07\ \mathrm{nm}^{-1}$ and $2.15\ \mathrm{nm}^{-1}$ show a peak localization ratio of 1:2, which is typical of a lamellar organization of the lipoplex. The additional spacing of 3.4 nm is attributed to the in-line spacing between parallel DNA helices, as described by Lasic et al. (1997).

After an incubation of either complex with anionic lipid containing phospholipid vesicles, the obtained diffraction

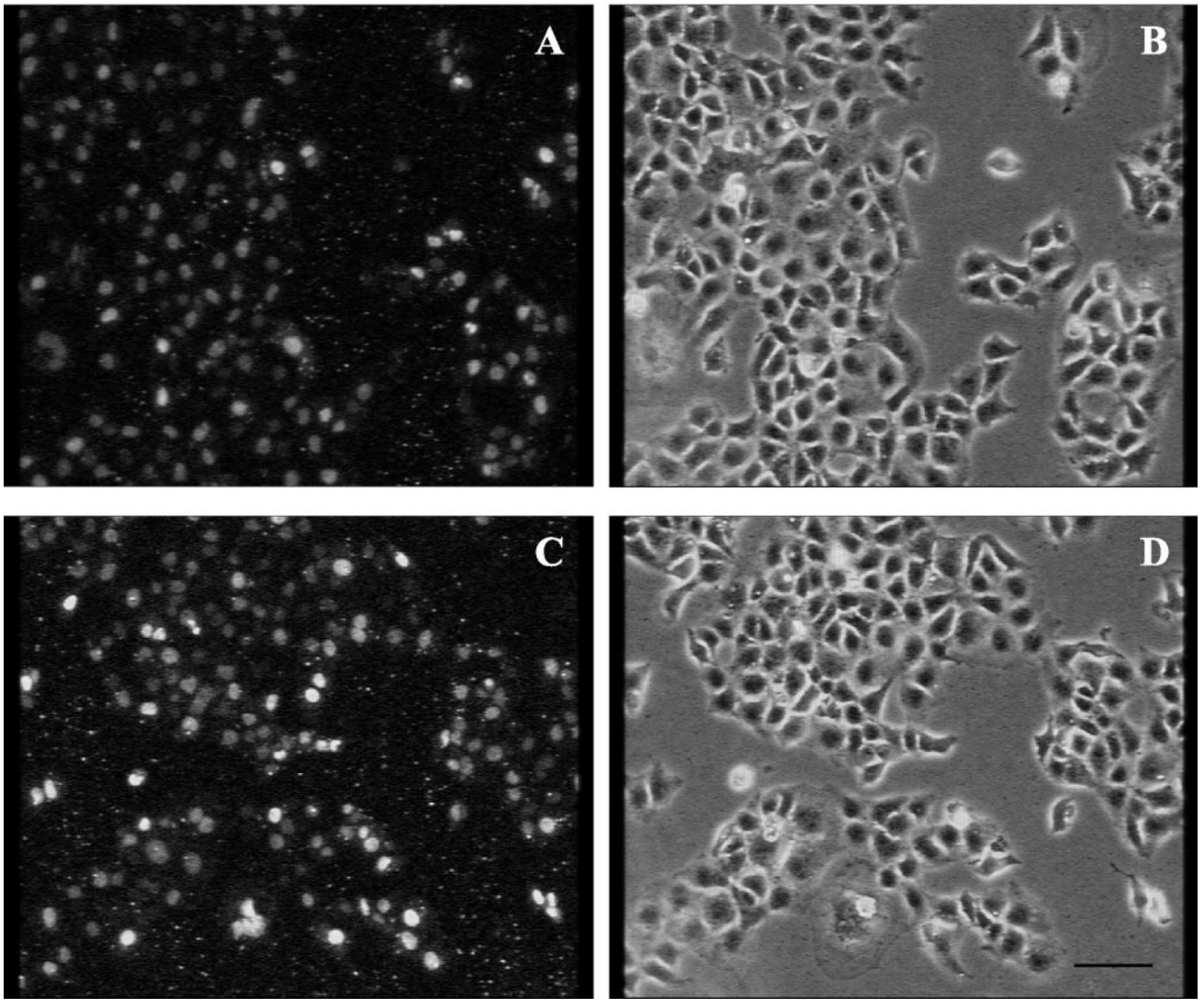

FIGURE 7 The nuclear accumulation of ODN is similar after delivery with SAINT-2/DOPE and SAINT-5/DOPE lipoplexes. (*A* and *C*) Nuclear accumulation of FITC-ODN was measured after a 5-h incubation with SAINT-2/DOPE and SAINT-5/DOPE lipoplexes, respectively. Note that the complexes of either cationic lipid effectively deliver FITC-ODN, because a nuclear accumulation of the oligonucleotide is apparent in essentially all cells. (*B* and *D*) Corresponding phase contrast images of *A* and *C*, respectively. (*A–D*) Size bar in *D* represents 30 μm.

pattern for the SAINT-5/DOPE complexes (Fig. 8 *B*) is distinctly different from that observed at control conditions (Fig. 8 *A*). In this case, the pattern is very reminiscent of the hexagonal pattern, obtained for the SAINT-2/DOPE complex after interaction with anionic vesicles (Fig. 8 *C*). In the latter case the q values of 0.093, 0.160, 0.185, 0.243, and 0.277 reflect the diffraction peaks that are localized in a relative ratio of $1:\sqrt{3}:\sqrt{4}:\sqrt{7}:\sqrt{9}$, whereas for SAINT-5/DOPE the diffractions peaks that can be discerned are localized in a similar ratio of $1:\sqrt{3}:\sqrt{4}:\sqrt{7}$. These ratios typically reflect a hexagonal phase for either lipoplex, with a unit cell spacing of 7.8 nm (SAINT-2) and 8.1 nm (SAINT-5), respectively. Clearly, both SAINT-2/DOPE and SAINT-5/DOPE lipoplexes display a hexagonal phase when they interact with anionic vesicles, which could explain the similarity in their efficiency of nuclear ODN delivery.

Importantly, these data indicate that also SAINT-5-containing lipoplexes can adopt a hexagonal phase, which moreover occurs under conditions (i.e., upon acquirement of anionic lipids) relevant to release at the level of endosomes. Consistently, ODNs are efficiently released from SAINT-5 complexes, in contrast to plasmids, which as shown above, become decondensed during complex assembly. The data may thus suggest that if the condensed state could be maintained, efficient release and hence transfection might also occur for plasmid-containing SAINT-5/DOPE lipoplexes. To examine this possibility, we condensed the

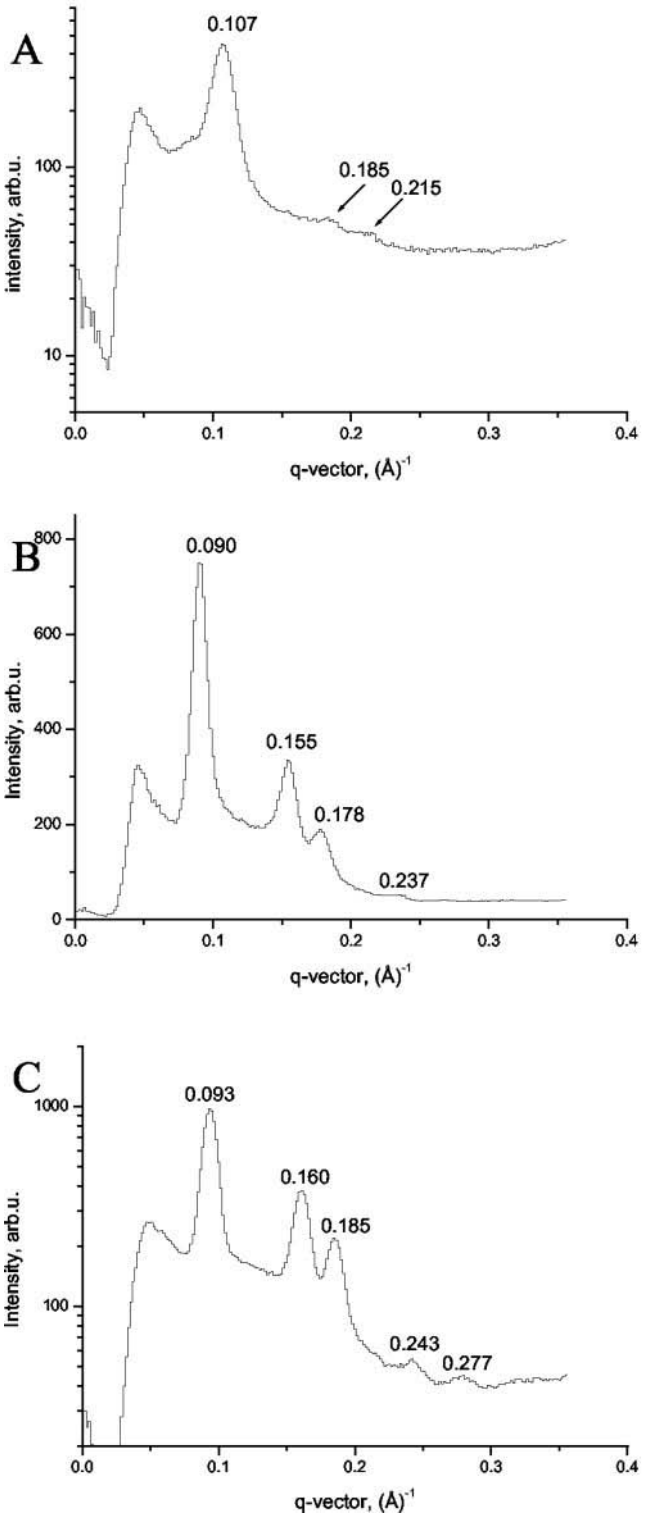

FIGURE 8 SAINT-2/DOPE and SAINT-5/DOPE lipoplexes show hexagonal phases after interaction with anionic target vesicles. The presence of a lamellar or hexagonal conformation in lipoplex after interaction with anionic vesicles was determined by SAXS. Whereas SAINT-5/DOPE lipoplexes (*A*) show a lamellar organization, the diffraction peaks of SAINT-5/DOPE (*B*) and SAINT-2/DOPE (*C*) lipoplex in combination with anionic vesicles both show a typical hexagonal spacing pattern, indicating the presence of a hexagonal phase.

TABLE 4 Percentage of GFP-positive COS7 cells after transfection with SAINT-5/DOPE lipoplexes, with and without previous treatment of the plasmid with poly-L-lysine

	SAINT-2/DOPE	SAINT-5/DOPE	No lipid
0 μg PLL	100 ± 2.6	9.8 ± 0.9	ND
0.75 μg PLL	n.d.	21.3 ± 0.5	0.2 ± 0.1
1.25 μg PLL	n.d.	37.4 ± 6.9	ND

COS7 cells were transfected with lipoplexes containing 15 nmol; lipid and 1 μg; DNA, coated or noncoated with poly-L-lysine (PLL). The transfection efficiency with SAINT-2/DOPE was set at 100%. Transfection with poly-L-lysine-DNA complexes was negligible.

plasmid by previous treatment with polylysine, and subsequently prepared the SAINT-5/DOPE lipoplexes and transfected the cells.

Precondensation of DNA in SAINT-5/DOPE lipoplexes with poly-L-lysine promotes transfection efficiency

COS7 cells that were transfected with poly-L-lysine-coated plasmids, assembled in SAINT-5/DOPE lipoplexes, showed a considerable enhancement in the amount of green fluorescent protein-positive cells compared with poly-L-lysine-free SAINT-5/DOPE lipoplexes (Table 4). Inclusion of 0.75 μg of poly-L-lysine in the lipoplex formulation resulted in a twofold increase in transfection efficiency, whereas inclusion of 1.25 μg poly-L-lysine gave rise to a fourfold increase. As a control, transfection was performed with poly-L-lysine-DNA complexes without lipid. The transfection that could be achieved with these complexes was negligible. Thus, provided that the condensed state is maintained, effective delivery and hence effective transfection can also be obtained with SAINT-5/DOPE complexes.

DISCUSSION

The present work revealed that transfection accomplished with the unsaturated pyridinium amphiphile SAINT-2 is two- to fivefold more efficient than that obtained with the saturated pyridinium amphiphile SAINT-5, whereas inclusion of the helper lipid DOPE strongly promotes SAINT-2 but failed to exert any effect on SAINT-5-mediated transfection efficiency.

The monolayer experiments suggest that the wrapping of plasmids by cationic lipids requires a distinct degree of monolayer (or membrane) flexibility that facilitates lipid recruitment onto the plasmids. This flexibility presumably depends on the tightness of the packing of the lipid-donating monolayer (or membrane), which in turn appears to be governed by chain saturation, saturation (SAINT-5) causing tighter packing than unsaturation (SAINT-2). Under conditions in which both SAINT-2 and SAINT-5 were fluid (Fig. 3), complexes were readily formed with the former

but not with its saturated counterpart. The data indicate that in the case of SAINT-5, the monolayer crystallizes out upon plasmid binding, as revealed by *N*-NBD-PE phase separation, suggesting that the process of crystallization occurs faster than lipid recruitment, thereby frustrating lipoplex assembly.

The promoting effect of membrane dynamics in conjunction with lipid fluidity on lipoplex formation was further supported by the influence of the (fluidizing) helper lipid DOPE on the interaction between the cationic lipids and DNA. When mixed with DOPE, SAINT-5 at least partly acquired the ability to form lipoplexes, the presence of DOPE presumably affecting local membrane curvature and hence the tightness of SAINT-5 packing properties, as reflected by an enhanced accessibility of picogreen toward the nucleic acid. During lipoplex formation the cationic lipid should be in a fluid phase and display sufficient dynamics to kinetically favor the formation of particles containing effectively condensed plasmid (that is, lipid-DNA interactions are favored over lipid-lipid interactions), both conditions being promoted by the presence of an appropriate helper lipid. Moreover, the data reveal that stability and transfectability require the lipoplexes to be kept at temperatures above the liquid-crystalline-phase transition temperature of the lipids.

Our data indicate that the difference in transfection efficiency between SAINT-2/DOPE and SAINT-5/DOPE might rely on the relative efficiencies of DNA release from the lipoplexes upon encounter with (intra)cellular membranes. Both SAINT-2/DOPE and SAINT-5/DOPE release ODNs and mediate their efficient translocation across the endosomal membrane, emphasizing that also SAINT-5/DOPE displayed an effective translocation capacity. The mechanism underlying the effectiveness of this translocation has been proposed to correlate with the ability of the lipoplexes to adopt the hexagonal phase (Smisterova et al., 2001; Lewis and McElhaney, 2000; Hafez et al., 2001). Indeed, although SAINT-2/DOPE lipoplexes are capable of adopting such a phase when suspended in physiological salt solutions (Smisterova et al., 2001), also SAINT-5/DOPE lipoplexes displayed the ability to form this phase, but only after an interaction with membranes that contained anionic phospholipids. These observations would be entirely consistent with the need of a recruitment of acidic phospholipids into the complex, which has been shown to promote the hexagonal phase (Lewis and McElhaney, 2000). Under physiological conditions, such lipids might become recruited into the complex when intimately interacting with the endosomal membrane, as proposed (Xu and Szoka, 1996). Importantly, given their relatively small size, ODNs can freely diffuse into the cell's nuclei, whereas plasmid DNA most likely does not acquire nuclear access by passive diffusion. Therefore, the extent of ODN accumulation does not necessarily reflect the extent of plasmid that will be integrated into the nucleus. Moreover, the passage of plasmid over the endosomal membrane could be frustrated compared with the passage of the much smaller oligonucleotide. Clearly, these relevant issues are not taken into account by the cell-free assays measuring the amount of DNA release induced with anionic vesicles. Uncondensed DNA, induced upon plasmid-SAINT-5 interaction, may still readily dissociate upon charge competition, as occurs when lipoplexes are incubated with anionic lipid vesicles. Yet, for steric reasons, it could be envisaged that translocation of uncondensed DNA from the endosome into the cytosol, in contrast to the much smaller supercoiled DNA, might be frustrated. The same impediment in intracellular plasmid transport may then occur when reaching the nucleus. In addition, the conformation of DNA is important for the efficiency of transcription, supercoiled DNA being more readily transcribed than linear DNA (Middaugh et al., 1998). The fact that transfection is rescued when decondensation of DNA is prevented by poly-L-lysine compaction of the plasmid before lipoplex formation is fully in line with these notions. Indeed we observed a two- to fourfold increase in transfection efficiency if the DNA in SAINT-5/DOPE lipoplexes has been preincubated with poly-L-lysine.

Taken together, we propose that the ability of the cationic lipid to rapidly wrap and condense the plasmid DNA, in conjunction with the facilitation of DNA release from the lipoplex by DOPE, allows for efficient lipoplex-mediated gene transfer Thus, the presence of DNA in a condensed state in lipoplexes, dictated by the physical and structural cationic lipid (membrane) properties as revealed in this study, seems crucial for obtaining efficient transfection, Direct determination of the nuclear accumulation of plasmid DNA, as delivered by poly-L-lysine-containing and poly-L-lysine-free SAINT-5/DOPE complexes, and transcriptional activity may provide direct support for the importance of the condensed state of DNA in its translocation across the endosomal and nuclear membrane and/or for transcriptional activity.

This work was supported by the the Netherlands Foundation for Chemical Research/Netherlands Technology Foundation (349-4001).

REFERENCES

Akao, T., T. Fukumoto, H. Ihara, and A. Ito. 1996. Conformational change in DNA induced by cationic bilayer membranes. *FEBS Lett.* 391: 215–218.

Balasubramaniam, R. P., M. J. Bennett, A. M. Aberle, J. G. Malone, M. H. Nantz, and R. W. Malone. 1996. Structural and functional analysis of cationic transfection lipids: the hydrophobic domain. *Gene Ther.* 3:163–172.

Bally, M. B., P. Harvie, F. M. Wong, S. Kong, E. K. Wasan, and D. L. Reimer. 1999. Biological barriers to cellular delivery of lipid-based DNA carriers. *Adv. Drug Deliv. Rev.* 38:291–315.

Bennett, C. F., D. Mirejovsky, R. M. Crooke, Y. J. Tsai, J. Felgner, C. N. Sridhar, C. J. Wheeler, and P. L. Felgner. 1998. Structural requirements for cationic lipid mediated phosphorothioate oligonucleotides delivery to cells in culture. *J. Drug Target.* 5:149–162.

Byk, G., C. Dubertret, V. Escriou, M. Frederic, G. Jaslin, R. Rangara, B. Pitard, J. Crouzet, P. Wils, B. Schwartz, and D. Scherman. 1998. Synthesis, activity, and structure-activity relationship studies of novel cationic lipids for DNA transfer. *J. Med. Chem.* 41:229–235.

Felgner, J. H., R. Kumar, C. N. Sridhar, C. J. Wheeler, Y. J. Tsai, R. Border, P. Ramsey, M. Martin, and P. L. Felgner. 1994. Enhanced gene delivery and mechanism studies with a novel series of cationic lipid formulations. *J. Biol. Chem.* 269:2550–2561.

Hafez, I. M., N. Maurer, and P. R. Cullis. 2001. On the mechanism whereby cationic lipids promote intracellular delivery of polynucleic acids. *Gene Ther.* 8:1188–1196.

Hagting, J. C., R. E. T. P. de-Vos, K. Sinkovics, E. J. Vorenkamp, and A. J. Schouten. 1999. Langmuir-Blodgett mono- and multilayers of (di)alkoxy-substituted poly(p-phenylenevinylene) precursor polymers. II. Stability, transfer, and multilayer structure of (di)alkoxy-substituted precursor PPVs. *Macromolecules.* 32:3939–3945.

Hansma, H. G., R. L. Sinsheimer, J. Groppe, T. C. Bruice, V. Elings, G. Gurley, M. Bezanilla, I. A. Mastrangelo, P. V. Hough, and P. K. Hansma. 1993. Recent advances in atomic force microscopy of DNA. *Scanning.* 15:296–299.

Harvie, P., F. M. Wong, and M. B. Bally. 1998. Characterization of lipid DNA interactions. I. Destabilization of bound lipids and DNA dissociation. *Biophys. J.* 75:1040–1051.

Lasic, D. D., H. Strey, M. C. A. Stuart, R. Podgornik, and P. M. Frederik. 1997. The sructure of DNA-liposome complexes. *J. Am. Chem. Soc.* 119:832–833.

Lewis, R. N. A. H., and R. N. McElhaney. 2000. Surface charge markedly attenuates the nonlamellar phase-forming propensities of lipid bilayer membranes: calorimetric and ^{31}P-nuclear magnetic resonance studies of mixtures of cationic, anionic, and zwitterionic lipids. *Biophys. J.* 79: 1455–1464.

Meekel, A. A. P., A. Wagenaar, J. Smisterova, J. E. Kroeze, P. Haadsma, B. Bosgraaf, M. C. A. Stuart, A. Brisson, M. H. J. Ruiters, D. Hoekstra, and J. B. F. N. Engberts. 2000. Synthesis of pyridinium amphiphiles used for transfection and some characteristics of amphiphile/DNA complex formation. *Eur. J. Org. Chem.* 2000:665–673.

Middaugh, C. R., R. K. Evans, D. L. Montgomery, and D. R. Casimiro. 1998. Analysis of plasmid DNA from a pharmaceutical perspective. *J. Pharm. Sci.* 87:130–146.

Möhwald, H. 1990. Phospholipid and phospholipid-protein monolayers at the air/water interface. *Annu. Rev. Phys. Chem.* 41:441–476.

Oberle, V., U. Bakowsky, I. S. Zuhorn, and D. Hoekstra. 2000. Lipoplex formation under equilibrium conditions reveals a three-step mechanism. *Biophys. J.* 79:1447–1454.

Reich, Z., R. Ghirlando, and A. Minsky. 1991. Secondary conformational polymorphism of nucleic acids as a possible functional link between cellular parameters and DNA packaging processes. *Biochemistry.* 30: 7929–7936.

Scherman, D., M. Bessodes, B. Cameron, J. Herscovici, H. Hofland, B. Pitard, F. Soubrier, P. Wils, and J. Crouzet. 1998. Application of lipids and plasmid design for gene delivery to mammalian cells. *Curr. Opin. Biotechnol.* 9:480–485.

Shi, F., A. Nomden, V. Oberle, J. B. Engberts, and D. Hoekstra. 2001. Efficient cationic lipid-mediated delivery of antisense oligonucleotides into eukaryotic cells: down-regulation of the corticotropin-releasing factor receptor. *Nucleic Acid Res.*29:2079–2087.

Smisterova, J., A. Wagenaar, M. C. A. Stuart, E. Polushkin, G. ten Brinke, R. Hulst, J. B. F. N. Engberts, and D. Hoekstra. 2001. Molecular shape of the cationic lipid controls the structure of cationic lipid/DOPE-DNA complexes and the efficiency of gene delivery. *J. Biol. Chem.* 276: 47615–47622.

Spector, S., and J. M. Schnur. 1997. DNA ordering on a lipid membrane. *Science.* 275:791–792.

Stegmann, T., and J. Y. Legendre. 1997. Gene transfer mediated by cationic lipids: lack of a correlation between lipid mixing and transfection. *Biochim. Biophys. Acta.* 1325:71–79.

Sternberg, B., F. L. Sorgi, and L. Huang. 1994. New structures in complex formation between DNA and cationic liposomes visualized by freeze-fracture electron microscopy. *FEBS Lett.* 356:361–366.

van der Woude, I., A. Wagenaar, A. A. P. Meekel, M. B. ter Beest, M. H. J. Ruiters, J. B. F. N. Engberts, and D. Hoekstra. 1997. Novel pyridinium surfactants for efficient, nontoxic in vitro gene delivery. *Proc. Natl. Acad. Sci. U.S.A.* 94:1160–1165.

Xu, Y., and F. C. Szoka, Jr. 1996. Mechanism of DNA release from cationic liposome/DNA complexes used in cell transfection. *Biochemistry.* 35:5616–5623.

Zuhorn, I. S., W. H. Visser, U. Bakowsky, J. B. F. N. Engberts, and D. Hoekstra. 2002. Interference of serum with lipoplex-cell interaction: modulation of intracellular processing. *Biochim. Biophys. Acta.* 1560: 25–36.