Surface Charge Density Determines the Efficiency of Cationic Gemini Surfactant Based Lipofection

Samppa J. Ryhänen,* Matti J. Säily,* Tommi Paukku,* Stefano Borocci,† Giovanna Mancini,† Juha M. Holopainen,* and Paavo K. J. Kinnunen*

*Helsinki Biophysics and Biomembrane Group, Institute of Biomedicine/Biochemistry, University of Helsinki, Finland and †CNR, ICCOM—Sezione di Roma c/o Dipartimento di Chimica, Universita degli Studi di Roma "La Sapienza," P. le A. Moro 00185, Rome, Italy

ABSTRACT The efficiencies of the binary liposomes composed of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine and cationic gemini surfactant, (2S,3R)-2,3-dimethoxy-1,4-bis(*N*-hexadecyl-*N,N*-dimethylammonium)butane dibromide as transfection vectors, were measured using the enhanced green fluorescent protein coding plasmid and COS-1 cells. Strong correlation between the transfection efficiency and lipid stoichiometry was observed. Accordingly, liposomes with $X_{SR-1} \geq 0.50$ conveyed the enhanced green fluorescent protein coding plasmid effectively into cells. The condensation of DNA by liposomes with $X_{SR-1} > 0.50$ was indicated by static light scattering and ethidium bromide intercalation assay, whereas differential scanning calorimetry and fluorescence anisotropy of diphenylhexatriene revealed stoichiometry dependent reorganization in the headgroup region of the liposome bilayer, in alignment with our previous Langmuir-balance study. Surface charge density and the organization of positive charges appear to determine the mode of interaction of DNA with (2S,3R)-2,3-dimethoxy-1,4-bis(*N*-hexadecyl-*N,N*-dimethylammonium)butane dibromide/1,2-dimyristoyl-*sn*-glycero-3-phosphocholine liposomes, only resulting in DNA condensation when $X_{SR-1} > 0.50$. Condensation of DNA in turn seems to be required for efficient transfection.

INTRODUCTION

Gene therapy and genetic engineering require safe and efficient vehicles for conveying foreign genetic material into eukaryotic cells. Compared to viral systems DNA-cationic lipid complexes (lipoplexes) possess a number of desirable properties and have become perhaps the most popular utilities for this purpose (Felgner et al., 1987). Importantly, such complexes are nonimmunogenic, nonpathogenic, biodegradable, and easy to prepare. They have also been shown to pass through the blood-brain barrier (Shi and Pardridge, 2000) and have reached clinical trials (Caplen et al., 1995; Alton et al., 1999). Yet, despite intensive efforts the mechanism(s) of liposome mediated transfection (lipofection) and therefore also the characteristics of an ideal transfection agent remain incompletely understood.

Several physical or chemical properties of different cationic lipids have been proposed to be responsible for their ability to transfect cells. The number of positive charges of the cationic lipid (Wheeler et al., 1996), the linkage between the hydrophobic and cationic portion of the molecule (Leventis and Silvius, 1990), and the structure of the hydrophobic moieties (Solodin et al., 1995) have been demonstrated to influence the measured transfection efficiencies. Also properties of the lipoplexes formed have been extensively studied to establish proper composition required for efficient and reproducible transfection. Presence of inverted hexagonal H_{II}-phase–forming dioleoylphosphatidylethanolamine (DOPE) (Farhood et al., 1995; Koltover et al., 1998; Mok and Cullis, 1997), diacylglycerol (DAG) (Paukku et al., 1997), or other nonlamellar phase promoting compounds (Pedroso de Lima et al., 1999) in the lipoplex has been shown to enhance transfection efficiency. However, this requirement is challenged by some previous studies (Hui et al., 1996; Simões et al., 1999), as well as by the findings presented here. The morphologies of cationic lipid-DNA complexes have been visualized by various electron microscopic techniques (e.g., Sternberg et al., 1994; Huebner et al., 1999; Gustafsson et al., 1995) and atomic force microscopy (Wheeler et al., 1996; Kawaura et al., 1998). These studies suggest several structures which could be responsible for transfection, e.g., "spaghetti-and-meatballs" (Sternberg et al., 1994), dense multilamellar complexes (Huebner et al., 1999), or complexes with a particular diameter (Kawaura et al., 1998). However, Xu et al. (1999) recently reported that the

Submitted March 26, 2002, and accepted for publication September 10, 2002.

Address reprint requests to Dr. Paavo K. J. Kinnunen, Helsinki Biophysics and Biomembrane Group, Institute of Biomedicine/Biochemistry, Biomedicum, P.O. Box 63 (Haartmaninkatu 8), FIN-00014 University of Helsinki, Finland. Tel.: 358-9-191 25400; Fax: 358-9-191 25444; E-mail: paavo.kinnunen@helsinki.fi.

Abbreviations used: c_A, concentration of compound A; CL, cationic lipid; DAG, diacylglycerol; DMEM-10, Dulbecco's modified Eagle's medium with 10% fetal calf serum; DMEM-SF, serum free Dulbecco's modified Eagle's medium; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; DPH, 1,6-diphenyl-1,3,5-hexatriene; DSC, differential scanning calorimetry; EDTA, ethylenediaminetetraacetic acid; EGFP, enhanced green fluorescent protein; EtBr, ethidium bromide; HADAB, *N*-hexadecyl-*N*-{10-[O-(4-acetoxy)phenylundecanoate]ethyl}dimethyl ammonium bromide; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; LUV, large unilamellar vesicle; MLV, multilamellar vesicle; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; PE, phosphatidylethanolamine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; *r*, fluorescence emission anisotropy; RFI, relative fluorescence intensity; SR-1, (2S,3R)-2,3-dimethoxy-1,4-bis(*N*-hexadecyl-*N,N*-dimethylammonium)butane dibromide; T_m, main transition temperature; T_p, pretransition temperature; X_A, mole fraction of compound A.

0006-3495/03/01/578/10 $2.00

morphology of complex as visualized by electron microscope does not have significant impact on the transfection efficiency.

Understanding the nature of cationic lipid-DNA interaction could have broader biological significance because of the natural cationic lipid sphingosine. The latter is ubiquitously present in mammalian cells and forms complexes with DNA (Kinnunen et al., 1993; Kõiv et al., 1994), revealing ''beads-on-a-string'' morphology as well as larger aggregates. It is, in principle, possible that sphingosine could be involved in the regulation of gene expression and signal transduction. To this end, the well established lipid second messenger, phosphaditic acid, is able to reverse DNA-sphingosine complex formation (Kinnunen et al., 1993).

Gemini surfactants, composed of two conventional surfactant molecules connected by a spacer, have acquired a lot of attention in colloid and surface chemistry (Menger and Keiper, 2000). Recently also the use of gemini surfactants as transfection vectors has been investigated (e.g., Fielden et al., 2001). Our preliminary experiments revealed the cationic gemini surfactant (2S,3R)-2,3-dimethoxy-1,4-bis(*N*-hexadecyl-*N*,*N*-dimethylammonium)butane dibromide (SR-1; chemical structure illustrated in Fig. 1) to be an efficient transfection vehicle. Moreover, there was no requirement for the presence of nonbilayer ''helper'' lipids such as DOPE. In our Langmuir-balance study we found that the properties of mixed monolayers of SR-1/POPC were strongly dependent on the content of SR-1 in the films (Säily et al., 2001). These results led us to explore the impact of X_{SR-1} on the interaction of this cationic gemini surfactant with DNA and the efficiency of the resulting complexes in cellular transfection. Surface charge density of mixed SR-1/phosphatidylcholine liposomes is demonstrated to represent an important determinant for the transfection efficiency and condensation of DNA.

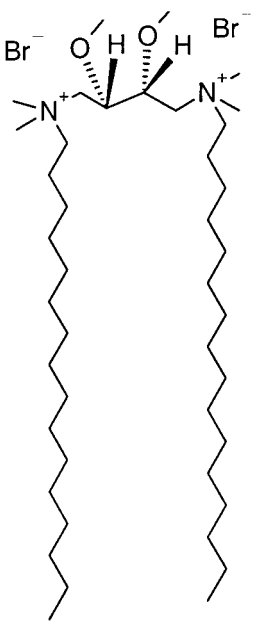

FIGURE 1 Structure of the cationic gemini surfactant SR-1.

MATERIALS AND METHODS

Materials

Calf thymus DNA, DMPC, DOPE, POPC, Hepes, ethidium bromide, and EDTA were from Sigma. DPH was purchased from EGA Chemie (Steinheim, Germany). The gemini surfactant (2S,3R)-2,3-dimethoxy-1,4-bis(*N*-hexadecyl-*N*,*N*-dimethylammonium)butane dibromide (SR-1) was synthesized as described previously (Cerichelli et al., 1996) and its purity was verified by NMR. The purity of other lipids was checked by thin-layer chromatography on silicic acid coated plates (Merck, Darmstadt, Germany) using chloroform/methanol/water (65:25:4, by vol.) as a solvent system. Examination of the plates after iodine staining or, when appropriate, by fluorescence illumination revealed no impurities. Concentrations of phospholipids and SR-1 were determined gravimetrically using a high precision electrobalance (Cahn, Cerritos, CA). DNA concentrations (expressed in mM basepairs) were determined by absorbance at 260 nm ($\varepsilon = 6600$ l/mol $\times$ cm). The 260/280 ratio of the calf thymus DNA was ~1.9. Freshly deionized filtered water (Milli RO/Milli-Q, Millipore Inc., Jaffrey, NH) was used in all experiments.

Preparation of liposomes

Multilamellar liposomes (MLVs) were prepared by mixing appropriate amounts of the lipid stock solutions in dry chloroform to obtain the desired compositions. Thereafter the solvent was removed by evaporation under a stream of nitrogen. For removal of residual amounts of solvent the samples were further maintained under high vacuum for at least 2 h. The resulting dry lipid films were then hydrated with 5 mM Hepes, 0.1 mM EDTA, pH 7.4 and thereafter incubated for 30 min at ~60°C, i.e., above the temperatures of the transition endotherms of the lipid components and their mixtures (Fig. 6 *A*). To obtain large unilamellar vesicles (LUVs) the hydrated lipid dispersions were vortexed vigorously and then extruded with a LiposoFast small volume homogenizer (Avestin, Ottawa, Canada) by subjecting to 19 passes through polycarbonate filter (100-nm pore size, Nucleopore, Pleasanton, CA). LUVs with diameters of ~100–115 nm are produced by this method (MacDonald et al., 1991; Wiedmer et al., 2001). Unless otherwise stated the liposomes were kept on an ice water bath for at least 12 h before their use. Importantly, to avoid interference with fluorescence spectroscopy resulting from light scattering due to varying lipid concentrations X_{SR-1} was varied in these measurements (as well as in DSC experiments) by altering both the amount of SR-1 and DMPC while keeping total lipid concentration constant. In the transfection experiments the total cationic charge (i.e., the amount of SR-1) was maintained constant and X_{SR-1} was varied by altering the amount of the DMPC in the liposomes.

Transfection experiments

The pEGFP-N1 expression vector (Clontech, Palo Alto, CA) consisting of the transcriptional regulatory domain of cytomegalo virus preceding the enhanced green fluorescent protein (EGFP) gene was used in all experiments and prepared using QIAGEN Plasmid Maxi kit (Qiagen GmbH, Hilden, Germany). Specific restriction endonuclease digestions confirmed the identity of the plasmid.

Transfection efficiencies were measured using COS-1 cells, derived from simian kidney cell line CV-1 transformed with a mutant simian virus 40 (Gluzman, 1981). Cells were grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum (DMEM-10) on 24-well plastic cell culture plates in an incubator with an atmosphere of 5% CO_2 in air. Three different kinds of liposome-DNA complexes were made. Accordingly, either

MLVs or LUVs, as indicated, were first formed and the plasmid was then added to these liposome solutions. Preformed MLVs and LUVs were prepared as described above. Complexes of DNA with these preformed liposomes were then formed by adding the indicated amounts of pEGFP-N1 plasmid at 23°C in 0.6 ml of serum free Dulbecco's modified Eagle's medium (DMEM-SF), followed by an incubation for 15 min at 23°C. Alternatively, the indicated amount of plasmid was included in the DMEM-SF buffer used to hydrate (at $T > T_m$ of the lipid constituents) the dry lipids to yield MLVs. Before transfection the total volume of the suspensions containing liposome-plasmid complexes was adjusted to 0.9 ml with DMEM-SF. DMEM-10 was removed from the wells of just confluent 24-well cell culture plates and the liposome-plasmid complexes were added to three separate wells, 0.3 ml to each. After a 6-h incubation at 37°C the transfection mixture was replaced by DMEM-10. Transfection efficiency was determined after 65 h of incubation at 37°C by measuring the fluorescence intensity of EGFP using SPECTRAFluor Plus fluorescence reader (Tecan AG, Hombrechtikon, Switzerland) with emission and excitation wavelengths set at 485 and 510 nm, respectively. The background measured for nontransfected cells serving as controls was subtracted from the values recorded for the transfected cells.

Cytotoxicity was assessed by Trypan Blue (Invitrogen, San Diego, CA) exclusion. Cells were cultured on 24-well culture plates in DMEM-10 and subsequently incubated at 37°C for 6 h with liposome-DNA complexes with the indicated compositions, whereafter the transfection mixture was replaced by DMEM-10 and the incubation continued for further 24 h. Subsequently, cells were detached from the wells by trypsinization and resuspended in one ml of DMEM-SF. An aliquot of 50 μl of the resulting cell suspension was mixed with 50 μl of 0.4% Trypan Blue and incubated at ambient temperature (~23°C) for 5 min. The amount of nonviable cells was determined by counting the stained cells with a hemocytometer.

Light scattering

Static light scattering due to the formation of complexes by the cationic liposomes and DNA was measured with a spectrofluorometer with both excitation and emission monochromators set at 500 nm. Two ml of 50-μM liposome solution of the indicated composition was placed into a magnetically stirred four-window quartz cuvette thermostated at 37°C. LUVs and DNA were mixed at the stated molar ratios. Scattering intensities were measured 5 min after the addition of DNA to the indicated concentrations and remained constant after this period.

Ethidium bromide intercalation assay

Fluorescence emission spectra of ethidium bromide in the 520–700-nm region were recorded with excitation at 500 nm. In brief, 1.8 ml of 16 μM EtBr was first applied into a magnetically stirred four-window quartz cuvette thermostated at 37°C whereafter calf thymus DNA was added to yield a final concentration of 34 μM (expressed in basepairs). Subsequently, LUVs of the given compositions were included to achieve the indicated molar ratios. Emission spectra were measured 5 min after the addition of liposomes.

Differential scanning calorimetry

MLVs were vortexed and maintained on an ice bath for at least 12 h X_{SR} before loading into the calorimeter cuvette (final concentration 1 mM). When indicated DNA was present in the hydration buffer and the resulting cationic lipid-DNA complexes were incubated on an ice water bath as described above. A VP-DSC microcalorimeter (Microcal, Northampton, MA) was operated at a heating rate of 0.5° per min. The instrument was interfaced to a 486 PC, and the data were analyzed using the routines of the software provided by the instrument manufacturer.

Fluorescence spectroscopy using DPH

Diphenylhexatriene (DPH) was included in LUVs to yield a 1:500 molar ratio of probe to lipid ($X_{DPH} = 0.002$). Fluorescence measurements were carried out with a Perkin-Elmer LS 50B spectrofluorometer interfaced to a Pentium PC. The excitation and emission wavelengths were set at 360 and 450 nm, respectively. Two ml of 50 μM SR-1/DMPC solution was applied into a magnetically stirred four-window quartz cuvette placed in the sample compartment thermostated at 37°C by a circulating water bath. When indicated DNA was added to the LUVs to yield the desired molar ratios of the cationic lipid and DNA (expressed in basepairs). Anisotropy values were measured 5 min after the addition of DNA to the given concentrations and were found to remain constant after this period.

RESULTS

Transfection experiments

Our preliminary studies revealed a significant impact of the lipid stoichiometry on the transfection efficiency of the lipoplexes. This finding was explored in further detail by monitoring the expression of the EGFP coding plasmid in COS-1 cells. In the first series of experiments plasmid DNA was added to MLVs at ambient temperature. The transfection efficiency of the resulting lipoplexes did depend on $X_{SR\text{-}1}$ of the liposomes (Fig. 2 *A*). Accordingly, neat DMPC liposomes ($X_{SR\text{-}1} = 0$) were ineffective and weak transfection was evident also for liposomes with $X_{SR\text{-}1} = 0.25$. However, a dramatic increase in transfection efficiency was observed at $X_{SR\text{-}1} = 0.50$, whereafter further increasing $X_{SR\text{-}1}$ toward neat SR-1 liposomes caused a minor decrement in the expression levels. Importantly, these changes cannot be explained by an increment in the total number of cationic charges because unlike in the physicochemical experiments this parameter was maintained constant in all transfection experiments (i.e., $X_{SR\text{-}1}$ was varied by altering the amount of DMPC in the liposomes). Different CL/DNA ratios (charge of the cationic lipid/negative charge of DNA) were used by varying the amount of the plasmid DNA (6.0 nmol, 3.0 nmol, 1.5 nmol, and 1.0 nmol, corresponding to CL/DNA ratios of 0.5, 1.0, 2.0, and 3.0), while the amount of cationic charge was kept constant. Variation of CL/DNA between 0.5 and 3.0 had an insignificant impact on the transfection efficiency of SR-1/DMPC MLVs, when $X_{SR\text{-}1} \geq 0.50$. The expression levels achieved by preformed SR-1/DMPC MLVs (at $X_{SR\text{-}1} \geq 0.50$) were comparable or higher than those obtained with the commercial cationic liposome vector Lipofectin™ (data not shown). Yet, inasmuch as our goal was to study the effect of surface electrostatics to the transfection efficiency rather than to compare the efficiency of SR-1 to the commercial lipofection systems it must be emphasized that the transfection experiments were conducted using conditions optimized for SR-1/DMPC liposomes only.

To allow for an unambiguous comparison of the transfection efficiencies with the physicochemical data on the liposomes we also measured the expression levels using preformed LUVs with the subsequently added plasmid

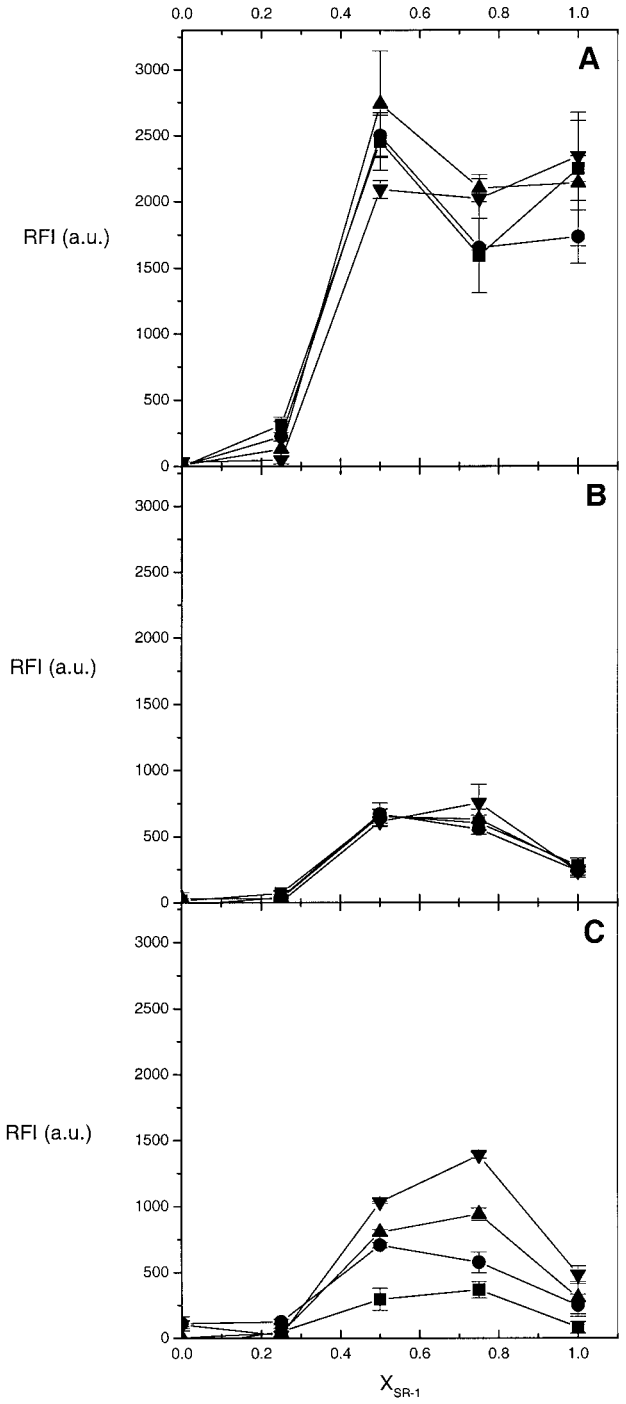

FIGURE 2 Comparison of the transfection efficiencies of pEGFP-N1 plasmid in COS-1 cells added to MLVs (*A*), LUVs (*B*), or in the buffer used to hydrate the lipids (*C*), illustrated as a function of X_{SR-1}. CL/DNA ratios in the lipoplexes were 0.5 (■), 1.0 (●), 2.0 (▲), and 3.0 (▼). See Materials and Methods for further details.

(Fig. 2 *B*). The dependence on X_{SR-1} in binary SR-1/DMPC LUVs (Fig. 2 *B*) was similar to that observed with preformed MLVs (Fig. 2 *A*) and, accordingly, LUVs having X_{SR-1} between 0.50 and 0.75 gave highest expression levels. Yet, in keeping with earlier studies (Patrick et al., 1998; Ross and Hui, 1999; Zuidam et al., 1999) complexes formed by adding DNA to preformed cationic MLVs were more efficient in transfection than those formed by adding the plasmid to LUVs with similar composition.

The third kind of lipoplexes were made by adding the plasmid into buffer, which was then used to hydrate the dried lipid residue. Adding the plasmid in the hydration buffer resulted in lower transfection efficiencies than measured for preformed MLVs (Fig. 2 *C*). However, the dependence of transfection efficiency on X_{SR-1} was evident also for these complexes. Further, these lipoplexes demonstrated sensitivity toward CL/DNA ratio in contrast to the other formulations employed in our study.

The above dependence of the transfection efficiency of the lipoplexes on X_{SR-1} could be due to different cytotoxities of the lipoplexes. To investigate this possibility we determined the cytotoxicity of SR-1/DMPC MLVs complexed with the plasmid (CL/DNA = 1) by Trypan Blue exclusion. However, the percentage of nonviable cells as a function of X_{SR-1} revealed only minor differences (Fig. 3). We may thus conclude that differences in transfection efficiencies are not due to cytotoxicity.

Static light scattering

LUVs without DNA scattered only weakly. Likewise, scattering due to the zwitterionic DMPC liposomes was unaffected by the addition of DNA. However, a rapid increase in light scattering upon the formation of complexes by DNA and the binary SR-1/DMPC liposomes was evident. Scattering intensity did depend on X_{SR-1} (Fig. 4) and increased slightly up to $X_{SR-1} = 0.50$. Upon exceeding this SR-1 mole fraction greatly augmented scattering was

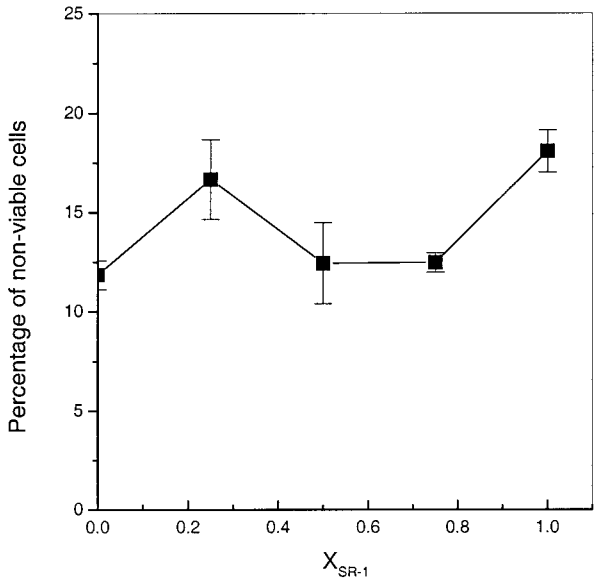

FIGURE 3 Cytotoxicity of SR-1/DMPC liposomes complexed with pEGFP-N1 plasmid, determined by Trypan Blue exclusion and expressed as the percentage of nonviable cells as function of X_{SR-1}.

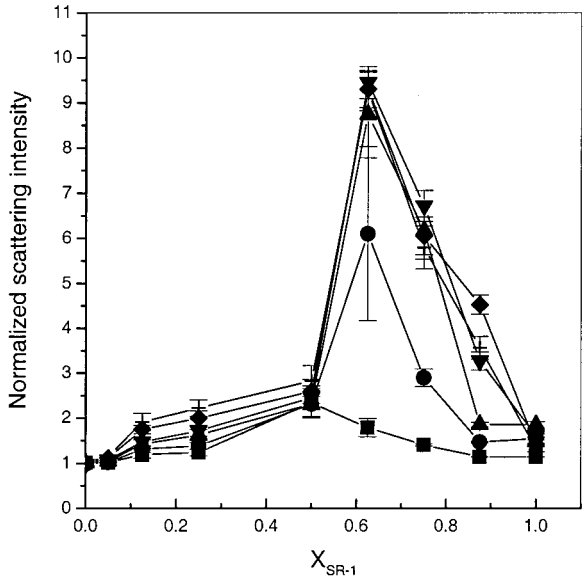

FIGURE 4 The static light scattering of liposome-DNA complexes as a function of X_{SR-1}. DNA concentration was 0.5 (■), 1.5 (●), 2.5 (▲), 5.0 (▼), 10.0 (◆), and 15.0 μM (+). Temperature was maintained at 37°C. Buffer was 5 mM Hepes, 0.1 mM EDTA, pH 7.4 and total concentration of lipid was 50 μM.

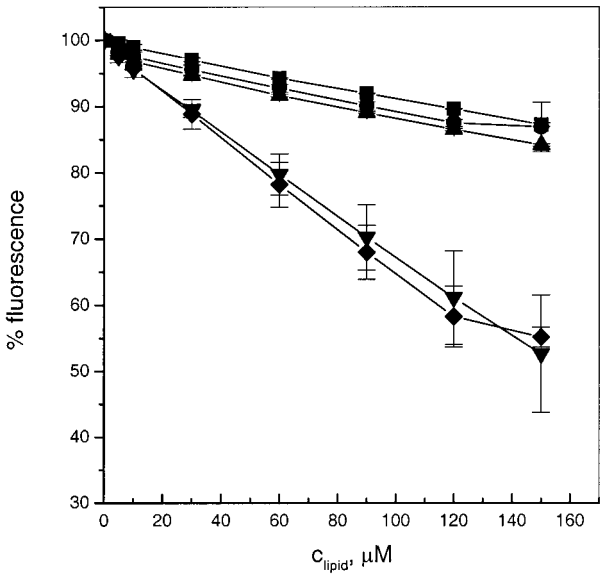

FIGURE 5 Normalized fluorescence emission intensity of DNA associated ethidium bromide at 590 nm as a function of lipid concentration. The mole fraction of SR-1 in DMPC liposomes was $X_{SR-1} = 0.00$ (■), 0.25 (●), 0.50 (▲), 0.75 (▼), and 1.00 (◆). Temperature was maintained at 37°C. Buffer was 5 mM Hepes, 0.1 mM EDTA, pH 7.4. Total concentrations of DNA and ethidium bromide were 34 μM (in basepairs) and 16 μM, respectively.

observed (Fig. 4), suggesting formation of more dense complexes, possibly due to the condensation of DNA (Bloomfield, 1996). Thereafter, with X_{SR-1} approaching 1.00 gradual decrement in scattering was measured. Liposomes with $X_{SR-1} = 0.75$ and 0.88 showed a weak tendency for precipitation, whereas no evidence for this was obtained at $X_{SR-1} < 0.75$. Precipitation due to DNA was evident for neat SR-1 liposomes, similarly to that observed for HADAB, a cationic lipid studied previously in our laboratory (Subramaniam et al., 2000). With X_{SR-1} from 0.50 to 0.88 maximal effect on scattering required only ≤ 2.5 μM DNA, corresponding to CL/DNA stoichiometries from 5.0 to 8.8.

Ethidium bromide intercalation assay

Ethidium bromide (EtBr) is an intercalating fluorophore whose emission intensity is ~10-fold higher when intercalated between the bases of DNA, compared to its fluorescence in water (Lakowicz, 1999). During condensation of DNA varying amounts of EtBr are forced to dissociate from DNA and partition into the aqueous phase, resulting in a decrease in fluorescence emission intensity (Cain et al., 1978; Bhattacharya and Mandal, 1998; Geall et al., 1999). We employed this method to verify condensation of DNA by SR-1/DMPC LUVs, suggested by static light scattering. The normalized emission intensity of DNA bound EtBr at 590 nm as a function of lipid concentration is illustrated in Fig. 5 and reveals two different patterns depending on X_{SR-1}. In brief, liposomes with $X_{SR-1} \leq 0.50$ caused only minor decrement in EtBr emission (~15%), which could result from augmented scattering due to liposomes. In contrast, liposomes with $X_{SR-1} > 0.50$ caused a much more pronounced attenuation in emission (maximally by ~47%), thus suggesting condensation of DNA by the added liposomes. Interestingly, the effect was not dependent on the total amount of cationic charge present inasmuch as the liposomes with $X_{SR-1} = 0.75$ and 1.00 show similar behavior despite significant difference in the amount of the added cationic surfactant.

Thermal phase behavior of SR-1/DMPC liposomes and the effect of DNA

The thermal phase behavior of lipids could be anticipated to bear a major influence on the interactions between liposomes and DNA, and be thus reflected also in the ability of the liposomes to cause condensation of DNA. To examine if the thermal phase behavior of SR-1/DMPC vesicles affects the mode of interaction between the cationic liposomes and DNA tentative phase diagrams were constructed based on the characterization of the cationic vesicles and their complexes by DSC. Representative DSC heating thermograms are shown in Fig. 6 *A*. Neat DMPC MLVs exhibited the pretransition at $T_p \approx 14$°C and the main transition at $T_m \approx 24$°C. At $X_{SR-1} = 0.05$ the value for T_p increased to 16.2°C and that for T_m to 26.0°C, in keeping with the saturated acyl chains of the cationic surfactant. When X_{SR-1} was increased to 0.13 the pretransition disappeared. Increasing X_{SR-1}

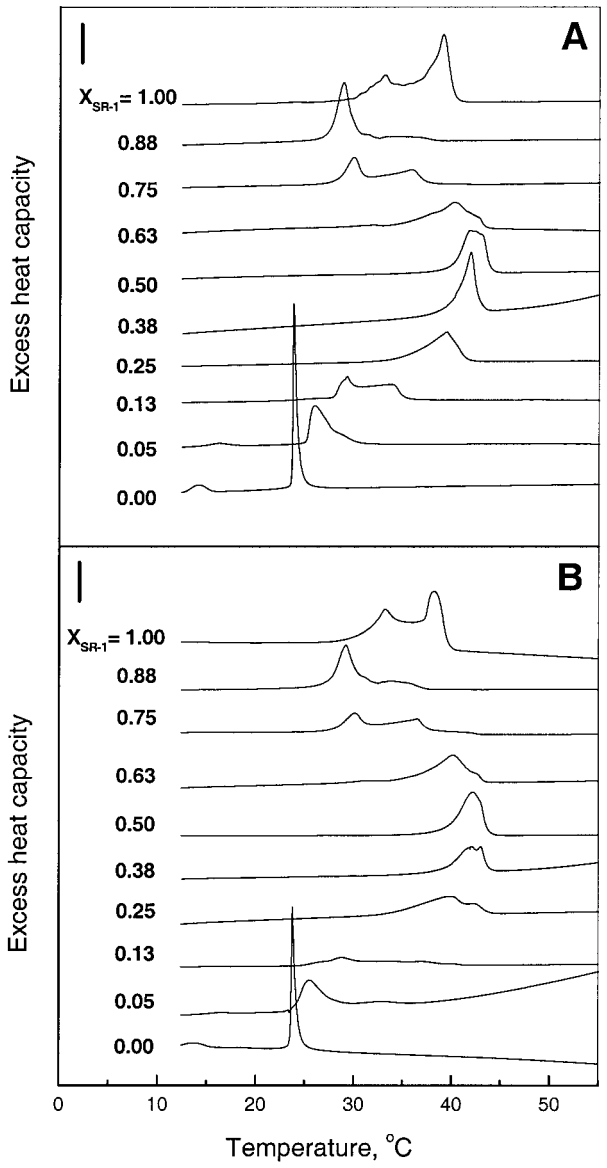

FIGURE 6 (*A*) DSC traces for SR-1/DMPC MLVs with the indicated mole fractions of the cationic gemini surfactant. Total lipid concentration was one mM in 5 mM Hepes, 0.1 mM EDTA, pH 7.4. (*B*) Thermograms of SR-1/DMPC MLVs with 0.05 mM (in basepairs) DNA. The calibration bars correspond to 5 mJ × °C^{-1}.

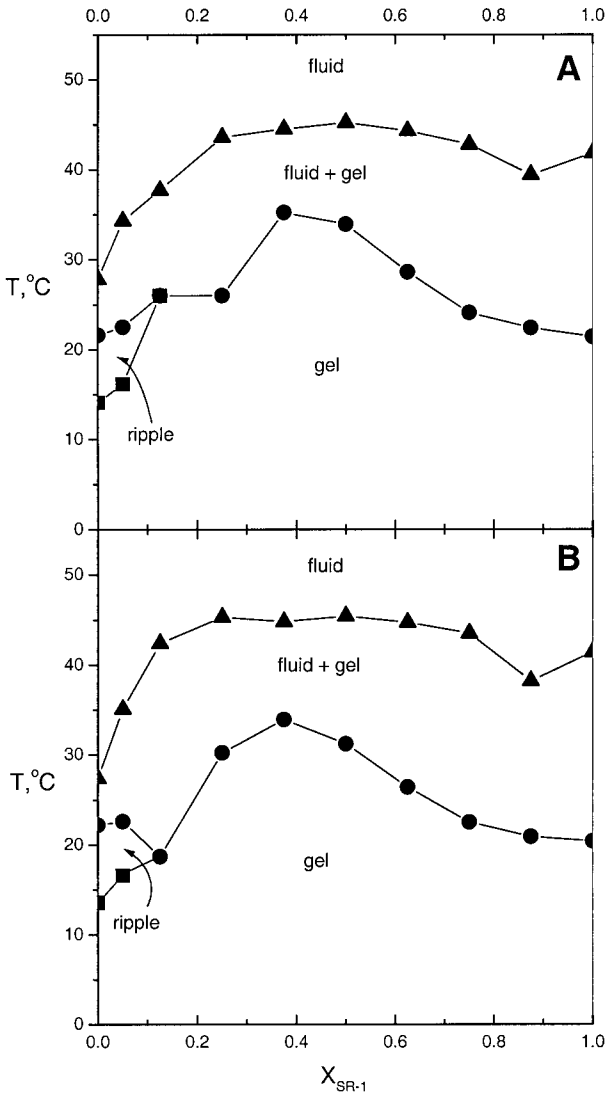

FIGURE 7 Tentative phase diagrams of binary SR-1/DMPC liposomes based on DSC data in the absence of DNA (*A*) and with 0.05 mM (in basepairs) DNA (*B*).

elevated T_m further until a maximum of 41.8°C was reached at $X_{SR-1} = 0.38$ and 0.50. At $X_{SR-1} = 0.63$ the main transition decreased to 40.2°C, with two smaller overlapping endotherms at 31.8 and 42.7°C. MLVs with $X_{SR-1} = 0.75$ revealed a broad transition with two separated peaks at 29.7 and 35.8°C. At $X_{SR-1} = 0.88$ a local minimum in T_m at 28.9°C was evident. Neat SR-1 revealed two endotherms at ~32 and 39°C, with a total enthalpy of ~73 kJ/mol. Their nature remains uncertain at this stage. Moreover, the endotherms for SR-1 were not fully reproducible, similarly to recent observations on other cationic amphiphiles (Zantl et al., 1999). Accordingly, although the beginning and the end of endotherm, as well as the total enthalpy remained almost the same in every measurement, the shape and the exact peak temperatures varied, the latter within ~4°C. Yet, despite this variation the composed tentative phase diagrams (Fig. 7 *A*) can be considered to be representative.

The effect of DNA on the thermal phase behavior of the binary SR-1/DMPC liposomes is illustrated in Fig. 6 *B*. Calf thymus DNA was added to the buffer used for the hydration of the lipids to a final concentration of 0.05 mM (in basepairs), corresponding to the lipid/DNA stoichiometry of 20 (mol/basepair). DNA did not influence the phase behavior of DMPC MLV*s*. This was true also for liposomes with $X_{SR-1} = 0.05$, whereas above this mole fraction effects of DNA became evident, with significant broadening of the

main endotherm at $X_{SR\text{-}1} = 0.13$. Similar broadening in the presence of DNA was evident also at $X_{SR\text{-}1} = 0.25$ with two smooth, overlapping endotherms, in contrast to one sharp peak measured without DNA. At $X_{SR\text{-}1} = 0.38$ the width of the endotherm was unaffected by the presence of DNA, yet in place of one sharp peak multiple smaller peaks were evident. Macroscopic aggregation was observed by visual observation of the samples having $X_{SR\text{-}1} = 0.13$, 0.25, and 0.38, although further increase in $X_{SR\text{-}1}$ abolished it. At $X_{SR\text{-}1} = 0.50$ narrowing of the transition peak due to DNA was observed, whereas the peak maximum was at 42.1°C, i.e., slightly higher than without DNA, 41.7°C. Liposome compositions with $X_{SR\text{-}1} = 0.63$, 0.75, 0.88, and 1.00 exhibited very similar thermal phase behavior both with and without DNA, with smoothening of the peaks and a minor increase in T_m being the only detectable differences produced by the nucleic acid, similarly to the data at $X_{SR\text{-}1} = 0.50$.

Tentative phase diagrams constructed from the above DSC data recorded for binary SR-1/DMPC liposomes both without DNA and with 0.05 mM DNA were compiled in Fig. 7, *A* and *B*, respectively. The only pronounced change due to DNA was the decrement in the solidus line at $X_{SR\text{-}1} = 0.13$. However, the shape of the endotherm at $X_{SR\text{-}1} = 0.13$ made it difficult to determine the exact onset of the transition, and therefore this point in the phase diagram is somewhat uncertain. The slight increment in the liquidus line of the phase diagram between $X_{SR\text{-}1} = 0.05$ and 0.38 was also evident. This effect disappeared at $X_{SR\text{-}1} > 0.50$.

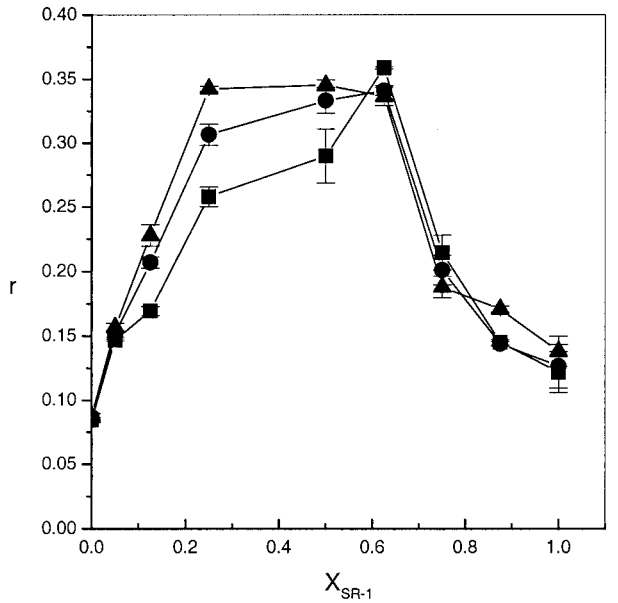

FIGURE 8 Changes in steady-state fluorescence anisotropy of DPH (X_{DPH} = 0.002) in SR-1/DMPC binary liposomes as a function of $X_{SR\text{-}1}$. Concentration of DNA was 0 (■), 1 (●), and 10 mM (▲). Temperature was maintained at 37°C. Buffer was 5 mM Hepes, 0.1 mM EDTA, pH 7.4 and the total lipid concentration was 50 μM.

Fluorescence spectroscopy

To observe possible effects of SR-1 and DNA on acyl chain order DPH was included into liposomes with different contents of SR-1 while recording the emission anisotropy *r* (Lakowicz et al., 1979a, b) both with and without DNA (Fig. 8). Values for *r* increased with increasing $X_{SR\text{-}1}$ up to 0.63. Above this SR-1 mole fraction anisotropy decreased significantly, yielding values similar to those measured for liposomes with $X_{SR\text{-}1} < 0.25$. The anisotropy measurements were done at 37°C, under the main transition temperatures of SR-1/DMPC liposomes with $0.25 \leq X_{SR\text{-}1} \leq 0.63$ (Fig. 6 *A*). In keeping with the DSC measurements these liposomes showed the highest anisotropy values.

Addition of DNA had little effect on DPH anisotropy for liposomes with $X_{SR\text{-}1} \leq 0.05$ (Fig. 8). When $X_{SR\text{-}1}$ was increased to 0.13, the effect of DNA became evident, with increased anisotropy at $X_{SR\text{-}1}$ from 0.13 to 0.50. The effect of DNA saturated at 5 μM, with no additional influence at higher concentrations. In keeping with the small changes observed by DSC, minor changes in *r* were caused by DNA at $X_{SR\text{-}1} \geq 0.63$.

DISCUSSION

The transfection efficiency of the binary SR-1/DMPC vesicles was observed to be highly dependent on the lipid stoichiometry. More specifically, liposomes with $X_{SR\text{-}1} = 0.25$ are strikingly poor in transfection when compared to compositions having $X_{SR\text{-}1} \geq 0.50$ (Fig. 2 *A*). Importantly, this difference is explained neither by variation in cytotoxicity (Fig. 3) nor by changes in the total cationic charge present in the liposomes, as the latter was maintained constant in the transfection experiments. Interestingly, biophysical parameters of both liposomes and DNA in the lipoplexes also demonstrated similar dependence on the lipid stoichiometry.

Increased static light scattering (Fig. 4) suggests condensation of DNA by the cationic liposomes, which has been previously shown to be required for efficient lipofection (Bloomfield, 1996; Tang and Szoka, 1998). Compared to the condensation of DNA by polycations, condensation caused by cationic amphiphiles is less thoroughly studied (Bloomfield, 1996). Moreover, the latter system is also inherently more complex, involving the association of a charged polymer with a colloid, pseudo-two-dimensional cationic surface. In our measurements with calf thymus DNA a maximum in light scattering was evident at $X_{SR\text{-}1} \approx 0.63$. Yet, it should be emphasized that aggregation could also contribute, thus making strict relationship between the observed scattering and DNA condensation somewhat uncertain. To investigate the possibility of DNA condensation further we measured fluorescence intensity of the DNA intercalating dye EtBr with SR-1/DMPC liposomes (Fig. 5). The quenching of EtBr emission evident at $X_{SR\text{-}1} > 0.50$ provides strong support for the condensation of DNA being

the major reason for the observed increase in scattering. Furthermore, our preliminary transmission electron microscopy studies (data not shown) revealed that the size of the complexes did not significantly change when $X_{SR\text{-}1}$ was increased from 0.25 to 0.75. Thus condensation and not aggregation is likely to represent the reason for the observed augmented scattering.

A slightly lower content of SR-1 is required to produce high transfection efficiency than to induce condensation of DNA as measured by light scattering and EtBr intercalation assay ($X_{SR\text{-}1} \geq 0.50$ and $X_{SR\text{-}1} > 0.50$, respectively). This could be due to use of the circular 4700 bp plasmid DNA in the transfection experiments, whereas longer calf thymus DNA was utilized in the other measurements. The length of DNA could influence complex morphology and possibly also condensation when comparing very short DNA fragments (with persistence length of DNA being 500 Å) to much longer molecules (Bloomfield, 1996; Bloomfield, 1998). Accordingly, this difference in $X_{SR\text{-}1}$ could relate to the difference in the lengths of the two types of DNA. More specifically, the larger decrement in the conformational entropy of the longer DNA upon its coil-globule transition due to complex formation requires higher positive charge density in the liposomes (Bloomfield, 1998). Yet, this difference in the DNAs used is unlikely to have a major qualitative impact on our data so as to undermine a meaningful comparison.

Binary SR-1/DMPC liposomes have maxima in T_m with $X_{SR\text{-}1}$ between 0.38 and 0.50 (Fig. 6 *A*), and these values for T_m exceed the main endotherm temperatures of the neat constituents. In addition, the enthalpy peaks for the mixtures are rather narrow thus resembling transitions of liposomes composed of a single lipid rather than a mixture. A plausible explanation for this counterintuitive impact of $X_{SR\text{-}1}$ on T_m of DMPC is provided by an electrostatically driven reorganization in the headgroup region of the bilayer. Accordingly, the addition of cationic SR-1 into zwitterionic phosphatidylcholine bilayer is anticipated to cause a reorientation of the P^--N^+ dipole of the PC headgroup (Fig. 9 *B*). More specifically, although the orientation of PC headgroup dipole in the neat phosphatidylcholine liposomes has been shown by NMR to be almost parallel to the plane of the bilayer (Seelig et al., 1987), the presence of cationic amphiphiles in the bilayer causes the P^--N^+ dipole to reorient nearly perpendicular to the surface because of the Coulombic repulsion (Scherer and Seelig, 1989). This results in a reduced average area occupied by the PC headgroup. Reduction in mean molecular areas occupied by POPC molecules in the presence of SR-1 was shown in our previous Langmuir-balance study (Säily et al., 2001). Reorientation of the hydrated phosphocholine moiety can be expected to result in reduced repulsive interactions at the headgroup level. As a consequence chain–chain interactions within the hydrocarbon phase must be augmented so as to balance the forces in the membrane lateral pressure profile.

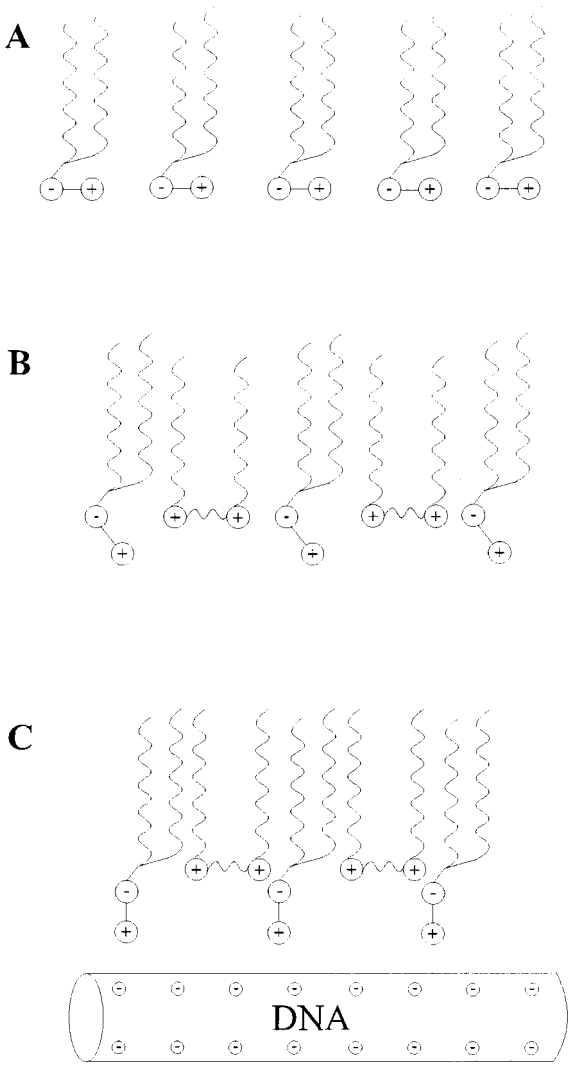

FIGURE 9 Schematic illustration of the postulated orientation of P^--N^+ dipole of the headgroup in neat PC liposomes (*A*), and in the presence of SR-1 (*B*) and DNA (*C*). See Discussion for further details.

The augmented packing of the acyl chains is described by reduction in the free volume of the bilayer in keeping with the elevated values for T_m and increased fluorescence anisotropy for DPH (Figs. 7 and 8). The above is in accordance with recent molecular dynamic simulations (Bandyopadhyay et al., 1999) and Langmuir-balance studies (Zantl et al., 1999; Säily et al., 2001) on cationic lipid-phosphatidylcholine mixtures. At $X_{SR\text{-}1} > 0.50$ Coulombic repulsion between the cationic headgroups of SR-1 is progressively enhanced, resulting in a lateral expansion of the bilayer, increase in membrane free volume, and thus decrement both in T_m and fluorescence anisotropy of DPH (Figs. 7 and 8, respectively).

The effects of DNA on the thermal phase behavior of SR-1/DMPC liposomes were surprisingly modest and only

a minor elevation in the liquidus line in the phase diagram ($X_{SR\text{-}1}$ between 0.05 and 0.38) due to the presence of DNA was evident. The simulation study by Bandyopadhyay et al. (1999) suggested that the cationic charge of the P^--N^+ dipole interacts directly with DNA. Accordingly, DNA could induce additional change in the orientation of P^--N^+ dipole due to Coulombic attraction (Fig. 9, *A–C*). In keeping with this notion the addition of DNA to the liposomes with $0.13 \leq X_{SR\text{-}1} \leq 0.50$ caused a significant increase in r (Fig. 8) thus suggesting that DNA induces tighter packing of the acyl chains. This mechanism would provide an explanation for the observed elevation in liquidus line in the phase diagram. Importantly, the above mechanism explains also why liposomes with $0.13 \leq X_{SR\text{-}1} \leq 0.50$ (and not liposomes with $X_{SR\text{-}1} > 0.50$ which bear more cationic net charge) demonstrate most pronounced changes due to the addition of DNA. In alignment with the above, compression isotherms for SR-1/POPC monolayers revealed film condensation in the presence of DNA (Säily et al., 2001).

The nature of the moiety bearing the cationic charge has been shown to be important in the condensation of DNA by cationic liposomes (Geall et al., 1999). Accordingly, the dependence of DNA condensation on the $X_{SR\text{-}1}$ could be explained by electrostatically driven molecular reorientations in the surface of liposomes suggested by DSC, fluorescence anisotropy of DPH, and monolayer (Säily et al., 2001) experiments. In the liposomes with $X_{SR\text{-}1} > 0.50$ the cationic charges of SR-1 are screened by the phosphates of the P^--N^+ dipoles thus causing association of DNA to liposomes to be mediated by the cationic charge of the tertiary ammonium group of the choline moiety (Fig. 9 *C*). However, the phosphocholine headgroup is strongly hydrated and because of its three methyl groups the cationic charge of the latter is anticipated to be incapable of as strong interaction with the phosphate residues of DNA as the sterically less shielded charges of SR-1. Interestingly, this raises the possibility that the enhanced transfection by the cationic liposomes containing PE may not relate only to the promotion of the formation of the inverted hexagonal phase H_{II} by this lipid but also to a more efficient Coulombic interaction of the weakly hydrated $-N^+H_3$ moiety of the PE headgroup with DNA. It seems feasible that in addition to the direct Coulombic interaction between SR-1 and DNA being required, also the cationic charge density is critical. The latter could be related to the lack of condensation of DNA by the divalent Mg^{2+}, in contrast to spermidine^{3+} and spermine^{4+}, for instance. The necessity for an interaction of SR-1 with DNA may also reflect the importance of the removal of hydration layers from the contacting molecular surfaces (Leikin et al., 1993). To this end, our preliminary transmission electron microscopy studies on negatively stained complexes of CT-DNA and SR-1/DMPC LUVs with $X_{SR\text{-}1} = 0.25$ and 0.75 revealed very different morphologies. Accordingly, at $X_{SR\text{-}1} = 0.25$ spaghetti-and-meatballs–like structure (Sternberg et al., 1994) was evident, whereas at $X_{SR\text{-}1} = 0.75$ more dense and irregular particles were observed (data not shown).

To conclude, our data demonstrate that the cationic charge density of liposomes is an important determinant of transfection efficiency and suggest that this is related to condensation of DNA. This observation further emphasizes the importance of compactly packed and well protected plasmid DNA for efficient lipofection. Surface electrostatics of the liposomes thus appear to have a more crucial role in the condensation of DNA and lipofection than previously anticipated (e.g., Wagner et al., 2000; Harries et al., 1998; Gelbart et al., 2000) involving complex rearrangements in the headgroup region of the bilayer. Experimental and theoretical efforts to elucidate more completely the role of electrostatics of liposomal surfaces in lipofection are in progress in our laboratory.

The authors thank Kaija Niva for excellent technical assistance, Juha-Matti Alakoskela, B.M, for rewarding discussions, and Ove Eriksson, Ph.D., and Oula Penate-Medina, M. Sc., for their contributions in the early stages of the study.

S.J.R. is supported by the M.D./Ph.D. program of the University of Helsinki. The Helsinki Biophysics and Biomembrane Group is supported by grants from TEKES and the Finnish State Medical Research Council.

REFERENCES

Alton, E. W. F. W., M. Stern, R. Farley, A. Jaffe, S. L. Chadwick, J. Phillips, J. Davies, S. N. Smith, J. Browning, M. G. Davies, M. E. Hodson, S. R. Durham, D. Li, P. K. Jeffery, M. Scallan, R. Balfour, S. J. Eastman, S. H. Cheng, A. E. Smith, D. Meeker, and D. M. Geddes. 1999. Cationic lipid-mediated CFTR gene transfer to the lungs and nose of patients with cystic fibrosis: a double-blind placebo-controlled trial. *Lancet*. 353:947–954.

Bandyopadhyay, S., T. Mounir, and M. L. Klein. 1999. Molecular dynamics study of a lipid-DNA complex. *J. Phys. Chem. B.* 103: 10075–10080.

Bhattacharya, S., and S. S. Mandal. 1998. Evidence of interlipidic ion-pairing in anion-induced DNA release from cationic amphiphile-DNA complexes. Mechanistic implications in transfection. *Biochemistry*. 37:7764–7777.

Bloomfield, V. A. 1996. DNA condensation. *Curr. Opin. Struct. Biol.* 6:334–341.

Bloomfield, V. A. 1998. DNA condensation by multivalent cations. *Biopolymers*. 44:269–282.

Cain, B. F., B. C. Baguley, and W. A. Denny. 1978. Potential antitumor agents. 28. Deoxyribonucleic acid polyintercalating agents. *J. Med. Chem.* 21:658–668.

Caplen, N. J., E. W. F. W. Alton, P. G. Middleton, J. R. Dorin, B. J. Stevenson, X. Gao, S. R. Durham, P. K. Jeffery, M. E. Hodson, C. Coutelle, L. Huang, D. J. Porteous, R. Williamson, and D. M. Geddes. 1995. Liposome-mediated CFTR gene transfer to the nasal epithelium of patients with cystic fibrosis. *Nat. Med.* 1:39–46.

Cerichelli, G., L. Luchetti, and G. Mancini. 1996. Surfactant control of the ortho/para ratio in the bromination of anilines. 3. *Tetrahedron*. 52: 2465–2470.

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

Felgner, P. L., T. R. Gadek, M. Holm, R. Roman, H. W. Chan, M. Wenz, J. P. Northrop, G. M. Ringold, and M. Danielsen. 1987. Lipofection:

a highly efficient, lipid-mediated DNA-transfection procedure. *Proc. Natl. Acad. Sci. USA*. 84:7413–7417.

Fielden, M. L., C. Perrin, A. Kremer, M. Bergsma, M. C. Stuart, P. Camilleri, and J. B. F. N. Engberts. 2001. Sugar-based tertiary amino gemini surfactants with a vesicle-to-micelle transition in the endosomal pH range mediate efficient transfection *in vitro*. *Eur. J. Biochem.* 268:1269–1279.

Geall, A. J., M. A. W. Eaton, T. Baker, C. Catterall, and I. S. Blagbrough. 1999. The regiochemical distribution of positive charges along cholesterol polyamine carbamates plays significant roles in modulating DNA binding affinity and lipofection. *FEBS Lett*. 459:337–342.

Gelbart, W. M., R. F. Bruinsma, P. A. Pincus, and V. A. Parsegian. 2000. DNA-inspired electrostatics. *Physics Today*. 9:38–44.

Gluzman, Y. 1981. SV40-transformed simian cells support the replication of early SV40 mutants. *Cell*. 23:175–182.

Gustafsson, J., G. Arvidson, G. Karlsson, and M. Almgren. 1995. Complexes between cationic liposomes and DNA visualized by cryo-TEM. *Biochim. Biophys. Acta*. 1235:305–312.

Harries, D., S. May, W. M. Gelbart, and A. Ben-Shaul. 1998. Structure, stability, and thermodynamics of lamellar DNA-lipid complexes. *Biophys. J*. 75:159–173.

Huebner, S., B. J. Battersby, R. Grimm, and G. Cevc. 1999. Lipid-DNA complex formation: reorganization and rupture of lipid vesicles in the presence of DNA as observed by cryoelectron microscopy. *Biophys. J*. 76:3158–3166.

Hui, S. W., M. Langner, Y.-L. Zhao, P. Ross, E. Hurley, and K. Chan. 1996. The role of helper lipids in cationic liposome-mediated gene transfer. *Biophys. J*. 71:590–599.

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

Kinnunen, P. K. J., M. Rytömaa, A. Kõiv, J. Lehtonen, P. Mustonen, and A. Aro. 1993. Sphingosine-mediated membrane association of DNA and its reversal by phosphatidic acid. *Chem. Phys. Lipids*. 66:75–85.

Kõiv, A., and P. K. J. Kinnunen. 1994. Binding of DNA to liposomes containing different derivates of sphingosine. *Chem. Phys. Lipids*. 72:77–86.

Kõiv, A., P. Mustonen, and P. K. J. Kinnunen. 1994. Differential scanning calorimetry study on the binding of nucleic acids to dimyristoylphosphatidylcholine-sphingosine liposomes. *Chem. Phys. Lipids*. 70:1–10.

Koltover, I., T. Salditt, J. O. Rädler, and C. R. Safinya. 1998. An inverted hexagonal phase of cationic liposome-DNA complexes related to DNA release and delivery. *Science*. 281:78–81.

Lakowicz, J. R. 1999. Principles of Fluorescence Spectroscopy. Kluwer Academic/Plenum Publishers, New York.

Lakowicz, J. R., F. G. Prendergast, and D. Hogen. 1979a. Differential polarized phase fluorometric investigations of diphenylhexatriene in lipid bilayers. Quantitation of hindered depolarizing rotations. *Biochemistry*. 18:508–519.

Lakowicz, J. R., F. G. Prendergast, and D. Hogen. 1979b. Fluorescence anisotropy measurements under oxygen quenching conditions as a method to quantify the depolarizing rotations of fluorophores. Application to diphenylhexatriene in isotropic solvents and in lipid bilayers. *Biochemistry*. 18:520–527.

Leikin, S., V. A. Parsegian, D. C. Rau, and R. P. Rand. 1993. Hydration forces. *Annu. Rev. Phys. Chem*. 44:369–395.

Leventis, R., and J. R. Silvius. 1990. Interactions of mammalian cells with lipid dispersions containing novel metabolizable cationic amphiphiles. *Biochim. Biophys. Acta*. 1023:124–132.

MacDonald, R. C., R. I. MacDonald, B. P. Menco, K. Takeshita, N. K. Subbarao, and L. R. Hu. 1991. Small-volume extrusion apparatus for preparation of large, unilamellar vesicles. *Biochim. Biophys. Acta*. 1061:297–303.

Menger, F. M., and J. S. Keiper. 2000. Gemini surfactants. *Angew. Chem. Int. Ed.* 39:1906–1920.

Mok, W. C. K., and P. R. Cullis. 1997. Structural and fusogenic properties of cationic liposomes in the presence of plasmid DNA. *Biophys. J*. 73:2534–2545.

Patrick, C., M. L. Hensen, R. Supabphol, and S. W. Hui. 1998. Multilamellar cationic liposomes are efficient vectors for in vitro gene transfer in serum. *J. Liposome Res*. 8:499–520.

Paukku, T., S. Laureus, I. Huhtaniemi, and P. K. J. Kinnunen. 1997. Novel cationic liposomes for DNA-transfection with high efficiency and low toxicity. *Chem. Phys. Lipids*. 87:23–29.

Pedroso de Lima, M. C., S. Simões, P. Pires, R. Gaspar, V. Slepushkin, and N. Düzgünes. 1999. Gene delivery mediated by cationic liposomes: from biophysical aspects to enhancement of transfection. *Mol. Mem. Biol*. 16:103–109.

Ross, P. C., and S. W. Hui. 1999. Lipoplex size is a major determinant of in vitro lipofection efficiency. *Gene Ther*. 6:651–659.

Säily, V. M. J., S. J. Ryhänen, J. M. Holopainen, S. Borocci, G. Mancini, and P. K. J. Kinnunen. 2001. Characterization of mixed monolayers of phosphatidylcholine and a dicationic gemini surfactant SS-1 with a Langmuir balance. *Biophys. J*. 81:2135–2143.

Seelig, J., P. M. MacDonald, and P. G. Scherer. 1987. Phospholipid head groups as sensors of electric charge in membranes. *Biochemistry*. 26:7535–7541.

Scherer, P. G., and J. Seelig. 1989. Electric charge effects on phospholipid headgroups. Phosphatidylcholine in mixtures with cationic and anionic amphiphiles. *Biochemistry*. 28:7720–7728.

Shi, N., and W. M. Pardridge. 2000. Noninvasive gene targeting to the brain. *Proc. Natl. Acad. Sci. USA*. 97:7567–7572.

Simões, S., V. Slepushkin, P. Pires, R. Gaspar, M. C. Pedroso de Lima, and N. Düzgünes. 1999. Mechanisms of gene transfer mediated by lipoplexes associated with targeting ligands or pH-sensitive peptides. *Gene Ther*. 6:1798–1807.

Solodin, I., C. S. Brown, M. S. Bruno, C. Y. Chow, E. H. Jang, R. J. Debs, and T. D. Heath. 1995. A novel series of amphiphilic imidazolinium compounds for in vitro and in vivo gene delivery. *Biochemistry*. 34:13537–13544.

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.

Subramaniam, M., J. M. Holopainen, T. Paukku, O. Erikson, I. Huhtaniemi, and P. K. J. Kinnunen. 2000. Characterization of three novel cationic lipids as liposomal complexes with DNA. *Biochim. Biophys. Acta*. 1466:289–305.

Tang, M. X., and F. C. Szoka, Jr. 1998. Characterization of polycation complexes with DNA. *In* Self-assembling Complexes for Gene Delivery: From Laboratory to Clinical Trial. A.V. Kabanov, P.L. Felgner, and L.W. Seymour, editors. John Wiley and Sons, Ltd. 169– 196.

Wagner, K., D. Harries, S. May, V. Kahl, J. O. Rädler, and A. Ben-Shaul. 2000. Direct evidence for counterion release upon cationic lipid-DNA condensation. *Langmuir*. 16:303–306.

Wheeler, C. J., L. Sukhu, G. Yang, Y. Tsai, C. Bustamente, P. Felgner, J. Norman, and M. Manthorpe. 1996. Converting an alcohol to an amine in a cationic lipid dramatically alters the co-lipid requirement, cellular transfection activity and the ultrastructure of DNA-cytofectin complexes. *Biochim. Biophys. Acta*. 1280:1–11.

Wiedmer, K. S., J. Hautala, J. M. Holopainen, P. K. J. Kinnunen, and M.-L. Riekkola. 2001. Study on liposomes by capillary electrophoresis. *Electrophoresis*. 22:1305–1313.

Xu, Y., S. W. Hui, P. Frederik, and F. C. Szoka, Jr. 1999. Physicochemical characterization and purification of cationic lipoplexes. *Biophys. J*. 77:341–353.

Zantl, R., L. Baicu, F. Artzner, I. Sprenger, G. Rapp, and J. O. Rädler. 1999. Thermotropic phase behavior of cationic lipid-DNA complexes compared to binary lipid mixtures. *J. Phys. Chem. B*. 103:10300–10310.

Zuidam, N. J., D. Hirsch-Lerner, S. Marqulies, and Y. Barenholz. 1999. Lamellarity of cationic liposomes and mode of preparation of lipoplexes affect transfection efficiency. *Biochim. Biophys. Acta*. 1419: 207–220.