Factors Governing the Assembly of Cationic Phospholipid-DNA Complexes

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ABSTRACT The interaction of DNA with a novel cationic phospholipid transfection reagent, 1,2-dioleoyl-*sn*-glycero-3-ethylphosphocholine (EDOPC), was investigated by monitoring thermal effects, particle size, vesicle rupture, and lipid mixing. By isothermal titration calorimetry, the heat of interaction between large unilamellar EDOPC vesicles and plasmid DNA was endothermic at both physiological and low ionic strength, although the heat absorbed was slightly larger at the higher ionic strength. The energetic driving force for DNA-EDOPC association is thus an increase in entropy, presumably due to release of counterions and water. The estimated minimum entropy gain per released counterion was 1.4 cal/mole-°K (about 0.7 kT), consistent with previous theoretical predictions. All experimental approaches revealed significant differences in the DNA-lipid particle, depending upon whether complexes were formed by the addition of DNA to lipid or vice versa. When EDOPC vesicles were titrated with DNA at physiological ionic strength, particle size increased, vesicles ruptured, and membrane lipids became mixed as the amount of DNA was added up to a 1.6:1 (+:−) charge ratio. This charge ratio also corresponded to the calorimetric end point. In contrast, when lipid was added to DNA, vesicles remained separate and intact until a charge ratio of 1:1 (+:−) was exceeded. Under such conditions, the calorimetric end point was 3:1 (+:−). Thus it is clear that fundamental differences in DNA-cationic lipid complexes exist, depending upon their mode of formation. A model is proposed to explain the major differences between these two situations. Significant effects of ionic strength were observed; these are rationalized in terms of the model. The implications of the analysis are that considerable control can be exerted over the structure of the complex by exploiting vectorial preparation methods and manipulating ionic strength.

INTRODUCTION

Cationic lipids as vehicles for delivery of DNA into cells have been shown to be effective both in vitro (Felgner et al., 1987) and in vivo (Fortunati et al., 1996; Lee et al., 1996; Templeton et al., 1997) and present an appealing alternative to viral vectors, given the disadvantages associated with the latter (Yang et al., 1995; McElvaney, 1996). Formation of the active complexes is accomplished by simply mixing DNA and cationic lipid, followed by incubation of the newly formed complex with the cells to be transfected. Current research suggests that DNA:cationic lipid (DNA:CL) complexes enter the cell by endocytosis (Friend et al., 1996; Zhou and Huang, 1994; Felgner et al., 1994; Zabner et al., 1995). DNA escapes from endosomes and is ultimately trafficked to the nucleus by an unknown mechanism. Because of the apparently low efficiency of transfection, many investigations have focused on improving transfection activity by examining the steps involved in lipofection, including DNA-lipid complex formation (Eastman et al., 1997; Gershon et al., 1993; Gustafsson et al., 1995; Mok and Cullis, 1997; Reimer et al., 1995), entry into the cell (Friend et al., 1996; Reimer et al., 1997; Tseng et al., 1997; Matsui et al., 1997), escape of DNA from endosomal compartments (Zelphati and Szoka, 1996; Xu and Szoka, 1996; Zabner et al., 1995), and entry into the nucleus (Zabner et al., 1995; Aronsohn and Hughes, 1998).

Despite their near-universal acceptance as gene transfer reagents, no consensus has been reached on the structure of the "active" DNA:CL complex. Numerous studies have revealed the presence of liposomes associated with DNA (Gershon et al., 1993; Eastman et al., 1997; Templeton et al., 1997; Mok and Cullis, 1997; Sternberg et al., 1994) and the presence of DNA encapsulated in tubelike bilayers (Sternberg et al., 1994; Gershon et al., 1993). In addition, multilamellar complexes of DNA and cationic lipid have been described (Rädler et al., 1997; Gustafsson et al., 1995; Lasic et al., 1997; Boukhnikachvili et al., 1997; Huebner et al., 1999), as well as structures containing nonbilayer elements (Mok and Cullis, 1997; Koltover et al., 1998; Labat-Moleur et al., 1996). The finding that the structures of complexes formed at DNA:CL ratios giving optimal transfection are dissimilar from those formed at suboptimal ratios (Mok and Cullis, 1997; Sternberg et al., 1994) and the proposition that a hexagonal arrangement of DNA-lipid complexes may promote transfection (Koltover et al., 1998) suggest that the organization of DNA:CL complexes is a key factor in transfection efficiency. Theoretical studies on the stability and organization of DNA:CL complexes are appearing in the literature (Dan, 1997, 1998; Harries et al., 1998; Bruinsma, 1998).

As a first step in studying the interaction between lipid transfection reagents and DNA, other investigators have examined the interaction of simple micellar cationic surfactants with DNA. Cationic surfactant binds to DNA (Spink

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and Chaires, 1997; Mel'nikov et al., 1995a; Hayakawa et al., 1983; Shirahama et al., 1987) and is capable of condensing DNA (Mel'nikov et al., 1995a,b), and binding is sensitive to ionic strength (Hayakawa et al., 1983; Shirahama et al., 1987). A calorimetric determination of the binding of cetyltrimethylammonium bromide revealed cooperative binding of the surfactant to DNA (Spink and Chaires, 1997). However, most transfection lipids are not micellar, precluding the extension of micelle studies to transfection lipids. A calorimetric analysis of the association of DNA and a lamellar transfection lipid has not yet been reported.

Here we propose a mechanism for the formation of DNA:CL complexes, based on studies with the novel cationic phospholipid 1,2-dioleoyl-*sn*-glycero-3-ethylphosphocholine (EDOPC), an analog of a natural phospholipid (MacDonald et al., 1999a) that has been shown to be an efficient and biodegradable transfection agent (MacDonald et al., 1999b). Dynamic light scattering, isothermal titration calorimetry (ITC), and vesicle contents release indicate that the formation of DNA:CL complexes is sensitive to ionic strength and can be controlled by the stepwise addition of lipid to DNA or DNA to lipid, resulting in particles of different sizes. Moreover, calorimetric analysis of the binding of EDOPC to DNA reveals that the interaction is endothermic and is driven by an increase in entropy.

MATERIALS AND METHODS

EDOPC was synthesized by reacting dioleoylphosphatidylcholine with ethyl trifluoromethylsulfonate in chloroform and purifying the product by silica gel chromatography (MacDonald et al., 1999a). Its molecular weight is 946 as the trifluoromethansulfonate salt. Alternatively, EDOPC is available commercially from Avanti Polar Lipids (Alabaster, AL), which was the source of N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt. YOYO-1 and RhPE were purchased from Molecular Probes (Eugene, OR). Plasmid DNA (pUC18, ~2.7 kb) was obtained from Bayou Biolabs (Harahan, LA). All phospholipid concentrations were verified by phosphate analysis (Bartlett, 1959), and DNA concentrations were determined by measuring the absorption at 260 nm, using the relationship 1 O.D. = 50 μg/ml.

Preparation of large unilamellar vesicles

Unilamellar liposomes were prepared by extrusion of lipid dispersions through 0.1-μm polycarbonate filters (Nuclepore, Cambridge, MA), using a small volume extrusion apparatus (Avanti Polar Lipids). The desired amount of lipid in chloroform was dried down under argon, then subjected to high vacuum for >45 min to remove any residual chloroform. The lipid film was resuspended in the appropriate amount of HE-S (20 mM HEPES, 0.15 M NaCl, 0.1 mM EDTA, pH 7.5) or HE (20 mM HEPES, 0.1 mM EDTA, pH 7.5) buffer and extruded 10 times, as described, except that a single filter was used (MacDonald et al., 1991). Modifications of the liposome preparation procedure required for the vesicle leakage and membrane mixing assays are described below.

Isothermal titration calorimetry

The enthalpy associated with the interaction between EDOPC and pUC18 was determined using a MicroCal isothermal titration calorimeter (Northampton, MA) at 30°C. The reference cell contained either HE-S or HE buffer, depending on the experiment. Approximately 1.5-ml samples of either EDOPC or DNA (~0.4 mM) were loaded into the sample cell and titrated with 10 μl-aliquots of buffered solution containing either DNA or EDOPC, using the instrument's automated injection system. Heats of dilution corresponding to added ligand were estimated from the final additions of ligand at saturating concentrations and were found to be equal to the injection of ligand into buffer alone. The enthalpies of each injection were corrected for this value. Both the addition of lipid to DNA and the addition of DNA to lipid were investigated. Raw data were processed using Origin graphing software and macros provided by MicroCal.

Light scattering measurements

Dynamic light scattering measurements were carried out using a Brookhaven Instruments (Brookhaven, NY) dynamic light scattering apparatus with a 300-mW argon ion laser at 488 or 514 nm, with equivalent results. In a quartz fluorescence cuvette, a 2-ml aliquot of either 2.5×10^{-5} M EDOPC or 2.9×10^{-5} M DNA was titrated with DNA or EDOPC, as appropriate. Correlation data were collected at 90° relative to the incident beam, and delay times from 5 ms to 1.0 s were explored. Correlation data were fitted, using the method of cumulants (Koppel, 1972), to the logarithm of the correlation function, yielding the diffusion coefficient, D. The effective diameter was calculated using D and the Stokes-Einstein relationship: $D = kT/6\pi\eta R$, where D is the diffusion coefficient of the particle, k is Boltzmann's constant, T is the temperature in degrees Kelvin, η is the solvent viscosity, and R is the radius of the particle. To assess polydispersity within each sample, data were also analyzed using the nonnegative least-squares (NNLS) (Lawson and Hanson, 1974) or CONTIN (Provencher, 1979, 1982) algorithms supplied with the instrument software.

For experiments examining the size of DNA:CL particles formed by rapidly mixing the reactants, the appropriate amounts of DNA and lipid were quickly mixed using an air-displacement pipette. This method differs from titration, in which small increments of titrant were added to the cuvette (containing either DNA or lipid, well stirred) at 5–10-min intervals.

Vesicle contents release assay

The release of vesicle contents was monitored using a modification of the terbium/dipicolinic acid (Tb/DPA) assay as described (Wilschut et al., 1980; Düzgüneş and Wilschut, 1993), by measuring the decrease in fluorescence of encapsulated Tb/DPA after its dilution into buffer containing EDTA. Briefly, dried EDOPC films were rehydrated in 20 mM HEPES, 2.25 mM $TbCl_3$, 5 mM dipicolinic acid, and NaCl, at either 0.15 M or 20 mM, pH 7.5. Attempts at resuspending the lipid in Tb/DPA buffer not containing NaCl resulted in lipid precipitation, in accord with previous observations using cationic amphipaths (Düzgüneş et al., 1989). The lowest concentration of NaCl sufficient to reverse precipitation was 20 mM. The resuspended lipid was then extruded as described above. Extruded Tb/DPA vesicles were then desalted over a Bio-Rad 10-DG column in HE containing 20 mM NaCl or HE-S, as appropriate, to separate encapsulated from free Tb/DPA. Control experiments in which vesicles were lysed with detergent in a solution containing 5 mM EDTA verified that all fluorescence arising from the vesicle suspension was contained within the encapsulated volume. Both Triton X-100 and Zwittergent 3–14 were used as detergents, but the latter is very preferable over the former, which exhibits an undesirably large amount of quenching of terbium fluorescence.

Typically, a 50-μg aliquot of Tb/DPA vesicles in 500 μl was titrated with DNA, and the fluorescence was monitored as a function of time (λ_{ex} = 277 nm, λ_{em} = 545 nm). The percentage Tb/DPA fluorescence after each addition of DNA (F) was calculated relative to the initial fluorescence (F_o), using % Tb/DPA Fluor = $F/F_o \times 100$. Effects of dilution arising from the addition of DNA to the cuvette were negligible.

As a result of a change in personnel, the titrations in the two directions were done by different investigators, and the procedure for titration of DNA by lipid differed slightly from that described above. Specifically, the extruded Tb/DPA vesicles were desalted by dialysis (Spectra/Por cellulose ester membrane with an 8-kDa pore size) against HE-S, to separate encapsulated from free Tb/DPA. Furthermore, fluorescence intensity was determined by integrating the entire 545-nm peak in the emission spectrum (λ_{ex} = 277 nm).

Because the titrations with lipid involve introducing fluorescence into the solution, and this is largely eliminated when the vesicles rupture, a control measurement is made to establish the unquenched levels of fluorescence for each aliquot of vesicles added to the DNA. These values (F^0) correspond to 0% rupture. Zwittergent 3–14 detergent was added to selected samples to assess the fraction (Y) of the total fluorescence that was present when all of the vesicles were lysed. The fluorescence intensities for each aliquot after the addition of Zwittergent 3–14 corresponding to 100% rupture are thus given by $YF^0 = F^{100}$. A 2-μg aliquot of DNA in 300 μl of HE-S was titrated with Tb/DPA vesicles and the fluorescence intensities for each addition. The magnitude of the signal from these aliquots (F^n) relative to the difference between the 0% and the 100% values gave the percentage rupture for each such addition, i.e., % rupture = $(F^{100} - F^n)/(F^{100} - F^0)$.

An AlphaScan spectrofluorometer (PTI, Princeton, NJ) was used for all fluorescence measurements.

Membrane mixing assay

We measured the mixing of lipids of initially separate vesicles, using a common fluorescence resonance energy transfer assay that measures reduction of energy transfer between a donor-acceptor pair in one set of vesicles as they are diluted if they fuse or otherwise exchange lipid with a second, unlabeled set of vesicles (Struck et al., 1981). The EDOPC mixture was prepared from hydrated and extruded EDOPC samples containing three parts unlabeled EDOPC and one part EDOPC labeled with 0.5 mol% each of *N*-(lissamine Rhodamine B sulfonyl)phosphatidylethanolamine and *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine. The lipids were hydrated at 1 mg/ml in HE-S buffer and diluted to 0.09 mg/ml in the same buffer. Two hundred twenty microliters of this dispersion was titrated with plasmid DNA (pUC18, 1 mg/ml, in TE buffer; 10 mM Tris-Cl, 1 mM EDTA). For the reverse titration, the 3:1 labeled/unlabeled EDOPC mixture at 1 mg/ml was used for titration of plasmid DNA (7.5 μl at 1 mg/ml in TE buffer added to 200 μl HE-S buffer); however, in this case, each aliquot generated an increment of fluorescence due to residual fluorescence that was not quenched by energy transfer. Hence a titration into buffer alone was used to generate these background values that were then subtracted from the experimental values obtained when DNA was titrated with lipid. The fluorescence intensity corresponding to 100% lipid mixing was obtained by treating the EDOPC mixture with an equal volume of methanol, evaporating the methanol under a stream of argon, and reconstituting the mixture at the appropriate volume.

For the titration of lipid with plasmid, the percentage of lipid mixing was calculated using $(F_n - F_0)/(F_{100} - F_0) \times 100\%$, where F_n is the fluorescence after each addition of DNA to EDOPC, F_0 is the fluorescence of the EDOPC mixture in the absence of DNA, and F_{100} is the fluorescence at 100% lipid mixing. Fluorescence intensities were corrected for sample dilution after the addition of each aliquot.

For the titration of plasmid with lipid, the percentage of lipid mixing after each addition of the EDOPC mixture to DNA was calculated using $[(F_n - F_{n0})/F_{n0}(\eta - 1)] \times 100\%$, where F_n is the fluorescence after each addition of EDOPC to DNA, F_{n0} is the background fluorescence after each addition of EDOPC to HE-S buffer (no DNA), and η is F_{100}/F_{n0} (F_{100} is fluorescence at 100% lipid mixing), which was constant and approximately equal to 2. The end point of these experiments was not as sharp as desirable, for as the ratio of lipid to DNA became quite high, the signal began to decrease. This may have been due to turbidity, which increases as more vesicles are added, and which would have reduced the fluorescence signal. Although, in principle, this is correctable, we deemed it unnecessary, because the pattern seen in this case of membrane mixing was completely consistent with the other characteristics of the complex. Thus we have simply not plotted points beyond where the signal began to decrease.

Samples were excited at 469 nm and measured at 535 nm with bandwidths of 2 nm.

RESULTS

Formation of DNA:EDOPC complexes by the addition of DNA to lipid

Calorimetry

It has been postulated that the thermodynamic driving force for DNA-lipid association is the entropy increase from the release of counterions and bound water associated with DNA and the lipid surface (Ross and Shapiro, 1974; Manning, 1978; Rädler et al., 1997; Bruinsma, 1998). However, a determination of the enthalpic and entropic contributions to the Gibbs free energy has not yet been reported. The filled circles of Fig. 1 show the results of a determination of the enthalpy for the interaction of pUC18 and EDOPC vesicles as a function of the DNA:lipid charge ratio. The data points represent the cumulative heats for the individual injections during the titration, normalized to the number of moles of the component present in the cell initially, in this case, lipid. As shown in the figure, the binding of DNA to EDOPC is endothermic, indicating that a significant increase in entropy must be the driving force for the association of DNA and cationic lipid. The binding of sonicated salmon sperm DNA to EDOPC was also examined and found to be endothermic as well, showing that a similar thermodynamic response occurs when the DNA is in a linear form (data not shown).

The slope of the heat absorption line of Fig. 1 is 860 cal/mol DNA. There was no further heat absorption after the DNA/cationic lipid ratio reached 0.33, indicating that the stoichiometry of the complex formed by adding DNA to extruded vesicles is ~3:1 lipid/DNA. The total heat absorbed was 280 cal/mole lipid. Based on the stoichiometry of complex, and for simplicity assuming one counter ion is released from each pair of neutralized charges (one from each nucleotide and from one out of every 3 lipid molecules), a total of 0.66 moles of counterions were released per mole of lipid in formation of the stoichiometric complex. Accordingly, the heat was 420 cal per mole of released counterion. Given the same assumption, the slope of the heat absorption line gives essentially the same value, i.e., 430 cal/mole counterion.

The calorimetry points all fall on a line until the break corresponding to the end point at which charge ratio ΔH no longer changes measurably. These characteristics indicate that each increment of DNA comes into contact with the same amount of lipid and that the complex is sufficiently

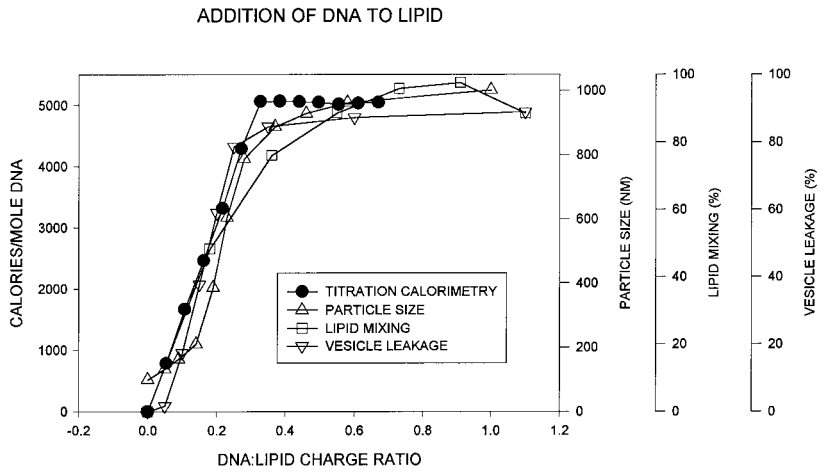

FIGURE 1 Interactions of DNA with cationic phospholipid during titration of EDOPC with pUC18. (*A*) EDOPC in the form of 100-nm extruded vesicles was titrated with pUC18 plasmid DNA. The aqueous phase was HE-S (0.15 M NaCl, 0.1 mM EDTA, and 20 mM HEPES buffer at pH 7.5). Formation of complexes was monitored by isothermal titration calorimetry (●), dynamic light scattering (△), vesicle contents release (▽), and membrane mixing (□). For calorimetry and dynamic light scattering, EDOPC equilibrated with HE-S was titrated with portions of DNA solution. In the former case, cumulative injection heats were normalized to the amount of lipid in the cell and plotted against the charge ratio as shown. For measurement of vesicle rupture, a suspension of lipid vesicles loaded with HE-S supplemented with Tb dipicolinate was titrated (in HE-S supplemented with 0.1 mM EDTA) with portions of DNA solution. Rupture was calculated from the decrease in fluorescence as released Tb dipicolinate became dissociated in the external solution. For lipid mixing measurements, a lipid dispersion that consisted of a mixture of vesicles of pure EDOPC and of EDOPC containing a NBD-rhodamine fluorescence resonance energy pair was titrated with portions of DNA solution. Membrane mixing was calculated from the change in energy transfer.

tight that little dissociation occurs at the concentrations used. Indeed, attempts to dilute the samples to the point where there was sufficient dissociation that an association constant could be measured were unsuccessful because of insufficient instrument sensitivity.

Size of the complex

To assess the size of the DNA:CL complex as DNA was added to lipid, formation of pUC18:EDOPC complexes was examined by dynamic light scattering. Titration of EDOPC large unilamellar vesicles (LUVs) with pUC18 resulted in particles of increased size (Fig. 1, *upright triangles*). The transition from particles the size of vesicles to the larger complex was complete at essentially the same charge ratio seen in the end point of the ITC titration, indicating that the complex increased in size until binding ceases. In contrast to the calorimetric titrations, however, the dynamic light scattering isotherms were nonlinear, and there was a distinct break at a charge ratio of about DNA/CL = 0.15, at which point the particle size was about twice that initially.

Average particle sizes were very reproducible, occurring over nearly identical DNA:CL ratios. The final sizes of the complexes, however, varied significantly from experiment to experiment, as much as 200–600 nm. Analysis of the correlation functions using the NNLS and CONTIN data fitting routines indicated that the samples were polydisperse throughout the transition range, which may account for the range of sizes observed in the final complexes.

Vesicle contents release

To define the point at which vesicle integrity is compromised, we employed a standard contents release assay to follow vesicle rupture as a function of added DNA. Fig. 1 (*inverted triangles*) shows the isotherm generated by titrating EDOPC vesicles containing Tb/DPA with pUC18. Like heat absorption and particle size change, vesicle rupture was also complete at a DNA:CL charge ratio of ~0.3. There may be a slight difference between the progression of leakage and heat absorbed; the two curves generally follow much the same path, as does membrane mixing (see next paragraph). This behavior, as will be described below, is much different from that in the case of the addition of lipid to DNA.

Membrane mixing

Given that the vesicles were clearly ruptured by contact with a relatively small amount of DNA, the question arose as to whether this was due to individual interactions of DNA molecules with a vesicle, or whether the DNA induced interactions among vesicles that gave rise to mutual rupture. That the latter occurred was shown by membrane mixing experiments in which two populations of vesicles were used, one of which contained a fluorescence energy transfer probe pair, while the other consisted only of EDOPC. This assay, commonly used for measurement of membrane fusion, relies on the reduction of quenching of the donor probe

by the acceptor probe when fusion and hence dilution of both probes occurs (Struck et al., 1981). The data from these experiments (*squares* in Fig. 1) show that membrane mixing is effectively coincident with the vesicle rupture data as well as with DNA-lipid interactions as measured by calorimetry. The curve describing particle size does differ somewhat from the other three; however, this is not unexpected and will be considered further in the Discussion.

Formation of DNA:EDOPC complexes by the addition of lipid to DNA

Because the structures of DNA (long, flexible strand) and lipid vesicles (spherical shells with distinct inner and outer surfaces) have very different symmetries, it would be expected that the structure of the complex formed by stepwise addition of lipid vesicles to DNA would differ from that formed by the reverse procedure. To explore this hypothesis, we have characterized the complex formed when DNA was titrated with EDOPC, using the methods described in the previous section.

Calorimetry

As shown in Fig. 2 (*filled circles*), reversal of the order of addition for formation of the DNA-lipid complex changed remarkably the calorimetric profile. Addition of EDOPC to pUC18 in HE-S resulted in an endpoint for the titration that was much different than that observed for the analogous titration of pUC18 into EDOPC. Moreover, the end point of the titration was much different, occurring in this case at a lipid/DNA charge ratio of 1.6:1. As in the case of addition of DNA to lipid, the points fall very close to a straight line (a very slight bend occurs at ~0.6) until the end point is reached. This sharp end point again indicates essentially complete association of the lipid in each aliquot with the DNA in solution. The heat absorbed at the end point is about 910 cal/mole DNA and the slope of the curve is 560 cal/mole lipid. Since the final complex contains excess lipid, about two moles of counterions are released per mole of DNA when the complex is formed this way. Accordingly, the heat absorbed per released counter ion (assuming one released counter ion per charge neutralized) is 460 cal/mole.

Size of the complex

Because the order of addition had a pronounced effect on the thermal titration equivalence point as well as the overall enthalpy, we also determined the size of DNA:CL particles generated by adding lipid to DNA. For this mode of mixing, we observe large differences between the interaction as measured by calorimetry and the characteristics of the complex. Instead of the characteristics of the particle changing in progression with the calorimetrically determined DNA-lipid interaction, there is little change until at least a 1:1 charge ratio is reached. This is seen in the case of particle size in Fig. 2 (*triangles*); the particle size remains roughly constant until the amount of lipid positive charge exceeds the nucleic acid negative charge, whereupon the particles grow rapidly. The decrease on the right side of the peak observed in the dynamic light scattering profile is a result of the addition of excess vesicles, which lowers the average size of particles in the population. This characteristic makes it difficult to determine the point at which complex growth ceases, but it appears that it is very close to the 1.6 charge ratio seen in the calorimetric end point.

Vesicle rupture

Like particle growth, the loss of contents from vesicles added to pUC18 DNA solution is much delayed relative to the interaction of lipid and DNA. As shown by the inverted triangles of Fig. 2, significant leakage of vesicle contents does not occur until the 1:1 charge ratio is exceeded. Up to that point, only about a quarter of the vesicles' contents is

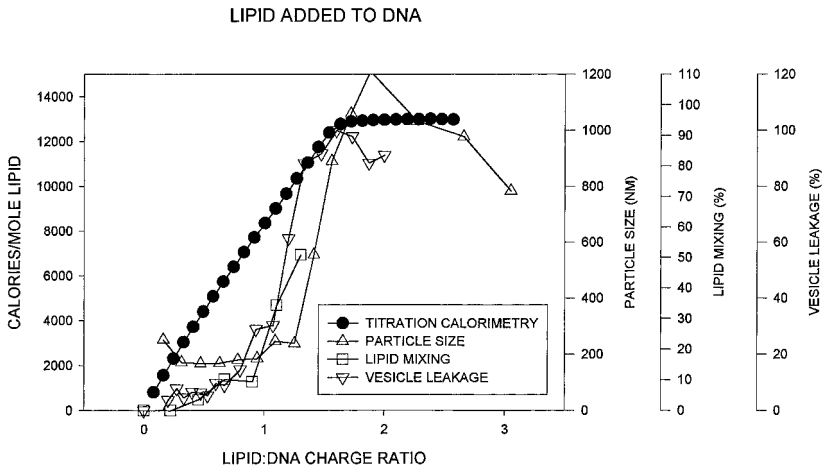

FIGURE 2 Interactions of cationic phospholipid with DNA during titration of pUC18 With EDOPC. Plasmid DNA (pUC18) was titrated with EDOPC in the form of 100-nm extruded vesicles. The aqueous phase was HE-S (buffered physiological saline). Procedures were essentially the same as described in the legend of Fig. 1, except that the lipid was added in small portions to the DNA and cumulative injection heats were normalized to the amount of DNA in the cell expressed as moles of nucleotide.

released, compared with almost all of the remainder, which is rather abruptly released when the charge ratio is further increased by only 20%, from 1:1 to 1.2:1.

Membrane mixing

Membrane mixing also generally follows the pattern of particle size change and vesicle rupture. As shown by the squares in Fig. 2, relatively little bilayer merging occurred at CL/DNA charge ratios below 1:1, but as this ratio was exceeded, the mixing increased abruptly, although the end point of the membrane mixing experiment was rendered somewhat uncertain because of the diminution of signal intensity at high charge ratios (see Materials and Methods). The exact onset and end point of the titration based on membrane mixing are not critical, because what is clear from the experiment is that there is a large and rather abrupt onset of membrane mixing that is greatly displaced from the lipid-DNA interaction detected by calorimetry.

Effect of low ionic strength on the interaction of DNA and EDOPC

Some of the interactions between lipid and DNA described above for solutions of physiological ionic strength were also examined at lower ionic strength to assess the effects of electrostatic parameters on complex formation. These measurements have been made with the simpler DNA-to-lipid mode of addition. With respect to titration calorimetry, there was a significant change in the complex stoichiometry, as the ionic strength was reduced (Fig. 3, *filled circles*). In contrast to titrations carried out in high ionic strength buffer (HE-S, Fig. 1, *filled circles*), where complex formation was complete at a DNA:CL charge ratio of ~0.33, similar titrations in low ionic strength buffer (Fig. 3) revealed an end point of ~0.65 DNA:CL (CL:DNA = 1.55). The effect of ionic strength on the difference in end points for the titration of lipid with DNA is likely to be a function of the reduced electrostatic interaction in the presence of a high concentration of Na^+ and Cl^- ions. In high ionic strength solution, the complex contains both negatively and positively charged regions that may charge-pair with complementary regions on other particles, thereby lowering the electrostatic free energy of the system, resulting in a lower DNA:CL ratio. Complex formation in low ionic strength apparently produces particles that are similarly charged on the exterior surface, resulting in charge-charge repulsion. Aggregation will proceed only after enough DNA has been added that the exterior surface of the particles is essentially neutral. Thus the end point may be determined by aggregation of DNA:CL particles, not saturation of the binding surface. An ITC run in which the titration reactants were diluted 10-fold (to $\sim 1 \times 10^{-6}$ M) produced similar results, indicating that the dissociation constant for this interaction is $< 1 \times 10^{-6}$ M (data not shown). Under these experimental concentrations, the instrument signal was close to baseline levels, thereby precluding further dilution of the reactants to more accurately estimate the binding constant of pUC18 to EDOPC.

Interestingly, the energetics of DNA binding to EDOPC are only weakly dependent upon ionic strength. The slope of the isotherm up to the end point in Fig. 3 is 720 cal/mol DNA (Fig. 3, *filled circles*). This value differs by at most by 40 cal/mol from the corresponding value for the titration at high ionic strength (Fig. 1). Most of the difference in the high and low ionic strength isotherms is in the stoichiometry; about twice as much lipid interacts with a DNA molecule at low ionic strength as at high ionic strength. This suggests that at even higher ionic strength, the amount of lipid interacting with DNA would decrease even further. Although we have not pursued this point in detail, it does seem to be the case that the complex is less tight at very high ionic strength. Using the assay for accessibility of DNA in the presence of cationic lipid described by Gershon

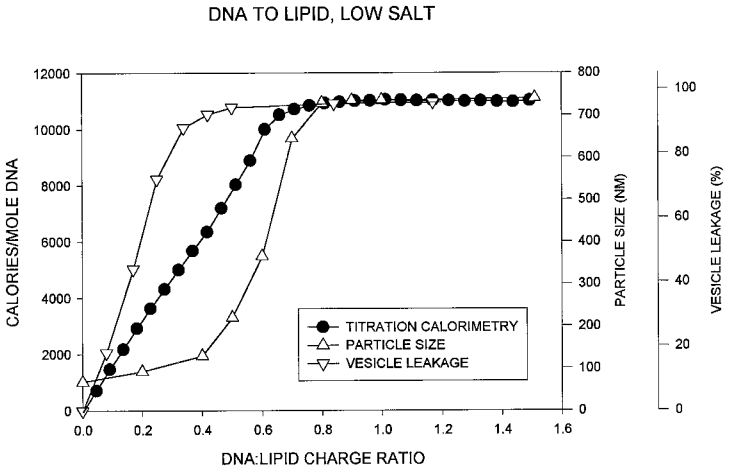

FIGURE 3 Interactions of DNA with cationic phospholipid during titration of EDOPC with pUC18 in the absence of added NaCl. Symbols are as in Fig. 1. With the following exceptions, the procedures were the same as those in Fig. 1: 1) The solution was HE (20 mM HEPES buffer at pH 7.5 containing 0.1 mM EDTA), except in the case of the leakage measurements, where it was HE supplemented with 20 mM NaCl. 2) Membrane mixing was not measured.

et al. (1993) as corrected by Eastman et al. (1997), we found that, in 0.5 M NaCl, lipid was ineffective in sequestering DNA from contact with DNA intercalating dyes (ethidium bromide and YOYO-1) added to the aqueous phase. The simplest interpretation of these results is that the complex is either much more open or much weaker under these conditions.

The enthalpy of DNA interaction with EDOPC was, according to Fig. 3, 500 cal/mol lipid. The stoichiometry indicates that about 1.3 (2×0.65) moles of counter ions are actually released per mole of lipid forming a complex with a stoichiometric amount of DNA. Assuming one counter ion per neutralized charge, the heat per mole of counterion released would then be 380 cal/mole.

Size of the complex

As in the experiments at physiological ionic strength, titration of EDOPC LUVs with pUC18 at low ionic strength (20 mM HEPES) generated particles of increased size (Fig. 3, *triangles*); however, the DNA:CL charge ratios over which the size of the particles grew most rapidly was significantly different under the two conditions. As shown in the figure, the transition was complete at a DNA:CL charge ratio of ~0.7. Thus the end points of the calorimetric and particle size titrations were both shifted to higher DNA/lipid charge ratios with decreasing ionic strength. Although the end points of the two titrations were similar, the progressions to the end points were very different. Whereas the calorimetric progression was almost linear, there was little change in particle size until the titration was ~5/7 complete, at which point the particles grew much more rapidly. This behavior was surprisingly similar to that where the complex was formed by adding lipid to DNA at high ionic strength, a condition under which there was also divergence between the paths of heat absorption and particle growth, even though the end points were similar. The small deviation from linearity (a 10% increase in slope) in the ITC trace occurred at a charge ratio of 0.4, which is about where the growth in particles occurs, as well as where vesicle rupture is complete (see below).

Vesicle rupture

As is also shown in Fig. 3 (*inverted triangles*), addition of DNA to EDOPC vesicles at low salt concentration leads to content loss at a rather low charge ratio, which is complete before the onset of particle growth at a DNA/lipid ratio of ~0.5 and well before the end point according to calorimetry at 0.65. (The leakage experiments had to be done with 20 mM NaCl present to prevent precipation of the vesicles in the presence of terbium dipicolinate. In a low-salt environment, vesicle rupture occurs immediately upon the addition of DNA to the lipid, whereas onset of rupture in the high-salt sample is at a slightly higher DNA:CL charge ratio and occurs over a narrower transition.

Particle size for complex generated by rapid, one-step mixing of DNA and lipid

Most of our characterization of the pUC18-EDOPC complex was done under controlled conditions of formation in which we slowly added one component to the other. Although this allowed us to monitor the progression of interaction of the components, transfection complexes are typically made simply by mixing DNA and lipid together. (But for evidence that this may not be optimal, see Boussif et al. (1996).) For comparison, we have also made some measurements of the complex formed by a single mixing step. As shown in Table 1, under these conditions of complex formation, the order of addition had essentially no effect on the resultant particle size. However, the size of the complex was dependent on the DNA:CL charge ratio used to form the complexes (Table 1). The dependence of size on DNA:CL ratio agreed well with the titration isotherm of lipid added to DNA—i.e., particles formed at a 1:1 DNA:CL charge ratio were small (~300 nm in diameter), whereas particles formed at a DNA:CL charge ratio of 1:2 were extremely polydisperse, consisting of primarily large (>3 μm) particles.

Because the complex was clearly different, depending upon the mode of formation, the obvious question arises as to the effects of the formation procedure on the transfection efficacy of the particle. Although we have just begun investigating this question and our data are incomplete, it is already clear that the simple operation of converting multilamellar dispersions into extruded vesicles itself led to transfection complexes of considerably reduced efficiency. Whether this reflects an inherent property of the complex or simply means that the smaller particle size of the complex formed from extruded vesicles makes it less likely to be taken up by cells is not yet clear, although we favor the latter explanation, because we suspect that the smaller particles are simply less likely to settle onto the cells. Indeed, there is evidence for such a hypothesis from studies with another amphipathic transfection agent (Ross and Hui, 1999).

TABLE 1 DNA:EDOPC complex sizes determined by dynamic light scattering

DNA:lipid charge ratio	Order of addition	Size (nm)
1:1	Lipid to DNA	320 ± 25
1:1	DNA to lipid	280 ± 29
0.5:1	Lipid to DNA	>3000
0.5:1	DNA to lipid	> 3000

DNA:EDOPC complexes were formed by quickly mixing DNA and EDOPC at the indicated molar ratios in HE-S buffer. Complexes were sized using dynamic light scattering as described in Materials and Methods. Data are presented as mean ± SEM of three independent trials.

DISCUSSION

Mode of formation and structure of the complex depend upon order of addition

Our most intriguing result was that the order of combination of DNA and EDOPC has a critical effect on the physical properties of the resultant DNA-CL complexes. The fundamental reason for the difference is the difference in symmetries of the two components, DNA and lipid vesicles. There are two characteristics of the order of addition that are important: 1) The end point of the interaction between DNA and lipid was nearly twice as large (DNA:CL charge ratio) for DNA into CL as for CL into DNA. 2) Particle growth, membrane mixing, and vesicle rupture all proceed immediately in the process DNA into CL, but are all delayed until approximately equal numbers of positive and negative charges are present in the case of the reverse addition. These two characteristics involve somewhat different phenomena. The differences in the physical characteristics of complex according to the mode of formation are a consequence of the fact that the lipid is organized into bilayer vesicles, and the stress on the walls of these vesicles differs, depending upon whether they interact with a small or a large amount of DNA. The difference in the stoichiometry according to the mode of complex formation is related to breakage of the vesicles but is also dependent on the relative availability of the two components as the vesicles collapse into the complex; when DNA is added to lipid, the latter is initially in excess and the stoichiometry favors a higher proportion of lipid in the complex than in the reverse addition, where DNA is initially in excess. In the following sections we consider the details of these processes.

Addition of lipid to DNA

Physical characteristics of complex formation

When vesicles are added to a DNA solution, until the end point of the titration, each vesicle faces an excess of DNA and hence becomes coated with DNA. This process is diagrammed in Fig. 4 *A*. Although binding of the DNA to the lipid bilayer surface may generate compressive stresses that could be large, such stresses would be isotropic, and the vesicle is not expected to rupture (at low ionic strength, the interaction with DNA alone is sufficient to rupture the vesicle—see the discussion of ionic strength effects below). This process is consistent with the absence of an initial change in vesicle size (Fig. 2, *squares*), because a coating of DNA would only add 4 nm to a 100 nm-diameter vesicle. Formation of stable DNA-coated vesicles has recently been observed by others in the electron microscope, albeit with less highly charged vesicles that would interact less strongly with DNA (Huebner et al., 1999). (Adhesion of ordered arrays of DNA to supported monolayers of cationic amphipaths has been known for some years (Fang and Yang, 1997).) After essentially all of the DNA in solution has been taken up, additional vesicles added to the dispersion will encounter vesicles that have negatively charged surfaces. The electrostatic attraction between these oppositely charged surfaces will give rise to anisotropic forces (Fig. 4 *A, structures in brackets*), with the result that the bilayer is likely to rupture. Such rupturing will generate unstable hydrophobic edges of bilayers, which will tend to fuse with like edges of other vesicles and hence allow transfer of fluorescent probes between vesicles. Unless they immediately rupture, added vesicles would be able to adhere to several (possibly six if close packing occurs) other DNA-coated vesicles. Each subsequent vesicle would induce aggregation of clusters already present, so the particle size should increase exponentially. It is thus expected that membrane mixing and vesicle rupture should follow a pattern similar to particle size increases, which was observed.

Although the three measures of particle properties (size, mixing, leakage) are not congruent (Fig. 2), they are quite similar, particularly given that all measures were made with different preparations at different times. These parameters change abruptly at a DNA:CL charge ratio between 1.0 and 1.2 moles of lipid charge per mole of DNA charge. It thus appears that the external surface of vesicles becomes saturated with DNA (at the aqueous phase concentrations in our experiments) at the same charge ratio, i.e., 1–1.2. Half of the lipid is in the inside monolayer, implying that saturation of a single lipid surface occurs at a point where there are actually about twice as many negative charges as positive charges associated with the surface as a whole. Because half of the DNA charges face the external solution, at the actual regions of contact between DNA and the bilayer, a situation close to electrical neutrality may obtain. Given an area of 0.6 nm^2 per lipid molecule (MacDonald et al., manuscript in preparation), this level of interaction would correspond to a 1.7-nm periodicity of DNA spacing. Because this is even smaller than the 2-nm diameter of the DNA, the implication is either that the surface is saturated and a significant proportion of many DNA strands are, in fact, "loose" and are not fully attached to the surface, or that enough vesicles have broken and exposed their inner surfaces to DNA to provide binding sites to what would otherwise be loose ends.

Stoichiometric end point by calorimetry

Given that coating the external surface of the vesicles with DNA consumes all of the free DNA, the heat absorbed between this point and the actual end point of 1.6 CL/DNA represents the interaction of lipid with the surface of predominantly intact, DNA-coated vesicles. Whether or not the calorimetric end point of 1.6 actually represents full interaction of all lipid with all DNA is difficult to judge; it is possible that steric barriers prevent equilibration, at least on the time scale of our experiments. The very fact that the calorimetric end point depends upon the order of addition

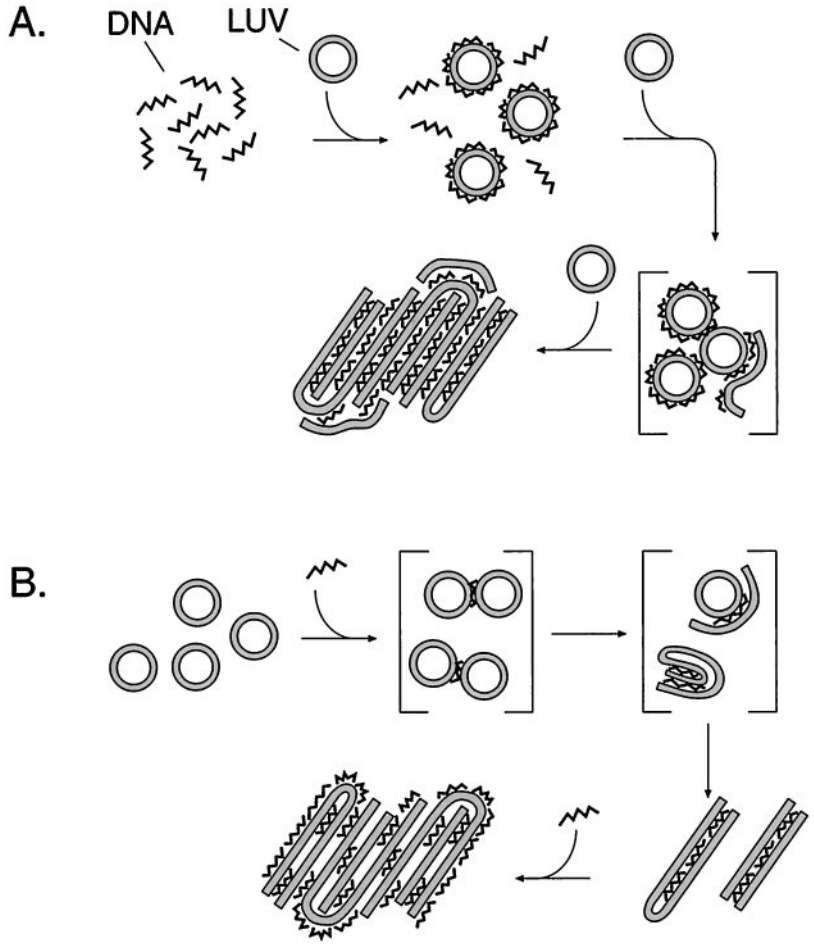

FIGURE 4 Proposed mechanisms for the formation of EDOPC:DNA complexes. (*A*) DNA-CL complex formation by titration of DNA with lipid (DNA initially in excess). DNA uniformly coats the surface of EDOPC vesicles—without their rupture—as they enter the DNA solution. After enough lipid has been added that all of the DNA in solution has become bound, subsequently added vesicles bind to vesicles previously coated with DNA, leading to, initially, clusters of a few DNA-coated vesicles surrounding a newly added vesicle. Because of the anisotropic stresses generated by vesicle-vesicle adhesion these small clusters are metastable (brackets) and they rupture. Subsequent addition of vesicles, along with association of free bilayer edges, leads to the additional growth of the particles. When enough lipid has been added to cover the surfaces of complexes in suspension with lipid, the end point has been reached and any added lipid simply coexists with complexes. It should be noted that the proportions of lipid and DNA have been distorted somewhat for clarity; the vesicles are 100 nm in diameter, and the DNA is a 900-nm (contour length) circle in the form of a flexible supercoil. LUV, Large unilamellar vesicle. (*B*) DNA-CL complex formation by the titration of lipid with DNA (lipid initially in excess). Although initially DNA may encounter a single vesicle, subsequent encounters with bare vesicles leads to dimerization (perhaps some trimerization) in which DNA associates at the interface of the two vesicles. Vesicle-DNA-vesicle adhesion generates asymmetrical stresses, which lead to rupture of at least one vesicle (brackets indicate metastable states). Additional DNA, along with edge-edge associations of previously ruptured vesicles, leads to continued aggregation and growth of the complex. After all the lipid has become associated in complexes, additional DNA coats the external surface and precludes further aggregation. This represents the calorimetric end point. Subsequently added DNA remains free in solution. The process differs from that shown for when the ionic strength is lower (no added NaCl), in which case the DNA-vesicle interaction is strong enough to lead to vesicle rupture upon contact of the vesicle with a DNA molecule. In addition, aggregation proceeds more slowly than at high ionic strength because the DNA interacts largely with one vesicle, not two, because of the larger vesicle-vesicle repulsive forces. Aggregation begins only when the external surface of the residue of a ruptured vesicle has essentially been neutralized by DNA.

indicates that equilibration does not occur on the time scale of our experiments.

The physical measures of particle size appear to reflect a cooperative behavior because they change rapidly with increasing charge ratio. Such cooperative behavior has previously been described for the binding of DNA to dioleoyldimethylammonium chloride (Wong et al., 1996). Because the appearance of cooperativity is based on multiple interactions of one component with the other, it seems appropriate to recognize that the behavior does not need to involve an actual structural change of either component.

Addition of DNA to lipid

When DNA was added to lipid, instead of lagging the calorimetric curve, all three measures of physical properties

of the complex (size, leakage, mixing) began changing significantly as soon as small amounts of DNA were added. As shown in Fig. 4 *B*, this difference in behavior is likely due to fact that DNA added to solution is immediately surrounded by cationic vesicles, an arrangement much different from that occurring during the reverse mode of mixing. The length of a pUC18 strand is 957 nm and, in a supercoiled and somewhat flexible form, would be a fuzzy rod of somewhat less than 400 nm. The DNA has ~5400 negative charges. The external surface of a vesicle has ~10 times as many charges as the plasmid, so interaction of a plasmid with the surface of a vesicle would leave the DNA spread out on the surface. Unless the vesicle immediately ruptures, the DNA on its surface would quickly encounter and bind to a second vesicle. (By fluorescence microscopy of bundles of lambda phage DNA and giant cationic vesicles, the DNA wraps entirely around a vesicle shortly after a contact occurs, but when DNA contacts two vesicles, the vesicles are brought together as the DNA maximizes its interaction with each vesicle. Sometimes the vesicles fuse, but rupture is more common after two vesicles come into contact in this way [Pantazatos and MacDonald, unpublished observations]). Alternatively, given the length of the DNA, it could contact two vesicles nearly simultaneously. When two or more vesicles adhere to one another, the DNA will become concentrated at the contact zone (which would amount to 5% of the vesicle area if it were neutral (equal charge density of DNA and lipid) and would be correspondingly larger if there remained uncompensated positive charges). In the adhesion of two or more vesicles, stresses on the bilayer become anisotropic and rupture and membrane mixing are favored (Pantazatos and MacDonald, 1999). Thus we anticipate that, at the point where about one plasmid has been added per two vesicles (corresponding to a charge ratio of ~0.06, DNA/CL), extensive rupture and membrane mixing will have occurred. These two processes were indeed observed to occur very early upon DNA addition. Experimentally, the particle size also increases, but there appears to be an initial linear change to about double the initial particle size, followed by a distinct change in slope as more DNA is added. The change slope occurs at a charge ratio of 0.15, which would indicate that two or three plasmids are actually required for vesicle dimerization, although this suggestion must be quite tentative, given the uncertainty that attends the determination of particle size of heterogeneous populations by dynamic light scattering. In any case, particle growth, vesicle rupture, and membrane mixing are all expected to occur as soon as a few plasmids per vesicle have been added.

The stoichiometric end point by calorimetry

The calorimetric end point corresponds reasonably well with the point at which particle size ceases to increase, the vesicles are essentially all broken, and the bilayers randomized. This end point is, however, at a CL/DNA charge ratio of 3:1, considerably different from the end point for the reverse titration. The difference in end points indicates that when DNA is added to lipid, not all of the lipid comes into contact with the DNA; some of it is merely carried along, increasing the CD/DNA ratio, but not actually binding to DNA. If we assume that the CL-into-DNA titration actually brings most lipid into contact with DNA (this could be an underestimate), then the difference in end points means that nearly half (1–1.6/3) of the lipid does not come into contact with the DNA during the DNA-into-CL titration.

Clearly there is additional DNA-binding capacity present at the end point of the DNA-into-CL titration, yet DNA added beyond that end point does not interact with lipid. At this point, the particle size is ~1 μm and, because the particles do not aggregate to a significant extent, the external surface of those particles must be negatively charged. We and others have observed that the complex formed by one-step mixing of DNA and lamellar phase-preferring amphiphiles is lamellar, consisting of bilayers glued together with DNA (MacDonald et al., 1999a). Furthermore, theoretical analyses show that under these conditions, a lamellar complex is expected (Dan, 1998; Harries et al., 1998; Bruinsma, 1998). Based on this evidence, if we assume a lamellar organization for a complex of this size with the measured spacing of 6 nm, each layer would represent the area of ~15 vesicles and there would be ~170 layers, representing the lipid of ~2500 vesicles. The surface of the particle would correspond to the area of only ~100 vesicles, so clearly, if there is limited exchange between the interior and the surface, the exterior could be saturated with DNA while the interior has a paucity of DNA. The end point of the titration is thus reasonably well established. As pointed out in the previous paragraph, it is likely that the initial steps of DNA-lipid interaction involve the adhesion of a pair or a few vesicles by one or a few plasmid molecules, followed by rupture of the vesicles.

Given a reasonably clear picture of the beginning and the end of complex formation, the next challenge is to understand the route between. Because lipid is in excess during formation of the complex, it is clear that the lipid will have an opportunity to become aggregated under conditions involving the minimum amount of DNA. The actual surface density of DNA between adherent bilayer surfaces will be set at the lower limit by repulsion between the bilayers; at some lower threshold, there is not enough DNA on the surface to withstand the inter bilayer repulsion (May and Ben-Shaul, 1997; Bruinsma, 1998). Thus two patches of membrane with DNA between them at less than the threshold amount would have disproportionately different amounts of DNA; adherent regions would contain most of the DNA at a density above that of the threshold surface, and nonadherent regions would have a lower surface concentration of DNA. We thus postulate that DNA added to solution associates with vesicles until they acquire some

tendency to adhere. Any excess DNA on the vesicles not involved in the initial contact can then migrate to the junction zone, a process that would be exergonic because of the opportunity of the DNA to bind to two surfaces. Adhesion must flatten the vesicles until the tension in the bilayers balances the adhesion force, after which the likelihood of at least one vesicle rupturing is greatly increased. After rupture, the newly created surface will acquire additional vesicles (or broken vesicles) and a stack will begin to build up. Such a process has been captured by cryoelectron microscopy in the case of vesicles composed of phosphatidylcholine mixed with a cationic derivative of cholesterol (Huebner et al., 1999). Because the broken edges of the membrane stacks are unstable in contact with water, these edges will promote the lateral growth of stacks. At some point, the particles will have accumulated all of the lipid in the suspension, and additional DNA added to the suspension will simply coat the external surface of the particle. This point would represent the calorimetric end point. It is important to note that the build-up of the final product requires adhesion of bilayers mediated by DNA, but the DNA need not be at the threshold concentration over all of the contacting surfaces; it must be at this concentration over sufficient portions of that area to maintain contact between vesicle fragments. In this way, the overall content of DNA may be less than that for stoichiometry with the lipid present, yet the charge on the particle as a whole may be negative and may be determined by DNA on the external surface.

The model for titration of DNA with lipid (Fig. 4 *A*) supports, in part, the model proposed by Eastman et al., who suggest that DNA:CL complexes retain vesicular shape, with the DNA either lying flat on the surface (low ionic strength) or projecting outward from the vesicle surface (high ionic strength) (Eastman et al., 1997). In our case, at the end point of titrations for either DNA to EDOPC or vice versa, the resultant complex is not vesicular because the content release assay indicates vesicle rupture.

Ionic strength effects

The major effect of lowering the ionic strength was to change the calorimetric stoichiometry from 3:1 to ~1.55:1 (+:−), at least for the mode of mixing we investigated, namely, titration of lipid with DNA. Thus each DNA molecule interacted with more lipid than at high ionic strength. This is what would be expected if, as was suggested, in this mode of mixing not all of the lipid "sees" the DNA, because reducing the ionic strength would certainly increase the electrostatic interaction between the oppositely charged components of the complex.

Such a consequence of increased interactions would appear to be the explanation for the divergence of the leakage curve from the ITC curve; leakage is complete at a charge ratio about half that of the stoichiometric end point measured by calorimetry, suggesting that leakage correlates with saturation by DNA of the external surface of the vesicles. Because the vesicles had essentially all lost their contents before the particle size began to change, we conclude that, with the more intense interaction at the lower ionic strength, contact with a DNA molecule is sufficient to rupture the vesicle, and vesicle-DNA-vesicle interactions are not required for rupture as they are at physiological ionic strength.

In contrast to the early rupture of vesicles, particle growth is delayed relative to the ITC curve until a −/+ charge ratio of ~0.4, at which point the particles grow rapidly, coming to a maximum size of ~700 nm at a DNA:CL charge ratio of 0.7, which was very similar to the calorimetric end point of 0.65. The charge ratio at which particle growth accelerates is a little higher than half the value of the calorimetric end point, suggesting that the residue after particle rupture is about half charged, that is, about half of the lipid is exposed and this is neutralized by bound DNA. With a near-neutral surface, particle aggregation should indeed proceed at the maximum rate. The fact that neutralization of EDOPC by DNA occurs at lower DNA:CL ratios in high-ionic-strength buffer is in agreement with research demonstrating that neutralization of other cationic lipid-DNA vesicles by DNA occurs at lower DNA:CL ratios in the presence of elevated ionic strength (Eastman et al., 1997).

Relationship of titration to one-step mixing

The titration procedure used here was chosen to obtain a thermodynamic measure of the stoichiometry of the complex. Generally this is not the procedure used for generating transfection complexes, although improved results have, in fact, been reported for a six-step addition protocol (Boussif et al., 1996). Because it is likely that the titration mode and one-step addition generate somewhat different structures, we did undertake a limited characterization of the size of particles generated by one-step mixing. In this case, the order of addition did not seem to have a large effect; it produced either very large (>3000 nm) or relatively small (~300 nm) complexes, independent of the order of addition; however, the size of the particles did strongly depend on the DNA:CL charge ratio (Table 1), an effect observed with other lipoplex systems (Xu et al., 1999).

Stabilization and structure of the complex

The ITC results indicate that the driving force for DNA:EDOPC association is entropic, given the experimentally measured positive enthalpy and the spontaneous nature of the association ($\Delta G < 0$). Positive values for ΔH were obtained regardless of the order of DNA and lipid addition. Binding of DNA to the micellar detergent cetyltrimethylammonium bromide has also been shown to be endothermic (Spink and Chaires, 1997). Moreover, the enthalpy of in-

teraction between DNA and EDOPC was independent of the ionic strength, as evidenced by nearly identical slopes of the ITC isotherms in low and high ionic strength. The increase in entropy most likely results from the dehydration and release of bound counterions and water from the interacting faces of DNA and EDOPC. One can estimate the lower limit for the entropy gain upon formation of the complex from the fact that the process is spontaneous, which means that $dG = dH - TdS < 0$, and hence that $dS > dH/T \sim 1.3–1.5$ cal/mole °K (dH varied from 380 cal/mole to 460 cal/mole, depending upon conditions), or at least 0.7 kT per released counterion. Theoretical studies in which the electrical energy of charged bilayers was examined confirm a high concentration of counterions near the bilayer (Cevc, 1990; Bruinsma, 1998), similar to the high concentration of cation within a short distance of the DNA surface (Manning, 1978) and that the entropy drives formation of DNA- cationic lipid complex (May and Ben-Shaul, 1997; Harries et al., 1998; Bruinsma, 1998).

Although release of counterions is clearly the driving force for the formation of DNA-CL complexes, for nearly every condition/lipid type studied, a wide array of DNA-lipid "neutralization points" have been reported. These values range from ~0.3 to 1.2 DNA:CL charge ratio (Gershon et al., 1993; Zuidam and Barenholz, 1998; Eastman et al., 1997). By calorimetry, neutralization of EDOPC by DNA occurs in an ionic-strength-dependent fashion with end points at ~0.3 and ~0.65 DNA:CL charge ratio for high and low ionic strengths, respectively (Figs. 1 and 3). For the cationic amphipaths 1,2-dioleoyloxy-3-(trimethylammonio)propane (DOTAP) (Rädler et al., 1997), dioctadecyldimethylammonium bromide (Lasic et al., 1997), dioctadecylamidoglycyl-spermine (Boukhnikachvili et al., 1997), as well as for EDOPC (MacDonald et al., 1999a), it has been determined by x-ray scattering that DNA-CL complexes formed by one-step mixing with these lipids contain DNA strands arranged between lipid bilayers. If enough dioleoylphosphatidylethanolamine is present, the structure may may assume a hexagonal arrangement (Koltover et al., 1998, Rakhmanova et al., 2000). A third structure, "spaghetti," has also been proposed (Sternberg et al., 1994). These two nonlamellar structures have been analyzed and found to be rather high energy structures, especially in the absence of high intrinsic curvature lipids (Dan, 1988; May and Ben-Shaul, 1997), and are therefore very unlikely to apply to the structure of the EDOPC-pUC18 complex generated by any of the methods that we have used.

Three theoretical studies of lamellar DNA-cationic lipid complexes have appeared. Different techniques, conditions, and compositions were used, but all agree that there are regimes in which the DNA-DNA spacing varies as a function of DNA:CL ratio, and on either side of that regime, the complex coexists with excess DNA or CL (Dan, 1998; Harries et al., 1998; Bruinsma, 1988). The upper limit of DNA-DNA spacing depends upon bilayer-bilayer repulsion and the lower limit upon DNA-DNA repulsion, although bilayer distortion may also have an influence. Experimentally, a variation in the DNA spacing of nearly threefold has been observed in a mixed-lipid system (Rädler et al., 1997). It therefore seems likely that there is sufficient latitude available to the DNA-DNA spacing to accommodate the variations in the DNA:CL ratio from 0.3 to 1.2 that have been observed. Our results suggest that part of this variation may well depend upon the conditions of complex formation, in addition to the structure of the cationic lipid.

Implications for transfection

The observed effects of DNA:CL charge ratio, order of addition, rate of addition, and ionic strength indicate that different complexes are generated when these variables are changed. The consequences for the transfection efficiencies of different complex structures, especially in the case of EDOPC, largely remain to be explored, but our results do suggest that manipulation of these variables may allow the tailoring of transfection complexes to particular applications. For example, in vitro, a large particle may be advantageous because it encourages maximum contact with cells, but in vivo, small particles are required for traversal of the capillary network in the lungs. By varying the order of addition and controlling the rate at which reactants are brought together, it may be possible to control the final size of the product, thereby optimizing the conditions for transfection. Analyses of the molecular arrangement of DNA and EDOPC in these complexes as well as their efficacy of transfection are currently under way.

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