Formation of Novel Hydrophobic Complexes between Cationic Lipids and Plasmid DNA†

Dorothy L. Reimer,* YuanPeng Zhang, Spencer Kong, Jeffery J. Wheeler,‡ Roger W. Graham,‡ and Marcel B. Bally

Division of Medical Oncology, British Columbia Cancer Agency, 600 West 10th Avenue, Vancouver, British Columbia V5Z 4E6

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ABSTRACT: An ability to generate a well defined lipid-based carrier system for the delivery of plasmid DNA *in vivo* requires the characterization of factors governing DNA/lipid interactions and carrier formation. We report that a hydrophobic DNA/lipid complex can be formed following addition of cationic lipids to DNA in a Bligh and Dyer monophase consisting of chloroform/methanol/water (1:2.1:1). Subsequent partitioning of the monophase into a two-phase system allows for the extraction of DNA into the organic phase. When using monovalent cationic lipids, such as dimethyldioctadecylammonium bromide, dioleyldimethylammonium chloride, and 1,2-dioleyl-3-*N*,*N*,*N*-trimethylaminopropane chloride, greater than 95% of the DNA present can be recovered in the organic phase when the lipid is added at concentrations sufficient to neutralize DNA phosphate charge. When the polyvalent cationic lipids 2,3-dioleyloxy-*N*-[2(sperminecarboxamido)ethyl]-*N*,*N*-dimethyl-1-propanaminium trifluoroacetate and diheptadecylamidoglycyl spermidine are used, efficient extraction of the DNA into the organic phase is also achieved when the charge ratio between lipid and DNA is approximately equal. Formation of the hydrophobic DNA complex can only be achieved with cationic lipids. In the absence of added cations or in the presence of excess Ca^{2+}, L-lysine, or poly(L-lysine), 100% of the DNA is recovered in the aqueous fraction. The monovalent cationic lipid/DNA complexes can also be prepared in the presence of detergent; however, low concentrations of NaCl (<1 mM) lead to dissociation of the complex. Importantly, these results clearly demonstrate that cationic lipid binding does not lead to DNA condensation. The methods described, therefore, enable DNA/lipid complexes to be characterized in the absence of DNA condensation. It is believed that this approach, where cationic lipids added in monomeric or micellar form are bound to DNA prior to condensation, will facilitate the preparation of DNA/lipid complexes with well defined surface characteristics and size.

The potential for utilizing gene therapies for clinical treatment of many diseases is enormous. In particular, clinical protocols have been developed for the treatment of cystic fibrosis (Rosenfeld *et al.*, 1992), adenosine deaminase deficiency (Culver, 1994), melanoma (Nabel *et al.*, 1993), brain tumors (Oldfield *et al.*, 1993; Yung, 1994), and renal cell carcinoma (Gansbacher *et al.*, 1992). A more comprehensive list of more than 50 clinical protocols authorized by agencies responsible for approving gene transfer clinical studies has recently been published [(1994) *Cancer Gene Ther. 1,* 289−295]. Despite the clinical progression of treatment modalities based on gene therapy, application of DNAs as pharmaceutical agents continues to represent a significant challenge.

Although gene transfection achieved through the use of viruses is extremely efficient, and this technology is becoming more sophisticated (Miller, 1992), concerns persist about potential toxicities associated with viral based methods of gene transfer. An alternative gene transfer method that has received significant attention has been referred to as "lipofection". In general, this term encompasses procedures where preformed small (≤100 nm) cationic liposomes are mixed with plasmid DNAs to generate transfection complexes (Felgner *et al.*, 1987). However, the physical characteristics of the resulting liposome/DNA complexes are poorly understood. *In vitro* studies have shown that liposome composition, in particular the type of cationic lipid used and the presence of dioleoylphosphatidylethanolamine (DOPE),[1] is important if optimal transfection efficiencies are to be achieved (Zhou & Huang, 1994; Farhood *et al.*, 1995). The role of the cationic lipid is, in part, to bind anionic phosphate groups in DNA and is also believed to be involved in binding of the cationic complex to cell membranes. DOPE is a zwitterionic lipid capable of adopting membrane structures that can engender membrane fusion (Cullis *et al.*, 1986). This process is thought to be required for intracellular delivery of DNA, an event that may occur at the cell surface membrane or within endocytic vesicles. The role of DOPE is, however, not clearly defined since it is well established that cationic peptides (e.g., polylysine, spermidine, spermine)

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‡ Present address: Inex Pharmaceuticals Corp., 1779 West 75th Ave., Vancouver, B.C. V6P 6P2.

* To whom correspondence should be addressed. Telephone: (604) 877-6098, ext 3191. Fax: (604) 877-6011.

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[1] Abbreviations: OGP, *n*-octyl β-D-glucopyranoside; DDAB, dimethyldioctadecylammonium bromide; DODAC, dioleyldimethylammonium chloride; DNase I, deoxyribonuclease I; lipofectin, DOTMA/DOPE; DOTMA, 1,2-dioleyl-3-*N*,*N*,*N*-trimethylaminopropane chloride; DOPE, dioleoylphosphatidylethanolamine; lipofectamine, DOSPA/DOPE; DOSPA, 2,3-dioleyloxy-*N*-[2(sperminecarboxamido)ethyl]-*N*,*N*-dimethyl-1-propanaminium trifluoroacetate; transfectam, DHGS; DHGS, diheptadecylamidoglycylspermidine; TBE, 89 mM Tris borate, 2 mM EDTA; TO-PRO-1, thiazole orange monomer; CMC, critical micelle concentration.

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as well as cationic lipids, added in the absence of DOPE, can also be used as transfecting agents (Wu *et al.*, 1989; Wagner *et al.*, 1990; Zhou *et al.*, 1991; Zhou & Huang, 1994).

One common attribute of transfection methods that use cationic peptides or liposomes is the generation of condensed DNA. Condensation of DNA is a phenomena that has been characterized extensively by Bloomfield (1991) and is defined as an energetically favorable reaction that occurs spontaneously when the DNA phosphate charge is 90% neutralized. Studies evaluating the physical characteristics of condensed DNA suggested that for DNAs ranging in size from 0.4 to 48 kb condensation yields a toroidal structure that has a uniform diameter of 40−60 nm (Bloomfield, 1991). These structures may contain 1−30 DNA molecules depending on the size of the DNA molecule used (Arscott *et al.*, 1990). Furthermore, condensation yields DNA that is inaccessible to small molecules, such as the DNA intercalating dye ethidium bromide, and is protected against degradation by DNase I (Gershon *et al.*, 1993). Whether DNA condensation is required for transfection has yet to be determined.

We propose that careful control of the DNA condensation reaction is crucial in obtaining a suitable lipid-based DNA particle for efficient delivery of genes to cells *in vitro* and *in vivo*. This report demonstrates for the first time that addition of monocationic lipids to plasmid DNA results in the formation of a hydrophobic complex. Futhermore, DNA in this complex is in an uncondensed state and can readily be isolated in organic solvents. The DNA/lipid complexes have the potential to be used as intermediates in the formation of DNA/lipid particles which are well suited for gene delivery.

MATERIALS AND METHODS

Materials

Transfecting reagents lipofectin and lipofectamine were purchased from Gibco/BRL (Grand Island, NY). Transfectam reagent was purchased from Promega Corp. (Madison, WI). The monocationic lipid DDAB, calcium chloride, L-lysine (free base), poly(L-lysine) hydrobromide (av MW 52 000), and DNase I (from bovine pancreas) were purchased from Sigma Chemical Co. (St. Louis, MO). The monocationic lipids DOTMA, DODAC, and [^{14}C]DODAC were synthesized and supplied by Steven Ansell of Inex Pharmaceuticals Corp. (Vancouver, BC). TO-PRO-1 was purchased from Molecular Probes Inc. (Eugene, OR).

The plasmid pCMVβ (GenBank Accession No. U02451) encoding *Escherichia coli* β galactosidase was obtained from Clontech Laboratories Inc. (Palo Alto, CA). Plasmid DNA was propagated and purified using standard techniques (Sambrook *et al.*, 1989). Radiolabeled DNA was prepared from *E.coli*/pCMVβ cultures by metabolic labeling using methyl-[^{3}H]thymidine-5′-triphosphate (Dupont NEN, Boston, MA). Specific activity of radiolabeled plasmid DNA was ~50 000 dpm/μg.

N-Octyl β-D-glucopyranoside (OGP) was obtained from Sigma Chemical Co. (St. Louis, MO) (Catalog No. O8001). Gas chromatography analysis indicated purity >98% with sodium <0.0023% and calcium <0.0005%. All other chemicals used in this study were of reagent grade, and all solvents used were HPLC grade.

Methods

Bligh and Dyer Extraction Procedure. Liposomes, lipid, and DNA were solubilized in chloroform/methanol/water (1:2.1:1) prior to the addition of plasmid DNA. This mixture of solvents and water is equivalent to that used in the preparation of a Bligh and Dyer monophase (Bligh & Dyer, 1959). Typically, DNA was added to achieve a final concentration of 10 μg/mL. Lipid was added at various concentrations. Trace quantities of ^{3}H-plasmid DNA were added such that 2000−4000 dpm was present per 10 μg of unlabeled DNA. The reaction mixtures were incubated at room temperature for 30 min in a total volume of 1 mL. Subsequently, the Bligh and Dyer monophase was partitioned into a two-phase system by the addition of water and chloroform (250 μL each). The samples were mixed by vortexing, and the separation of the lower organic and upper aqueous phases was facilitated by centrifugation at 600*g* for 5 min at room temperature. The aqueous phase was removed and retained for scintillation counting. The solvent phase was dried using a stream of nitrogen gas, and the resulting film was resuspended in Solvable (Dupont NEN, Boston, MA) and incubated at 50 °C for 1 h. The incubation with Solvable was necessary to solubilize the dried DNA/lipid complex since the addition of the scintillation cocktail alone was not sufficient to dissolve the complex. Picofluor scintillant (Canberra Packard) was added to all samples, and the radioactivity ([^{3}H]DNA) was measured using a Packard TR 1900 Scintillation Counter.

Assays evaluating the stability of cationic lipid/DNA complexes were done in the presence of varying concentrations of NaCl and OGP. Briefly, cationic lipid/DNA complexes were prepared under conditions where 100% of the DNA was expected to be recovered in the organic phase. NaCl or OGP was then added to the monophase system, and incubations were carried out at room temperature for 15 min. Bligh and Dyer extractions were performed as described above.

The binding of calcium, L-lysine, and poly(L-lysine) to DNA was evaluated using a modification of the above procedure. These compounds were dissolved at various concentrations in sterile distilled water and incubated with the DNA (10 μg/mL final in water) at room temperature for 30 min in a final volume of 250 μL. Reaction volumes were adjusted to 1 mL with chloroform/methanol (1:2.1) to produce the monophase. Bligh and Dyer extractions were performed as previously described.

Dye Intercalation Assay. The fluorochrome TO-PRO-1 was used to evaluate the state of condensation of the plasmid DNA in the DNA/lipid complex. TO-PRO-1 was used in this study due to its stable intercalation into the DNA as well as the high sensitivity in fluorescence detection compared with the more common intercalator ethidium bromide (Hirons *et al.*, 1994). DNA was dissolved in either the Bligh and Dyer monophase or 100 mM OGP. Poly(L-lysine) or DODAC was added to 10 μg of DNA at a 1:1 charge ratio. Complexes were prepared at room temperature for 30 min followed by the addition of TO-PRO-1 to a final concentration of 1 μM. Spectrofluorometric readings were performed using a Luminescence Spectrometer 50B (Perkin Elmer). All sample readings were recorded using an excitation wavelength of 509 nm and emission wavelength of 533 nm, and values were expressed as arbitrary fluorescence units.

Agarose Gel Electrophoresis. Complexes involving DNA and poly(L-lysine) were formed at a DNA concentration of 10 μg/mL and a 1:1 charge ratio in the presence of 100 mM OGP. Complexes involving the cationic lipid DODAC and DNA were formed at a DNA concentration of 10 μg/mL and increasing concentrations of DODAC (10−320 nmol/mL). The mixtures were incubated at room temperature for 30 min prior to loading onto a 0.8% agarose gel. Electrophoresis was carried out in TBE buffer according to standard techniques (Sambrook *et al.*, 1989). DNA was visualized after staining the gel with ethidium bromide (0.5 μg/mL, 20 min) by photography with UV transillumination.

DNase I Assay. To evaluate the protective effect of cationic lipids on DNA, the complexes formed in the presence of OGP were incubated with DNase I. Preformed DNA/DODAC complexes (1:1 charge ratio) were mixed with DNase I at a concentration where DNA alone was susceptible to degradation at 37 °C for 10 min (0.33 unit). The reactions were stopped by the addition of 25 mM EDTA and the samples extracted using the Bligh and Dyer extraction procedure in the presence of 150 mM NaCl. Under these conditions the cationic lipid/DNA complexes dissociate and DNA can be efficiently recovered in the aqueous fraction. This DNA was precipitated with one-tenth volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of 95% ethanol and recovered by centrifugation in a microfuge at 14 000 rpm for 30 min at 4 °C. The DNA pellet was resuspended in sterile distilled water and subjected to electrophoresis on a 0.8% agarose gel.

RESULTS

In preliminary experiments, the interaction of lipofectin with DNA was investigated. Lipofectin, originally developed through the work of Felgner *et al.* (1987), consists of sonicated unilamellar vesicles composed of DOTMA and DOPE (50:50 mole ratio). The liposomes were prepared in water and were provided (Gibco/BRL) at a final lipid concentration of 1 mg/mL. Typically these preformed liposomes are added to plasmid DNA at ratios established through empirical studies depending on the cell line to be transfected. After mixing the DNA with liposomes in water, we attempted to recover plasmid DNA using a simple Bligh and Dyer extraction procedure (Bligh & Dyer, 1959). Surprisingly, in the presence of lipofectin there was a concentration-dependent reduction in DNA recovered from the aqueous phase (Figure 1). Addition of 80 nmol of total lipid to 10 μg of DNA resulted in greater than 95% loss of DNA from the aqueous phase. This effect could not be achieved using liposomes prepared from egg phosphatidylcholine/DOPE (50:50 mole ratio) and, therefore, appeared to be mediated by the cationic lipid DOTMA. The initial objective of this study was to characterize this cationic lipid-dependent change in DNA partitioning characteristics.

The location of the plasmid DNA within the two-phase partitioning system was examined by first adding purified monovalent cationic lipids to DNA in the Bligh and Dyer monophase and subsequently partitioning this solution into a two-phase system by the addition of water and chloroform. Plasmid DNA levels in the aqueous and organic phases were then determined. These studies, shown in Figure 2, were completed with three different monovalent cationic lipids: DOTMA, DDAB, and DODAC. Consistent with the results

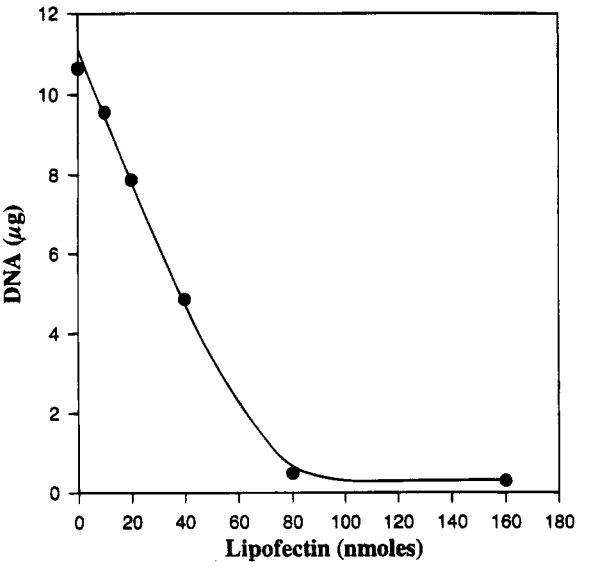

FIGURE 1: Effect of increasing amounts of lipofectin (DOTMA/DOPE; 50:50 mole ratio) on the recovery of pCMVβ plasmid DNA in the aqueous phase following Bligh and Dyer extraction of the DNA/lipid complexes (see Methods).

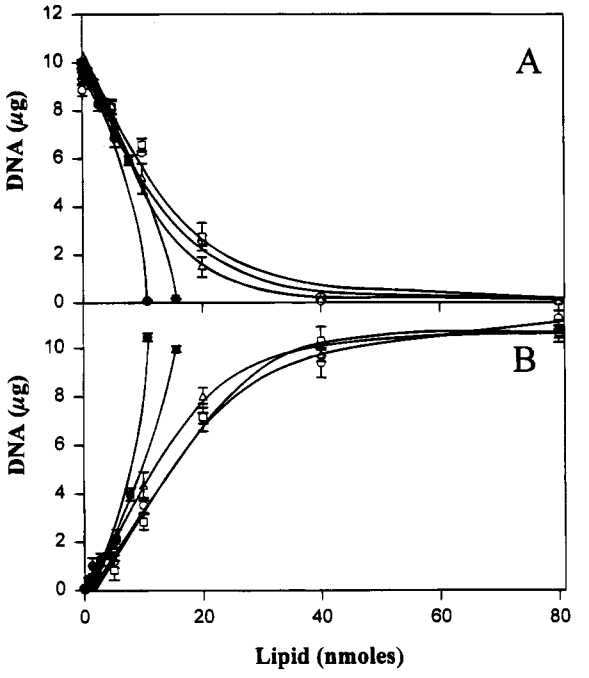

FIGURE 2: Effect of increasing amounts of lipid on the recovery of plasmid DNA in the aqueous (A) and organic (B) phase following Bligh and Dyer extraction of the DNA/lipid complexes. The amount of DNA used was 10 μg. Monocationic lipids used were DDAB (○), DOTMA (□), and DODAC (△). Lipopolyamines used were lipofectamine (●) and transfectam (■). All data points are averaged from three replications and expressed ±SEM.

shown in Figure 1, there was a cationic lipid-dependent loss of DNA from the aqueous phase (Figure 2A). There was no visible evidence of precipitated material at the aqueous/organic interface, and quantification of the DNA in samples collected to include the interface did not account for appreciable DNA levels (results not shown). The DNA was shown to be quantitatively transferred to the organic phase (Figure 2B). Greater than 95% of the DNA in the monophase could be recovered in the organic phase when 40 nmol monovalent cationic lipid was added. This value is identical to results presented in Figure 1 where 80 nmol of lipofectin

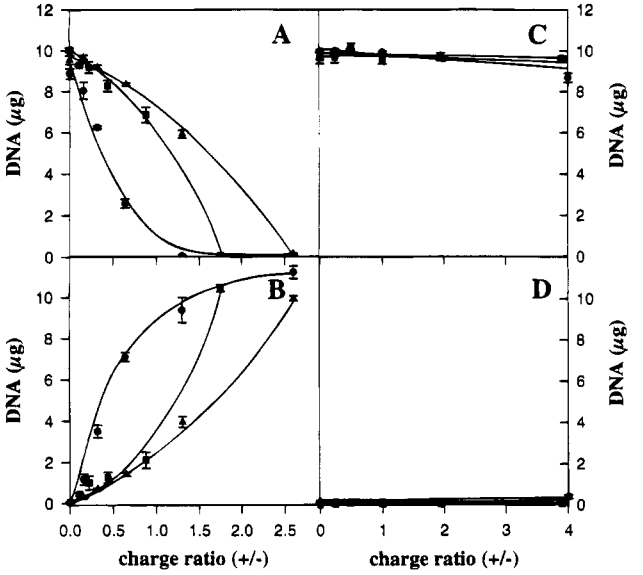

FIGURE 3: Recovery of plasmid DNA from aqueous (A and C) and organic (B and D) fractions following Bligh and Dyer extractions and expressed as a function of charge ratio (+/−). (A and B) DDAB (●), Lipofectamine (■), and Transfectam (▲). (C and D) Other cations: calcium (●), L-lysine (■), and poly(L-lysine) (▲). The amount of DNA used was 10 μg, and all data points were averaged from three experiments and presented ±SEM.

(50:50 DOTMA/DOPE mole ratio) resulted in the complete loss of DNA from the aqueous phase. The partitioning data shown in Figure 2 indicate that the three different monovalent lipids behave in a similar fashion under the conditions used.

In order to assess the influence of multivalent cationic lipids on DNA partitioning, studies were also performed using lipofectamine (Figure 2, ●) and transfectam (Figure 2, ■). Lipofectamine (DOSPA/DOPE; 75:25 mole ratio) and transfectam (100% DHGS) were added as preformed liposomes (lipofectamine) or lipids dissolved in ethanol (transfectam). DOSPA and DHGS contain headgroups derived from spermine and they exhibit five and four positive charges, respectively, at pH <7. As expected, significantly lower amounts of these lipids (calculated on the basis of the cationic lipid) were required to mediate DNA partitioning into the organic phase. Complete partitioning of the DNA into the organic phase was achieved after addition of approximately 10 nmol of DOSPA and DHGS.

Previous studies by Bloomfield demonstrated that DNA condenses into small toroid or rod-shaped structures when the DNA phosphate charge is at least 90% neutralized (Wilson & Bloomfield, 1979). The data presented in Figure 2 were therefore expressed as a function of cation/phosphate charge ratio (Figure 3A,B). For comparison, results obtained after the addition of the nonlipid-based monovalent (lysine), divalent (calcium), and multivalent [poly(L-lysine)] cations are included (Figure 3C,D). The results shown in Figure 3 suggest that, for monovalent cations, greater than 95% of the DNA partitioned into the organic phase when a +/− charge ratio >1 was achieved. Similar results were observed when the polyvalent lipids DOSPA and DHGS were used, although a slightly greater charge ratio was required to mediate efficient DNA transfer. In addition, DNA partitioning into the organic phase did not occur as a result of simple charge neutralization. When the DNA was mixed with the nonlipid cations at +/− charge ratios up to 4, >95% of the DNA was recovered in the aqueous phase.

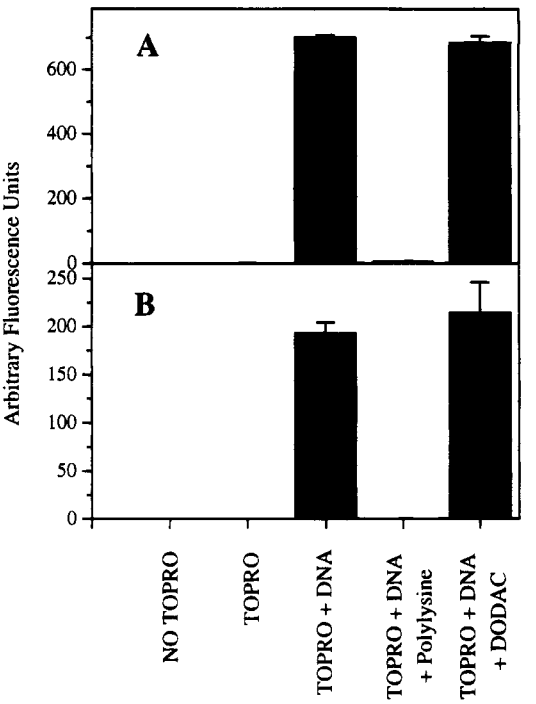

FIGURE 4: Evaluation of DNA condensation by poly(L-lysine) and DODAC assayed by TO-PRO-1 dye intercalation. Condensation state of the DNA was assessed in Bligh and Dyer monophase (A) and 100 mM OGP (B). Data are expressed as arbitrary fluorescence units and represent values obtained using an excitation wavelength of 509 nm and an emission wavelength of 533 nm. Data points were averaged from three experiments and presented ±SEM.

The data presented suggest that binding of cationic lipid to DNA results in the formation of a hydrophobic complex. This complex is unlike those formed as a result of DNA condensation by poly(L-lysine). It is important, therefore, to assess whether the DNA in this hydrophobic complex is in a condensed state. To evaluate this, we examined the ability of the DNA to bind a small fluorescent probe TO-PRO-1. This method is similar to approaches demonstrating that condensed DNA cannot bind ethidium bromide (Gershon *et al.*, 1993). TO-PRO-1 is a more sensitive, membrane impermeant, nucleic acid intercalating dye and, therefore, provides a more stringent test of DNA dye binding. DNA was mixed with either a monovalent cationic lipid or poly(L-lysine) in the Bligh and Dyer monophase (Figure 4A). Subsequently, TO-PRO-1 was added to a final concentration of 1 μM, and fluorescence was measured at 533 nm under conditions where the probe was excited at 509 nm. In the absence of DNA no fluorescence was observed. However, when plasmid DNA was added (10 μg/mL) there was a >600-fold increase in fluorescence at 533 nm. When TO-PRO-1 was added to DNA that had been mixed with poly(L-lysine), no fluorescence was observed, consistent with the fact that the fluorochrome cannot access DNA when it is in a condensed state. In dramatic contrast, following the formation of the cationic lipid/DNA complex (DODAC) in the Bligh and Dyer monophase, TO-PRO-1 binding to DNA was not excluded. This result is compatible with the concept that the DNA within the complex does not exist as a condensed structure. Figure 4B shows that similar results were obtained when TO-PRO-1 was added to plasmid DNA mixed with either poly(L-lysine) (condensed DNA) or the cationic lipid DODAC (uncondensed DNA) in the presence of 100 mM OGP, a nonionic detergent. Differences in initial

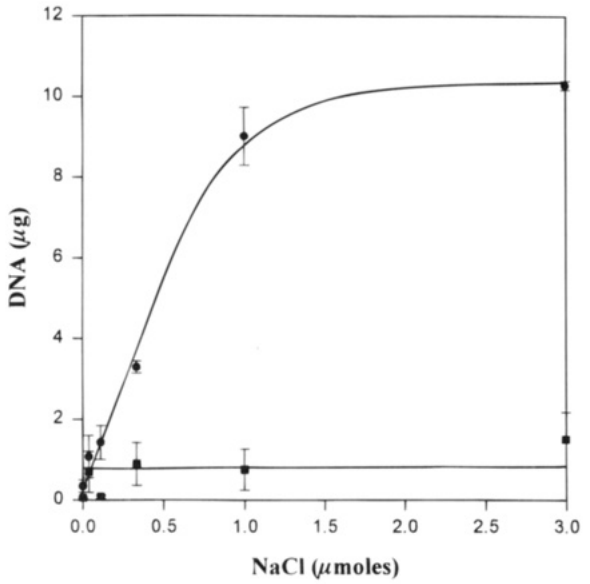

FIGURE 5: Effects of increasing amounts of NaCl on the recovery of plasmid DNA from the aqueous phase following Bligh and Dyer extraction of DNA/lipid complexes. The amount of DNA used was 10 μg. DODAC (●). Lipofectamine (■). Data are averaged from three experiments and expressed ±SEM.

flourescence were noted between the two systems and were likely caused by the changes in the environments in which the dye was introduced.

The data presented in Figure 4B provided an initial assessment of cationic lipid/DNA complex formation in the presence of detergent rather than a solvent-based solution. We have demonstrated that the DNA/lipid complex was formed in the presence of OGP by using the Bligh and Dyer extraction method (data not shown). Here, 10 μg of plasmid DNA was mixed with 40 nmol of DODAC, sufficient monovalent cationic lipid to promote complete partitioning of the DNA into the organic phase (see Figure 2). This mixture was prepared in the Bligh and Dyer monophase and OGP was added to achieve concentrations up to 20 mM (20 μmol in 1 mL) prior to separating the sample into the two phases. This concentration of OGP is above the CMC (17 mM) and was the maximum amount that could be added from a 2 M stock solution without disrupting the monophase system. Regardless of the OGP concentration, greater than 95% of the DNA partitioned into the organic phase. These results strongly suggest that the cationic lipid/DNA complex is stable in the presence of a neutral detergent such as OGP.

The ability to generate a heteromolecular complex between DNA and cationic lipids in solvent or detergent-based solutions provides an opportunity to further characterize the complex. It is known that transfection procedures can be affected by the presence of high NaCl concentrations. The effect of increasing concentrations of NaCl on the stablility of the cationic lipid/DNA complexes was therefore evaluated. As illustrated in Figure 5, monovalent cationic lipid binding to DNA, as measured by the partitioning assay described in Figure 2, was completely inhibited in the presence of 1 μmol of NaCl. At this level, Na^+ is present in a 25-fold molar excess relative to the amount of cationic lipid added. As expected, the complex between DNA and the polyvalent lipid DOSPA was more stable in the presence of NaCl. In fact, addition of Na^+ in a 300-fold molar excess relative to DOSPA did not increase the amount of DNA measured in the aqueous phase.

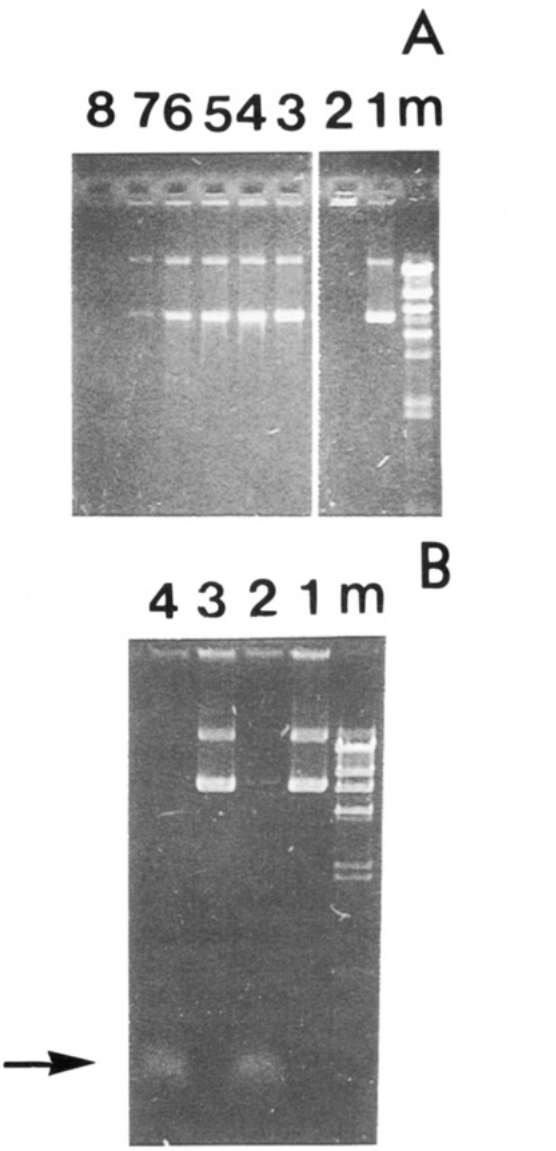

FIGURE 6: (A) Effect of poly(L-lysine) and DODAC on the electrophoretic mobility of plasmid DNA. 10 μg/mL pCMVβ plasmid (lane 1) was complexed in a final volume of 30 μL in OGP with 1:1 (+/−) ratio of poly(L-lysine) (lane 2), and 10, 20, 40, 80, 160, and 320 nmol/mL DODAC (lanes 3−8, respectively). The complexes were subjected to electrophoresis on a 0.8% agarose gel in TBE buffer. DNA was visualized by staining with ethidium bromide. (B) Effect of DNase I on the mobility of plasmid DNA when complexed with DODAC in OGP. 10 μg/mL plasmid (lane 1) was complexed with 40 nmol/mL DODAC (lane 3) in 100 μL. DNase I (0.33 unit) was added to the DNA control (lane 2) and the DNA/lipid complex (lane 4) and incubated at 37 °C for 10 min. Subsequently, the DNA was extracted by the Bligh and Dyer procedure in the presence of 150 mM NaCl and precipitated from the aqueous fraction. The resulting DNA was subjected to electrophoresis on a 0.8% agarose gel and stained with ethidium bromide. "m" represents the DNA size marker. The arrow represents digested DNA fragments.

A method now used to characterize liposome/DNA complexes is to evaluate the influence of liposome binding on DNA migration in agarose gel electrophoresis (Bertling *et al.*, 1991; Zhou *et al.*, 1991). Figure 6A shows the gel mobility characteristics of the lipid/DNA complexes made in the presence of OGP compared to that of the poly(L-lysine) condensed DNA control. Lane 2 shows that the nonlipid-based DNA/poly(L-lysine) complexes exhibit significantly reduced mobility in an agarose gel. This result is consistent with previous studies demonstrating that DNA condensed

with cationic liposomes adopts a macromolecular structure that does not move within an applied electric field (Bertling *et al.*, 1991). This may be a consequence of charge neutralization and/or increases in molecular size. In contrast, when DNA is mixed with cationic lipids in a micellar form, there is no indication that the migration of DNA has been altered (Figure 6A, lanes 3−5). We would expect that DNA will migrate into the gel under conditions when charge neutralization is not achieved or when the lipid/DNA complex has dissociated. Regardless, these studies provide further evidence suggesting that cationic lipid binding to DNA does not result in the condensation of DNA. Changes in DNA mobility, however, were observed when the cationic lipid concentration was increased beyond cationic lipid to DNA phosphate charge ratios of 2 (lanes 6−8). For example, addition of 320 nmol of DODAC resulted in a decrease in DNA migrating into the gel and a small proportion of the DNA migrating near the top of the gel, perhaps an indication that stable, neutral DNA/lipid complexes can be achieved in the presence of excess cationic lipids.

To determine the ability of cationic lipids to protect plasmid DNA from enzymatic digestion, DNase I mediated degradation of the cationic lipid/DNA complex was also evaluated using agarose gel electrophoresis (Figure 6B). In these experiments, DNA in OGP was mixed with a sufficient amount of DNase I to generate small DNA fragments after a 10 min incubation at 37 °C (lane 2). Lane 1 shows undigested DNA (control). Using identical conditions, the DNA complexed with the monocationic lipid DODAC was not protected against the enzymatic activity of DNase I (lane 4). DNA extracted from the complex in the absence of DNase I (lane 3) shows intact DNA. This also suggests that the DNA in these DNA/lipid complexes exists in an uncondensed state and is susceptible to degradation.

DISCUSSION

The results summarized identify the formation of a hydrophobic DNA/lipid complex which can readily be isolated in organic solvents. These complexes can also be prepared in neutral detergents such as OGP and the DNA exists in a form that is not condensed. We believe that this complex will serve as a basic building block that can be used in the preparation of well defined lipid-based carrier systems for plasmid DNA. Therefore, this discussion focuses on the characteristics of the DNA/lipid complex as well as procedures that can potentially be considered in the preparation of defined DNA carriers based on such DNA/lipid complexes.

It has been demonstrated through the work of Bloomfield and co-workers (Bloomfield, 1991) that polyvalent cations when mixed with DNA can lead to a condensation reaction. A wide variety of agents are capable of mediating DNA condensation including polyvalent peptides (e.g., polylysine, histone H1, spermidine, spermine), inorganic cations [e.g., $Co(NH_3)_6^{3+}$] and mono- and polycationic lipids incorporated into liposomes (Behr, 1986; Shapiro *et al.*, 1969; Arscott *et al.*, 1990; Plum *et al.*, 1990; Barthel *et al.*, 1993). All of these agents have been used in transferring selected genes to cells in culture, and it can be suggested that DNA condensation is a characteristic associated with all nonviral based gene transfection methods.

It is not clear, however, what physical and chemical attributes of particles formed through the condensation reaction are important for transfection. In particular, polyvalent cation mediated condensation reactions lead to the formation of not only small toroidal structures but also large aggregates (Bloomfield, 1991). Aggregation, due to intermolecular cross-linking between DNA strands in a condensed or partially condensed state, is typically observed when DNA concentrations are high or when there is an excess of polyvalent cations. Regardless of the size of the resulting particle, it can be suggested that aggregates may be more effective in mediating DNA delivery *in vitro*. In addition, *in vitro* lipid and liposome-based transfection rates are optimal under conditions where the cation to DNA phosphate charge ratio is greater than 1 (Behr, 1993). This excess positive charge may also promote association of the particle with cells *in vitro* since the cell membrane possesses an overall negative charge. Properties of polyvalent cation/DNA particles required for optimal *in vitro* transfection, including excess positive charge and formation of structures larger than 100 nm, however, are not necessarily well suited for *in vivo* applications. In particular, pharmacokinetic studies of liposomes that exhibit a surface charge and large size distribution (>200 nm) indicate that such particles will be rapidly eliminated from the central blood compartment (Hwang, 1987). In addition, liposomes exhibiting a positive surface charge are also known to bind serum proteins (Chonn *et al.*, 1992) that can promote accumulation of particles in phagocytic cells of the reticuloendothelial system and can lead to complement activation and platelet aggregation (Reinish *et al.*, 1988; Chonn *et al.*, 1991).

We initiated the studies summarized here in an attempt to characterize DNA−lipid binding in the absence of DNA condensation or aggregation. It is proposed that intermolecular aggregation reactions can be minimized by using monovalent cationic lipids that are added to DNA in a monomeric or a micellar form rather than incorporated in preformed liposomes. As shown in Figures 1 and 2, when monovalent or polyvalent cationic lipids are added to plasmid DNA in a Bligh and Dyer monophase, a hydrophobic complex is formed that facilitates the extraction of DNA into the organic phase. The extraction efficiency approaches 100% when the charge ratio of cationic lipid to DNA phosphate is above 1. The DNA in this complex is not in a condensed state, as measured by DNA binding dye TO-PRO-1 intercalation with DNA (see Figure 4). Consistent with the absence of condensed structures, cationic lipid/DNA complexes formed following the addition of lipid in a monomeric or micellar form do not exhibit changes in migration behavior following agarose gel electrophoresis (see Figure 6A). In addition, formation of the complex does not protect DNA from the enzymatic activity of DNase I (Figure 6B). These data are in contrast to results obtained with polylysine, an agent known to promote DNA condensation (Laemmli, 1975).

As illustrated in the model shown in Figure 7, it is believed that heteromolecular complexes between DNA and lipid are achieved initially through electrostatic interactions. Provided sufficient lipids are bound, the resulting complex will exhibit hydrophobic characteristics. However, we suggest that under our experimental conditions there are no inter- or intramolecular interactions occurring. Rather, the DNA molecules exist in a form that is coated by fatty acid chains, the composition of which is determined by the cationic lipids used. The properties of the resulting hydrophobic complex

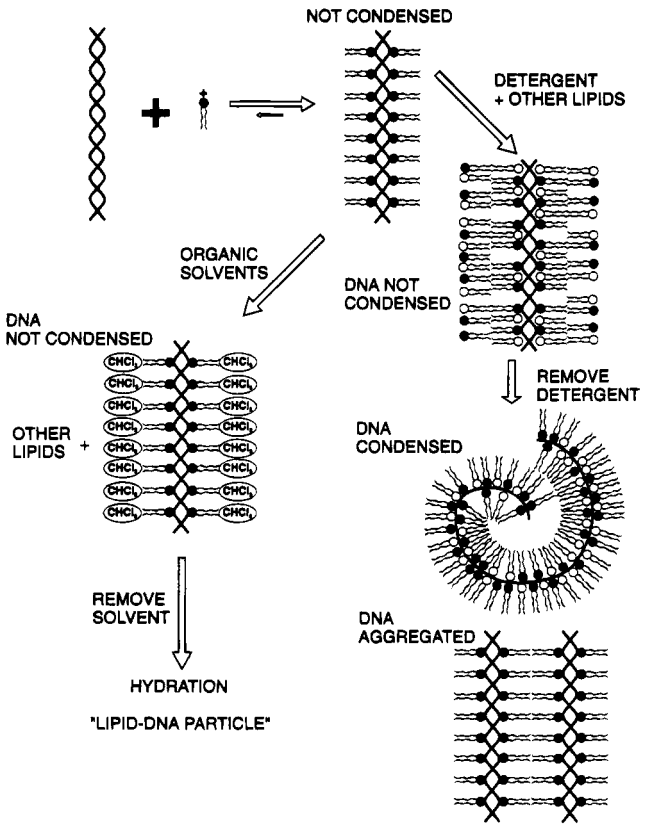

FIGURE 7: Model for the binding of monocationic lipids to DNA resulting in the formation of a hydrophobic DNA/lipid complex.

would thus be driven by simple and predictable hydrophobic interactions. It would, therefore, be expected that such a complex could readily be extracted into organic solvents. In detergent solutions it is possible that the complex would bind additional lipids. The interaction of these lipids with the cationic lipid/DNA complex would be mediated by hydrophobic interactions rather than charge−charge interactions. It is anticipated, therefore, that other lipids could readily be incorporated into this complex. The stability of the complex would be dependent on the concentration of the added lipid as well as DNA concentration. Excess lipid or detergent removal may promote DNA condensation or aggregation which could be facilitated through intramolecular acyl chain interactions.

Alternatively, DNA coated with a bilayer of lipid may adopt the energetically favorable toroidal structure achieved through DNA charge neutralization, with an intrahelical spacing defined by the thickness of the surrounding bilayer. Importantly, the outermost lipid surface would consist of a headgroup composition defined by the excess lipids added. If the DNA concentration is too high, one would predict DNA aggregation mediated through intermolecular acyl chain interactions. Regardless of the method proposed to achieve particle formation, the primary interaction between the cationic lipid and the DNA must be maintained. Stronger interactions are possible when forming the complex with polyvalent cationic lipids, as shown by the results in Figure 5.

In conclusion, we have established the conditions for the formation of a hydrophobic DNA/lipid complex. This complex can be isolated in organic solvents and is also formed in the presence of neutral detergents. The results also demonstrate that lipid binding to DNA does not result in DNA condensation. Experiments to develop and characterize particles prepared from defined DNA/lipid complexes are currently in progress.

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