ELSEVIER

Biochimica et Biophysica Acta 1461 (1999) 27–46

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.elsevier.com/locate/bba

A multi-step lipid mixing assay to model structural changes in cationic lipoplexes used for in vitro transfection

Ellen K. Wasan [a,b], Pierrot Harvie [a], Katarina Edwards [c], Göran Karlsson [c], Marcel B. Bally [a,b,*]

[a] *Advanced Therapeutics-Medical Oncology, BC Cancer Agency, 600 West 10th Ave., Vancouver, BC V5Z 4E6, Canada*
[b] *Department of Pathology and Laboratory Medicine, Faculty of Medicine, University of British Columbia, Vancouver, BC, Canada*
[c] *Department of Physical Chemistry, Uppsala University, Uppsala, Sweden*

Received 7 May 1999; received in revised form 6 August 1999; accepted 12 August 1999

Abstract

Formation of liposome/polynucleotide complexes (lipoplexes) involves electrostatic interactions, which induce changes in liposome structure. The ability of these complexes to transfer DNA into cells is dependent on the physicochemical attributes of the complexes, therefore characterization of binding-induced changes in liposomes is critical for the development of lipid-based DNA delivery systems. To clarify the apparent lack of correlation between membrane fusion and in vitro transfection previously observed, we performed a multi-step lipid mixing assay to model the sequential steps involved in transfection. The roles of anion charge density, charge ratio and presence of salt on lipid mixing and liposome aggregation were investigated. The resonance-energy transfer method was used to monitor lipid mixing as cationic liposomes (DODAC/DOPE and DODAC/DOPC; 1:1 mole ratio) were combined with plasmid, oligonucleotides or Na_2HPO_4. Cryo-transmission electron microscopy was performed to assess morphology. As plasmid or oligonucleotide concentration increased, lipid mixing and aggregation increased, but with Na_2HPO_4 only aggregation occurred. NaCl (150 mM) reduced the extent of lipid mixing. Transfection studies suggest that the presence of salt during complexation had minimal effects on in vitro transfection. These data give new information about the effects of polynucleotide binding to cationic liposomes, illustrating the complicated nature of anion induced changes in liposome morphology and membrane behavior.

Keywords: Liposome/DNA complex; Lipid mixing; Aggregation; Cryo-transmission electron microscopy; Transfection; Cationic liposome

Abbreviations: DODAC, dioleyldimethylammonium chloride; DOPE, dioleoylphosphatidylethanolamine; DOPC, dioleoylphosphatidylcholine; DOPS, dioleoylphosphatidylserine; NBD-PE, (*N*-7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine); Rh-PE, lissamine-rhodamine phosphatidylethanolamine; CHE, cholesteryl hexadecyl ether; FBS, fetal bovine serum; CAT, chloramphenicol acetyltransferase; HBS, HEPES buffered saline (25 mM HEPES, 150 mM NaCl); QELS, quasi-elastic light scattering; RT, room temperature; BCA, bicinchoninic acid; CTEM, cryo-transmission electron microscopy

* Corresponding author, at address a. Fax: (1) (604) 877-6011; E-mail: mbally@bccancer.bc.ca

0005-2736/99/$ – see front matter
PII: S0005-2736(99)00144-3

1. Introduction

Gene therapy using nonviral means of DNA delivery is facilitated by the use of cationic lipids [1]. These amphipathic lipids, typically alkylammonium compounds, readily form liposomes, which can then be mixed with plasmid DNA to form a self-assembling supramolecular complex ('lipoplex'). DNA within the complex is protected from nucleases present in the serum and the lipids facilitate uptake of DNA into cells by promoting cell binding and delivery into the cytoplasm. Various neutral or zwitterionic phospholipids (most notably dioleoylphosphatidylethanolamine (DOPE)) are usually included in the formulation along with the cationic lipid to improve transfection ability. Much effort has been devoted in the past decade to studying these liposome/DNA complexes to determine their mechanism of action and intracellular behavior during transfection in vitro or in vivo [2,3]. In addition, investigators have attempted to correlate how the physical properties of the lipids, liposomes and lipoplexes influence biological activity [4–6]. The relative importance of particular physicochemical properties of lipids and lipid-lipid interactions are still not fully elucidated in this context.

To illustrate the problems in assigning mechanisms of activity of lipid components it is useful to review the roles that have been assigned to the helper lipid DOPE. DOPE forms nonbilayer structures involved in the fusion of membranes [7] and to this property its transfection-enhancing abilities are usually attributed [8,9]. Alternatively, we have suggested that the amine group of phosphatidylethanolamine (PE) can interact with the DNA phosphate groups and that such an interaction serves to weaken the binding reaction between cationic lipids and DNA. This, in turn, may promote the eventual disassembly of the lipoplex, a process that has been defined as a critical step required for transgene expression [10]. It can also be predicted, however, that PE-containing lipoplexes will be more susceptible to factors that promote disassembly prior to accessing a target cell population. This is consistent with results demonstrating that phosphatidylethanolamine-containing lipoplexes afford less nuclease protection to DNA than those prepared with phosphatidylcholine [11].

It has been a challenging task to assign specific roles to individual lipids, partly because the lipid components have a significant influence on the physicochemical properties of the lipoplexes even prior to addition to cells or parenteral administration. As cationic liposomes and DNA interact to form a complex, increases in particle size are observed, which we refer to here in a general sense as 'aggregation'. Complicated vesicle-vesicle interactions that lead to increased particle size occur as a consequence of electrostatic and possibly hydrophobic interactions between the cationic liposomes and polynucleotides [10]. This may or may not involve complete fusion of lipid membranes, which is a specific rearrangement event occurring after aggregation (contact) and lipid mixing (an intermediate step) of the liposomal membranes resulting in the formation of one new bilayer where there once were two. These interactions facilitate cross-linking of vesicles between polynucleotide bridges and/or may trigger changes in the liposomal membranes that favor fusion, hemifusion or other forms of bilayer destabilization. Aggregation of liposomes, resulting in increases in particle size, can readily be monitored by light scattering techniques. Membrane destabilization can be examined using resonance energy transfer (RET) techniques [12]. These data can then be placed into context by use of electron microscopy-based visualization methods [13].

The RET technique has proven to be particularly useful in assessing polynucleotide induced membrane destabilization. It is useful to note, however, that this assay was developed to study lipid mixing mediated by addition of cations to anionic membranes. Various studies of anionic liposome lipid mixing following addition of monovalent or divalent cations [14,15] and polycations [16,17] have formed a basis for studies assessing polynucleotide binding to cationic liposomes [18,19]. There are similarities in the two systems considering that electrostatic interactions are the primary driving forces promoting adhesion of the liposomes. Perhaps the most important lesson that can be learned when reviewing these previous studies is that it is difficult to control fusion reactions between liposomes. This, in turn, makes it difficult to form homogenous membrane structures after inducing fusion.

In this report we have investigated the effects of plasmid DNA, oligonucleotides and phosphate on cationic liposome aggregation and membrane destabilization. Unlike previous studies, however, we have attempted to analyze the behavior of these systems sequentially as they are prepared, diluted in salt containing buffers and eventually added to anionic vesicles as model cell membranes. While the interaction of cationic liposomes with anionic vesicles or biological membranes has been investigated before [18,19], we believe this is the first study examining the lipid mixing behavior of lipoplexes in a multistep manner that represents a transfection-relevant scenario. We observe that the potential for cationic liposomes to undergo lipid mixing reactions generally decreases sequentially following addition of polynucleotides and salts, such that much of the original 'fusogenic potential' of the cationic liposomes is reduced by the time they come into contact with the model cell membranes. In addition, observation of the structural features of the polynucleotide/liposome complexes by cryo-transmission electron microscopy (CTEM) demonstrates the high degree of heterogeneity in the complexes. It is clear that advancement of this technology will require development of structurally and chemically well-defined lipid-based formulations.

2. Materials and methods

2.1. Materials

The cationic lipid DODAC and purified plasmid encoding the firefly luciferase reporter gene (pInexL018) were provided by Inex Pharmaceuticals (Vancouver, BC, Canada). Plasmid encoding the chloramphenicol acetyltransferase (CAT) gene from *Escherichia coli* (pInexCATv2.0), described elsewhere [20], was prepared in-house by standard techniques using the Qiagen anion exchange plasmid preparation kit (Qiagen, Santa Clarita, CA). The 4.5 kb CAT plasmid and the 5.65 kb luciferase plasmid were based on the pCMVβ plasmid from Clonetech (Palo Alto, CA) and thus contain the same CMV promoter/enhancer element and SV40 polyadenylation signal. DOPE and DOPC were purchased from Northern Lipids (Vancouver, BC, Canada). DOPS was obtained from Avanti Polar Lipids (Alabaster, AL). NBD-PE and Rh-PE, both of which have the fluorescent moiety attached to the phospholipid headgroup, were obtained from Molecular Probes (Eugene, OR). ^{3}H-Cholesteryl hexadecyl ether (CHE) was purchased from DuPont-NEN (Markham, ON, Canada). The Extruder device was provided by Lipex Biomembranes (Vancouver, BC, Canada). Polycarbonate membranes used with the Extruder were purchased from Poretics (Mississauga, ON, Canada). Oligonucleotides (18-mer nonsense sequence) were provided by Genta (San Diego, CA). The B16/BL6 mouse melanoma cell line was obtained from the National Cancer Institute (NCI Tumor Repository, Bethesda, MD) and maintained in RPMI 1640 with 10% fetal bovine serum (FBS). Minimum essential medium Eagle (modified) with Earle's salts (MEM) and FBS for tissue culture were purchased from ICN Biomedical (Aurora, OH). Triton X-100 was purchased from Bio-Rad (Richmond, CA). All other chemicals were of reagent grade from Sigma (St. Louis, MO). Tissue culture plates (96-well) were from Falcon (Becton-Dickinson, Franklin Lakes, NJ). Fluorometric analyses were performed on a Perkin-Elmer Model LS50B (Vancouver, BC, Canada). Liquid scintillation counting was performed in a Model TR1900 Tri-Carb from Canberra Packard (Meriden, CT) using Pico-Fluor liquid scintillation fluid (Canberra Packard).

2.2. Liposome preparation

DODAC/DOPE (50/50 mol%), DODAC/DOPC (50/50 mol%), DOPS/DOPC (50/50 mol%), DODAC/DOPE/NBD-PE/Rh-PE (50/49/0.5/0.5 mol%) and DODAC/DOPC/NBD-PE/Rh-PE (50/49/0.5/0.5 mol%) liposomes were prepared by the extrusion method [21]. Lipids were dissolved in chloroform at 20 mg/ml and dried down under a stream of N_2 gas, followed by drying under vacuum for 4 h. The dried lipid film was hydrated with 300 mM filtered lactose with agitation. The resulting liposomes were then passed ten times at approx. 400 psi through an extrusion device containing three stacked filters of 0.08 μm pore size. Final lipid concentration was determined through the use of a nonexchangeable radioactive lipid tracer, ^{3}H-CHE.

2.3. Preparation of lipoplexes

Separately, DNA and liposomes were centrifuged at 900×*g* for 2 min prior to mixing, to pellet any debris that could promote aggregation. This process does not affect lipid or DNA concentration. Lipoplexes were from the pre-formed liposomes and plasmid or oligonucleotides by mixing 1 vol. of DNA into 1 vol. of liposomes at the desired concentrations, usually for a total volume of less than 1.0 ml. Mixing was accomplished by slow, repeated pipetting of the suspension. For samples containing HBS, liposomes were prepared initially in lactose, and DNA was diluted in HBS prior to mixing of the liposomes and DNA. The final concentration of NaCl after mixing was 150 mM. Lipid concentration of the complexes was 1 mM unless otherwise indicated. Complexes were allowed to form for 30 min at RT prior to experiments unless otherwise indicated.

2.4. Preparation of liposome/Na_2HPO_4 mixture

A 2% volume of 50×Na_2HPO_4 solution was added to the preformed liposomes to achieve the appropriate phosphate concentration (0–5 mM). Lipid concentration was 1 mM. The mixture was allowed to incubate at RT for 30 min unless otherwise indicated.

2.5. Particle sizing

Mean diameters of liposomes and lipoplexes were assessed at ambient temperature by quasi-elastic light scattering (QELS), using a Nicomp Model 270 Submicron Particle Sizer (Pacific Scientific, Santa Barbara, CA) with an argon laser operating at a wavelength of 632.8 nm. Size analysis typically required about 10 min to perform. The resulting Nicomp analysis fitted the data to a normal distribution, with mean, standard deviation (S.D.), coefficient of variation, and χ^2 values. We report here the Gaussian mean ± S.D. *within* single samples, which is more representative of sample polydispersity than the mean ± S.D. among several different samples.

2.6. Assessment of salt-induced aggregation

DODAC/DOPE or DODAC/DOPC liposomes were prepared in lactose as described above. Liposomes were diluted into NaCl solution (0–150 mM final concentration). Liposome mean diameter was assessed by QELS within 10 min of dilution. Lipoplexes were prepared with DODAC/DOPE or DODAC/DOPC liposomes and pInexCATv2.0 plasmid using 100 μg DNA/ml (lipid:DNA = 10 nmoles lipid/μg DNA). Liposome/DNA complex formation was allowed to proceed for 30 min at RT, at which time 20 μl of lipoplexes were diluted into 250 μl of NaCl solution, followed by QELS analysis within 10 min of dilution.

2.7. CTEM

Thin sample films (100–500 nm) were prepared under controlled temperature (25°C) and high humidity in a custom-built chamber. A small volume of sample was applied to a copper grid that had been coated with the polymer cellulose acetate butyrate The sample was then blotted to produce a thin sample film across the holes in the polymer film and immediately vitrified by rapid freezing in liquid ethane. This technique is described in detail elsewhere [22,23]. The grid was then transferred to a Zeiss EM902 transmission electron microscope for viewing. Samples were kept cool to prevent formation of ice crystals on the sample surface by maintaining the temperature below 108 K during the transfer and examination procedures. A zero-energy loss bright-field mode was used and the accelerating voltage was 80 kV. It should be noted that in viewing the images the two-dimensional projection of a closed liposome appears as a circle with high contrast around the edge, because the projected thickness of the bilayer is greatest at the edges. A flat bilayer disk appears to have even contrast around the edges. Larger particles will tend to cluster at the edges of the film where the thickness is greater.

2.8. Lipid mixing assay

The resonance energy transfer technique was used to assess lipid mixing. Briefly, as the bilayers of NBD-PE/Rh-PE fluorescently labeled liposomes and unlabeled liposomes mix due to the presence of some fusion-inducing compound or conditions, the NBD- and rhodamine-lipids are diluted within the bilayer

as unlabeled lipids are introduced. Subsequently, the resonance energy transfer occurring between NBD and rhodamine decreases, resulting in an increase in fluorescence at the emission wavelength of NBD. Thus under these conditions, an increase in fluorescence reflects the degree to which lipid mixing is occurring. In our assay, the initial fluorescence (F_o) of the liposomes in the absence of the fusion-promoting compound was measured (excitation $\lambda = 465$ nm, emission $\lambda = 538$ nm, cutoff $\lambda = 530$ nm). The molar ratio of fluorescent to nonfluorescent liposomes was 1:1. Triplicate samples were prepared in which 1 vol. of plasmid or oligonucleotides (0–100 μg/ml final concentration) or 2% volume of 50× sodium phosphate (0–5 mM final concentration) was added to the liposomes for a final lipid concentration of 1 mM. Complex formation was allowed to proceed at room temperature for at least 5 min followed by fluorescence measurement (F). Finally, a 10% volume of 3% Triton X-100 was added and the samples were incubated at 100°C for 2 min followed by vortexing. The samples were allowed to cool to RT then fluorescence was read (F_t). Percent maximal lipid mixing (%maxLM) was calculated by the formula:

$$\%\text{maxLM} = [(F - F_o)/(F_t - F_o)] \times 100\%$$

2.9. Multi-step lipid mixing assay

Similarly to the lipid mixing assay described above, liposomes were prepared in 300 mM lactose and diluted with either 300 mM lactose or HBS to 2 mM with a molar ratio of fluorescent to nonfluorescent liposomes of 1:1. An aliquot of this dilution was used immediately to prepare lipoplexes in lactose or HBS (10:1 lipid:DNA ratio. Charge ratio (+/−) = 1.6). The fluorescence of the liposomes alone was read for approx. 200 s, then the fluorescence of lipoplexes prepared from the same sample of liposomes for another 200 s. One volume of tissue culture medium with or without 10% FBS was then added and the sample was mixed by rapid inversion, followed by fluorescence reading for another 200 s. Anionic liposomes (DOPS/DOPC, 50/50 mol%) in a small volume were then added such that the molar ratio of DODAC/DOPE or DODAC/DOPC to DOPS/DOPC was 1:1, followed by mixing by inversion and a continuation of fluorescence readings for another 400 s. Finally, a 10% volume of 3% Triton X-100 was added, the sample was mixed by inversion, and the fluorescence was read for another 400 s. Controls consisted of samples prepared without DNA to account for changes in fluorescence due to sample dilutions in the course of the assay, and samples prepared without the fluorescent liposomes (same total lipid concentration) to account for light scattering. The values obtained from the light scattering control were subtracted as background. Dilution factors were calculated from the fold signal reduction of the liposome-only control values at each step of the assay. The data plotted were representative experimental samples corrected for both light scattering and dilutions.

2.10. In vitro transfection

B16/BL6 murine melanoma cells were plated at 4×10^3 cells/well in a 96-well plate. They were transfected in replicates of six using lipoplexes containing plasmid encoding either CAT or luciferase as the reporter gene. Plasmid in the absence of liposomes ('free DNA') and liposomes alone were included as controls in all experiments. DNA and lipid concentrations were 25 μg/ml and 250 nmoles/ml, respectively. Twenty μl of lipoplexes were added to each well (0.5 μg DNA/well). Transfection proceeded for 4 h in media ±10% FBS. CAT activity was measured based on published methods [24,25]. Briefly, the assay involves a two-phase extraction in mixed xylenes and addition of ^{14}C-labeled chloramphenicol (D-*threo*-[dichloroacetyl-1,2-^{14}C]CAT, assay grade, DuPont NEN) as substrate, followed by detection of the radioactive product of the enzyme and comparison to a standard curve using purified CAT (Sigma). Luciferase activity was quantified using a luciferase assay kit from Boehringer Mannheim (Germany), following the manufacturer's instructions. Arbitrary light units were measured at ambient temperature in a luminometer within 1 min of adding ATP substrate. For purified luciferase used for standard curves, 1 mg luciferase protein yields approx. 10^7 light units. Total cellular protein for all transfection experiments was measured using the bicinchoninic acid (BCA)/CuII protein assay kit (Sigma) standardized with bovine serum albumin. The BCA/Cu I precipitate, produced by reduction of Cu

II by protein, was measured spectrophotometrically at 570 nm in a microplate reader.

3. Results

3.1. Effects of salt on aggregation of lipoplexes

Lipoplexes containing DOPE are often prepared in a nonionic or low ionic strength solution due to their well-known tendency to precipitate out of solution as the salt concentration is increased. This is illustrated by the data shown in Table 1. Cationic liposomes prepared using DOPC were stable in salt within the time they were used here, but slightly aggregated (mean diameter = 486 ± 335 nm) when mixed with serum-containing media. In contrast, the DODAC/DOPE liposomes aggregated when the NaCl concentration was ≥ 50 mM. Addition of DNA to DODAC/DOPE or DODAC/DOPC resulted in lipoplexes with mean diameters at least 2–3-fold larger than liposomes alone. Further increases in particle size were induced following addition of salt; however, the DODAC/DOPC lipoplexes aggregated less than the DODAC/DOPE lipoplexes. Although significant aggregation was observed when lipoplexes were diluted into tissue culture media, this effect was less pronounced when the media contained 10% FBS.

To further assess the effects of DNA concentration and the presence of NaCl on the mean diameter of lipoplexes, DODAC/DOPE and DODAC/DOPC cationic liposomes were prepared in 300 mM lactose (nonionic but physiologically iso-osmotic) and mixed with a solution of either plasmid or oligonucleotides as described in Section 2. The polynucleotides were in either lactose solution or in HBS. With the lipid concentration held constant at 1 mM, complexes were prepared with varying amounts of plasmid (0–125 μg/ml), or oligonucleotides (0–100 μg/ml) corresponding to charge ratios (CR) (+/−) ranging from 16 (10 μg/ml) to 0.8 (125 μg/ml) as indicated in the figure legends. At higher concentrations of polynucleotides under these conditions, visible particulates formed. The mean diameters of the liposomes and lipoplexes were measured by QELS within 1 h of complex formation and the results have been summarized in Fig. 1. The liposomes typically had a mean diameter of 120–130 nm in the absence of NaCl. As shown in Fig. 1, particle size increased with increasing polynucleotide concentration, and this increase was more pronounced when the lipoplexes were formed in the presence of HBS (filled triangles). The minimum diameter of liposome-nu-

Table 1
Effect of NaCl on liposome size with and without added plasmid

NaCl (mM)	Mean diameter ± S.D. (nm)[a]			
	DODAC/DOPC		DODAC/DOPE	
	Liposomes	Lipoplexes[b]	Liposomes	Lipoplexes
0	123 ± 43	325 ± 93	114 ± 36	205 ± 71
10	85 ± 22	710 ± 399	91 ± 23	167 ± 53
25	84 ± 23	939 ± 606	93 ± 25	1000[c]
38	85 ± 22	453 ± 189	95 ± 31	nd[d]
50	86 ± 19	555 ± 296	1000	nd
100	85 ± 23	704 ± 346	nd	nd
150	84 ± 24	585 ± 292	nd	nd
Medium[e]	87 ± 27	665 ± 355	1000	1000
Medium+serum[f]	486 ± 335	344 ± 610	1000	228 ± 101

[a] Measured by QELS.
[b] Lipoplex: liposome/DNA complex.
[c] 1000 = aggregated to > 1 μm diameter.
[d] nd, not done because next-lowest NaCl concentration resulted in aggregation.
[e] Medium was RPMI 1640.
[f] Medium contained 10% FBS.

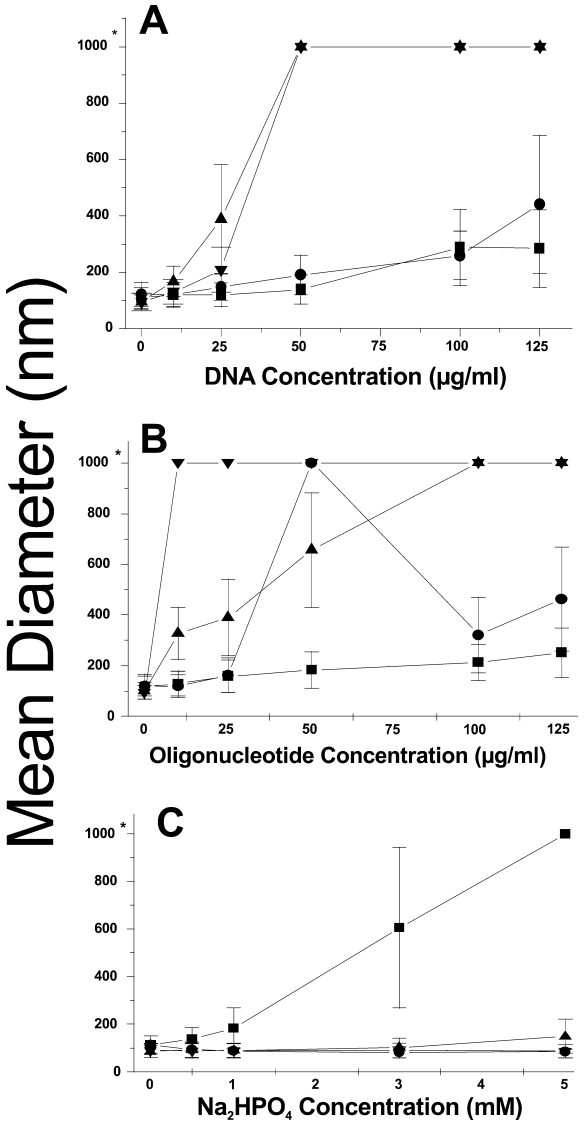

Fig. 1. Mean particle diameter of liposomes as measured by QELS at increasing concentrations of (A) plasmid; (B) 18-mer oligonucleotides; (C) sodium phosphate. ■, DODAC/DOPE in lactose; ●, DODAC/DOPC in lactose; ▲, DODAC/DOPE in HBS; ▼, DODAC/DOPC in HBS. Values represent mean ± intra-sample S.D. for individual samples. *Mean diameter ≥1000. Corresponding charge ratio (+/−) for A and B: 10 μg/ml = 16, 25 μg/ml = 6.4, 50 μg/ml = 3.2, 100 μg/ml = 1.6, 125 μg/ml = 0.8. Corresponding charge ratio for C: 0.5 mM = 1, 1 mM = 0.5, 3 mM = 0.17, 5 mM = 0.1.

cleic acid complexes as measured by QELS was approx. 200 nm. Interestingly, there was little difference between the 18-mer oligonucleotide (Fig. 1B) and the 4.5 kb plasmid (Fig. 1A) in their ability to promote vesicle aggregation as measured by light scattering techniques. For comparison, we also investigated the effects of Na_2HPO_4 (Fig. 1C), a divalent anion, on cationic liposome aggregation. Phosphate caused little aggregation, except when added to DODAC/DOPE liposomes prepared in lactose, where there was an increase in mean diameter as phosphate concentration increased. When DODAC/DOPE liposomes were mixed with a Na_2HPO_4 concentration >5 mM (CR (+/−) <0.1), visible particulates formed. When DODAC/DOPE liposomes were diluted into HBS first, however, and Na_2HPO_4 was then immediately added, aggregation was not observed even at concentrations >5 mM phosphate. This is in contrast to DODAC/DOPE liposomes in HBS without phosphate present (e.g. Table 1).

To facilitate the assessment of the aggregation state of multiple samples, we also measured sample turbidity was measured spectrophotometrically by reading absorbance at 570 nm. A 96-well tissue culture plate was loaded with 50 μl of complexes prepared in triplicate at various concentrations of plasmid, oligonucleotides, or phosphate. Changes in turbidity following addition of the various anions were comparable to the results shown in Fig. 1 (data not shown). This simple technique may therefore provide an alternative to investigators who do not have access to a particle sizer.

3.2. CTEM analysis

While determination of mean particle diameter by QELS and gross aggregation by turbidity give valuable information about the bulk sample, these methods tell little about the structure of the lipid/polynucleotide complexes formed. Significant morphological changes take place as the lipid vesicles interact with polyanions. We used CTEM to qualitatively assess the morphology of the lipoplexes (Figs. 2–5). In the case of DODAC/DOPE liposomes (Fig. 2a), dense polymorphic complexes formed when mixed with either plasmid (Fig. 2b) or separately, oligonucleotides (Fig. 2c).

In the case of liposome/plasmid complexes, we observed striated regions of stacked lipid layers consistent with the distinctive 'fingerprint' morphology also described in the CTEM studies of (1,2-dioleoyloxy-3-(trimethylammonium)propane (DOTAP)/DOPE lipoplexes by Gustafsson and Almgren [26] and of 1,2-dimyristoyl-*sn*-glycero-3-phosphocho-

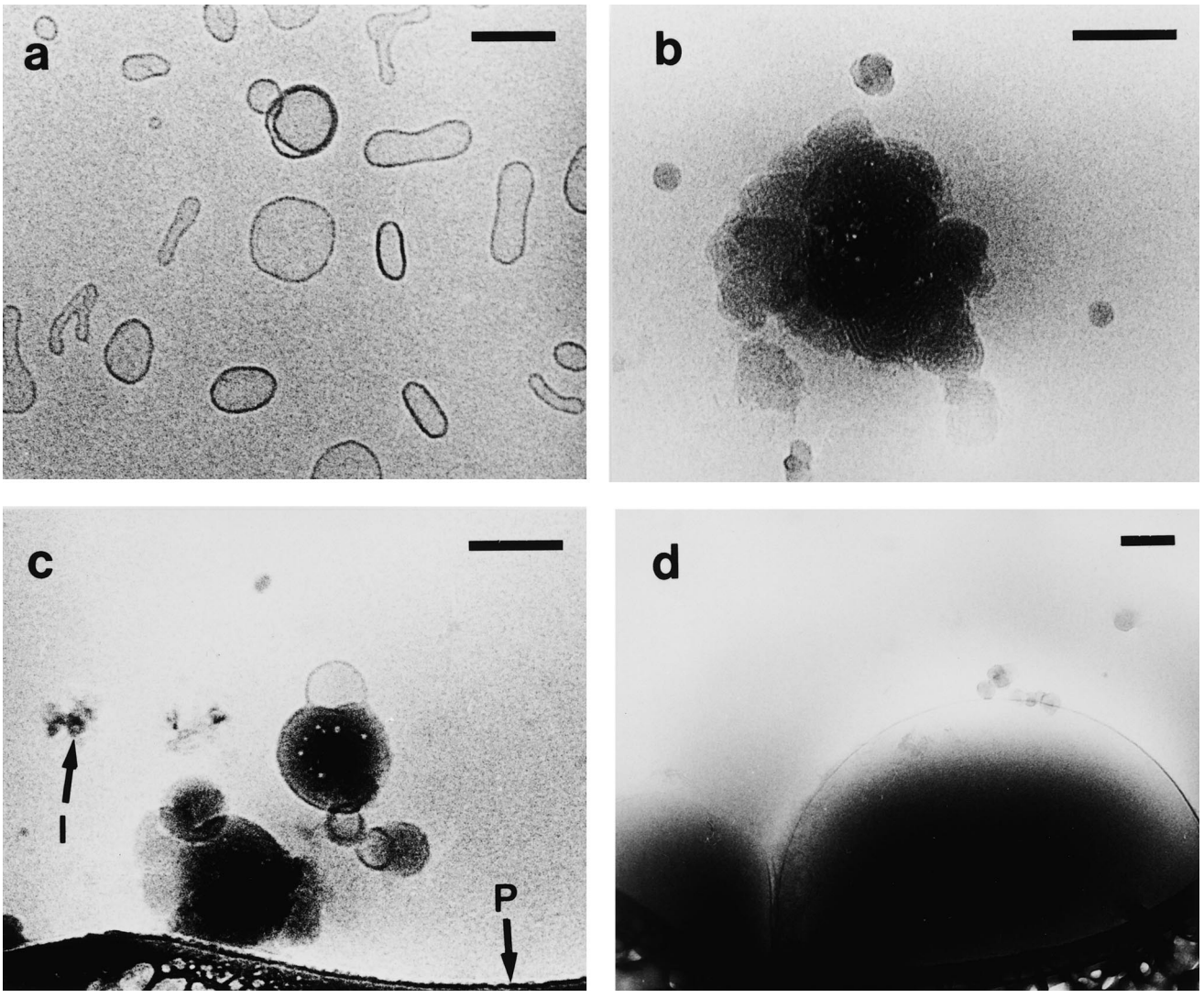

Fig. 2. Cryo-transmission electron micrographs of DODAC/DOPE liposomes (0.5 mM) in lactose. (a) Liposomes alone; (b) with added plasmid DNA (50 μg/ml, charge ratio (+/−) = 1.6); (c) with added oligonucleotides (50 μg/ml, charge ratio = 1.6); (d) with added Na_2HPO_4 (3 mM, charge ratio = 0.17). I, ice crystals; P, edge of polymer film. Scale bar indicates 100 nm.

line (DMPC)/3β-[*N*,*N*′,*N*′-dimethylaminoethane)carbamoyl]cholesterol (DC-Chol) by Battersby et al. [27]. The periodicity of the striations is about 6.5 nm, which is in agreement with the aforementioned studies. This indicates that it is likely that these are lipid bilayers with DNA sandwiched in between the layers. We observed the fingerprint morphology only when using cationic liposomes prepared with DOPE.

Addition of oligonucleotides to DODAC/DOPE liposomes in lactose resulted in the formation of dense complexes of indistinct morphology or closely associated liposomes (Fig. 2c). When 3 mM Na_2HPO_4 was added to the DODAC/DOPE liposomes instead of polynucleotides, very large vesicles were observed (Fig. 2d). This is consistent with the data in Fig. 1C, where an increase in mean diameter was observed in DODAC/DOPE liposomes in lactose to which sodium phosphate was added.

When this same set of anions was added to DODAC/DOPE liposomes in the presence of 150 mM buffered NaCl (HBS), the instability of the bilayer structure of these liposomes was even more evident (Fig. 3). The liposomes alone formed mostly clumps

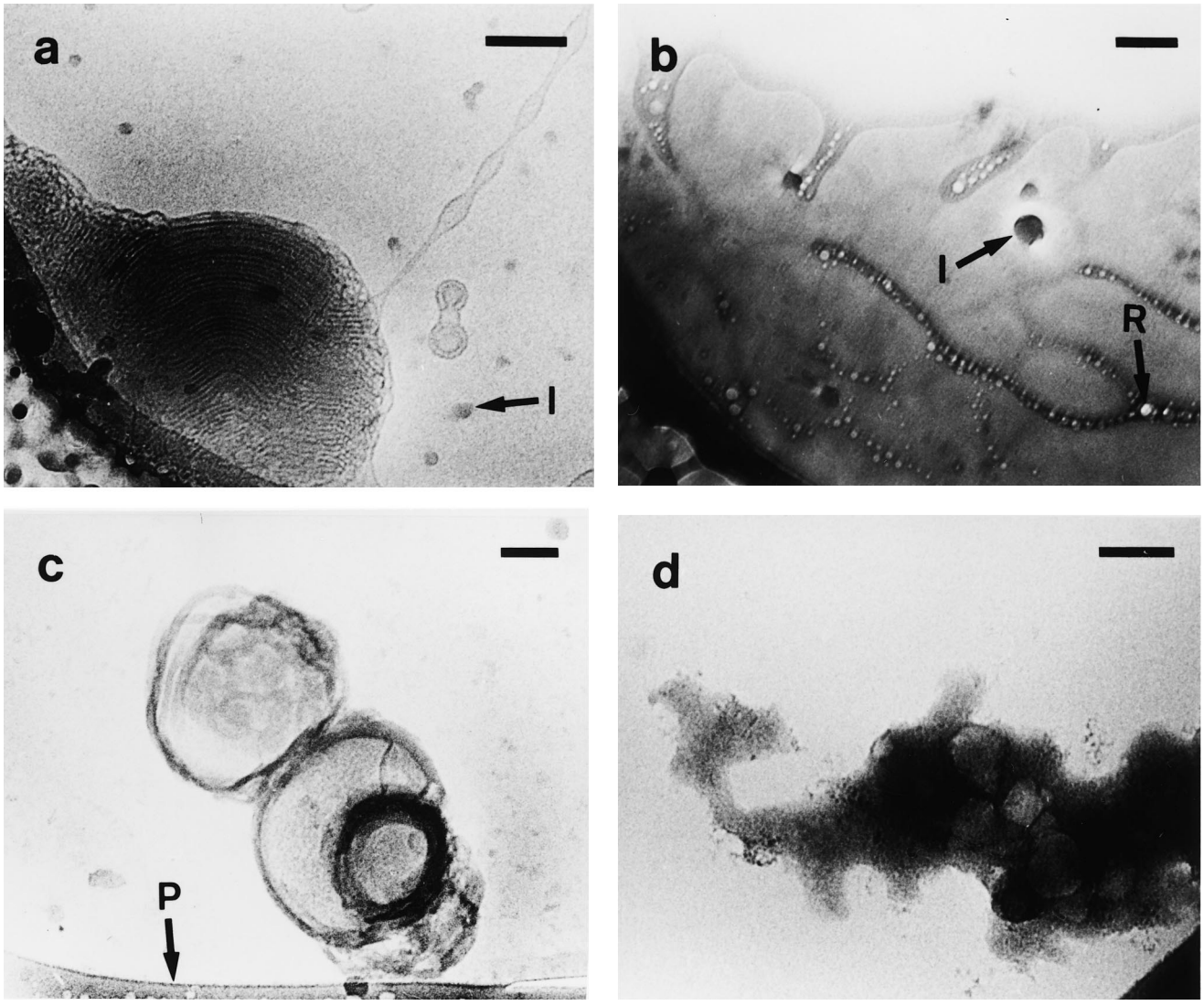

Fig. 3. Cryo-transmission electron micrographs of DODAC/DOPE liposomes prepared initially in lactose then diluted into HBS to a final lipid concentration of 0.5 mM. (a) Liposomes alone; (b) with plasmid DNA added at the same time as HBS diluent (final [DNA] = 50 μg/ml, charge ratio (+/−) = 1.6); (c) with oligonucleotides in HBS added (final [oligo] = 50 μg/ml, charge ratio = 1.6); (d) with Na_2HPO_4 in HBS added (final [Na_2HPO_4] = 3 mM, charge ratio = 0.17). I, ice crystals; R, radiation-damaged area; P, edge of polymer film.

of hexagonal phase lipid (Fig. 3a). When plasmid DNA was added at the same time as HBS (i.e. prior to equilibration of the liposomes in HBS), no vesicles or dense complexes were found, but rather sheets of lipid with what appeared to be strands of radiation-sensitive material (Fig. 3b). The lipoplexes were, in general, quite sensitive to radiation damage from the electron beam of the microscope, which is noted on the electron micrographs as 'R'. These sensitive areas may be DNA-rich regions, based on the observation that radiation sensitivity was not exhibited by any of our samples containing only lipid.

When oligonucleotides instead of plasmid were added to the DODAC/DOPE liposomes at the same time as HBS, the vesicles appeared to be fused and aggregated, with concentric, thickened or multiple lamellae observed in some regions (Fig. 3c). When DNA in either oligonucleotide or plasmid form was present, formation of NaCl-induced H_{II} phase formation (such as in Fig. 3a) was not ob-

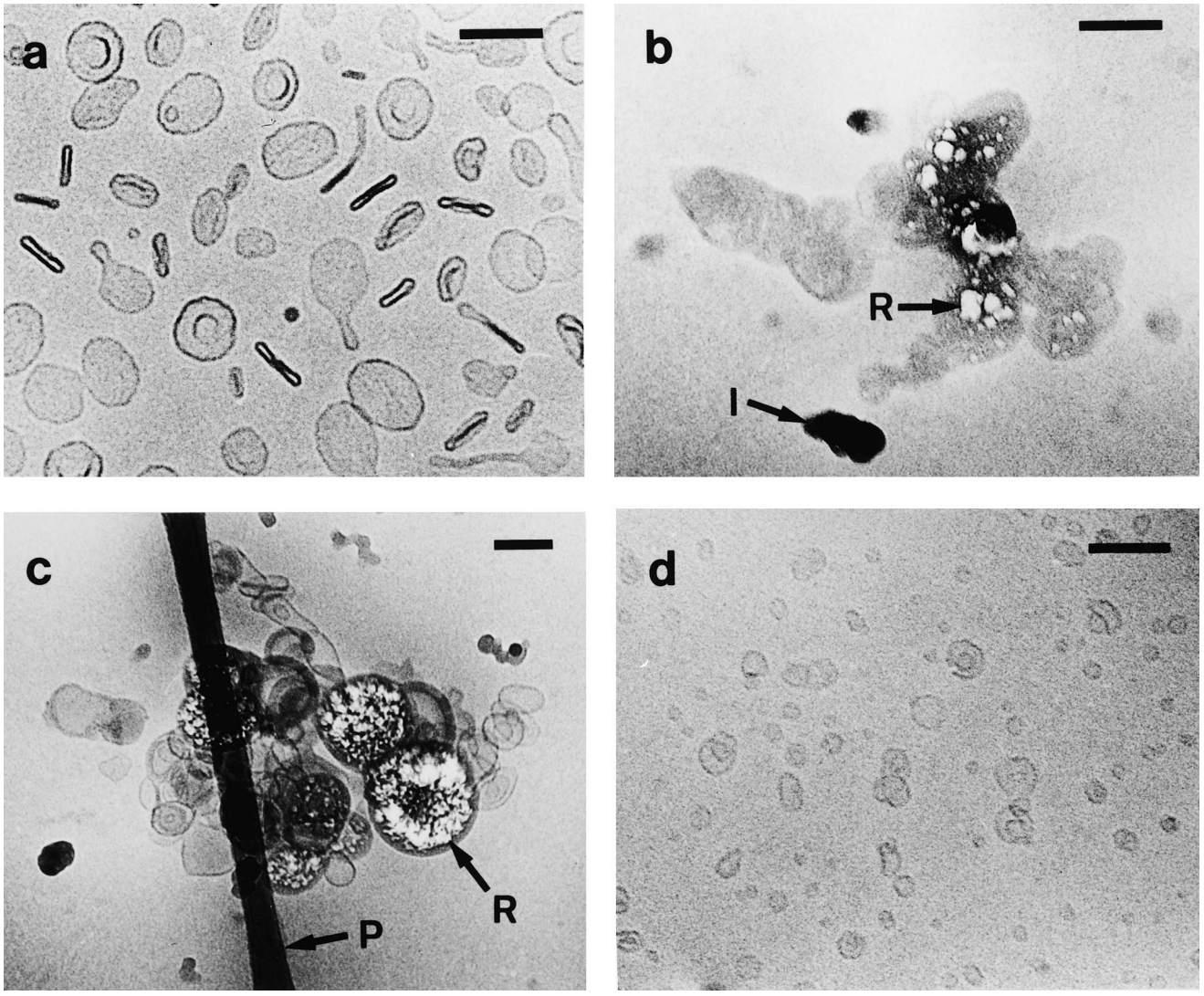

Fig. 4. Cryo-transmission electron micrographs of DODAC/DOPC liposomes (0.5 mM) in lactose. (a) Liposomes alone; (b) with added plasmid DNA (50 μg/ml, charge ratio (+/−) = 1.6); (c) with added oligonucleotides (50 μg/ml, charge ratio = 1.6); (d) with added Na_2HPO_4 (3 mM, charge ratio = 0.17). I, ice crystals; R, radiation-damaged area; P, edge of polymer film.

served. When Na_2HPO_4 was added to the liposomes instead of polynucleotides, it induced formation of highly irregular, nonvesicular structures, but without observable H_{II} phase formation (Fig. 3d).

In the case of DODAC/DOPC liposomes in 300 mM lactose (Fig. 4), somewhat different morphology was noted on CTEM analysis as either plasmid, oligonucleotides or Na_2HPO_4 were added separately. Liposomes alone, prepared by extrusion, were of the expected 100 nm diameter (Fig. 4a). The appearance of slightly flattened or invaginated vesicles, which also appeared in a few DODAC/DOPE liposome samples not shown here, may be due to slight osmotic stress incurred during sample preparation (i.e. partial evaporation of buffer in the brief time between when the sample is blotted on the copper grid and when it is frozen in the liquid ethane). Upon addition of plasmid (Fig. 4b) or oligonucleotides (Fig. 4c), large aggregates of fused liposomes were observed which were particularly sensitive to radiation damage. As expected, no structures consistent with H_{II}-phase lipid were observed in any of the DODAC/DOPC samples. Non-aggregated liposomes were present in the polynucleotide-containing sam-

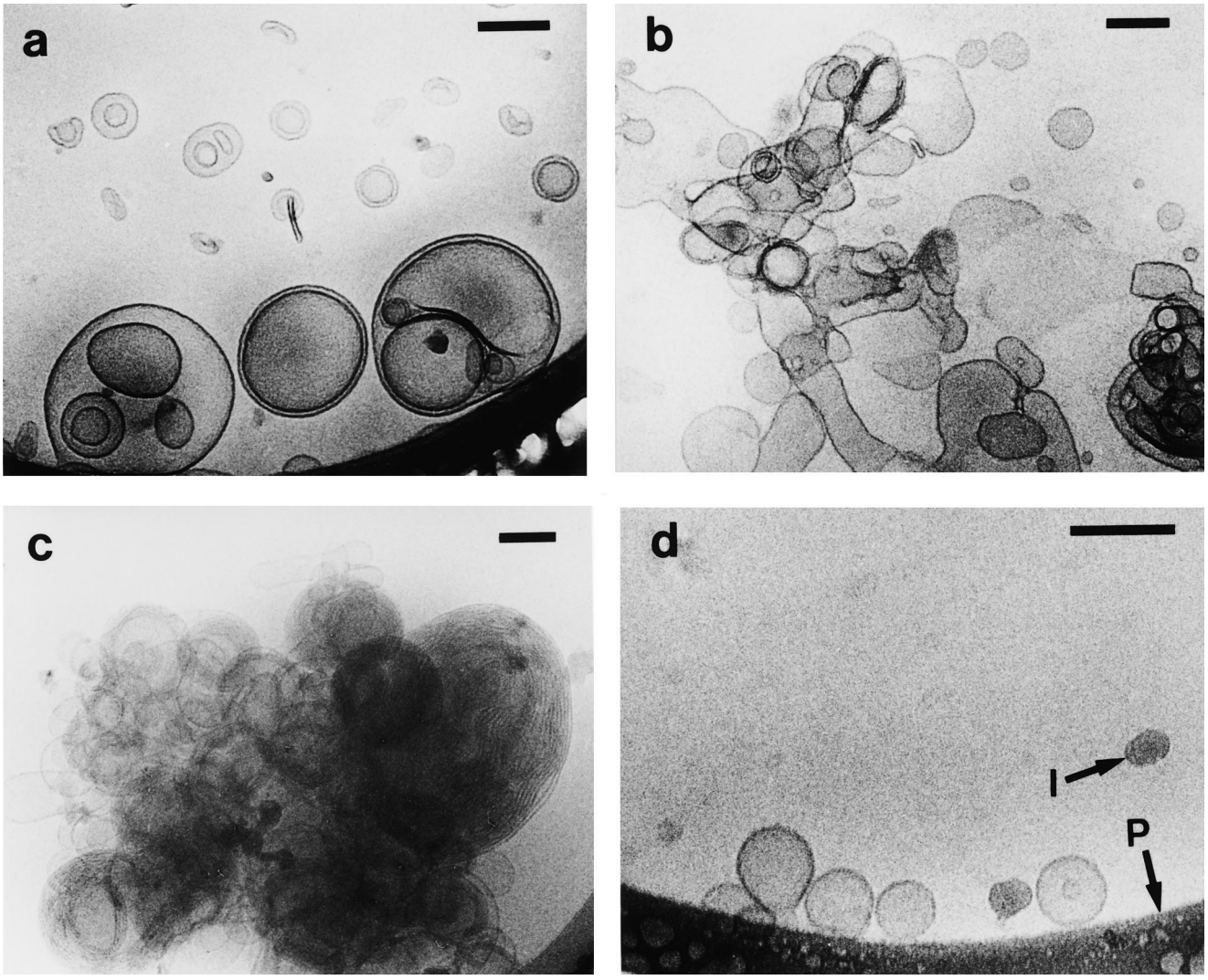

Fig. 5. Cryo-transmission electron micrographs of DODAC/DOPC liposomes prepared initially in lactose then diluted into HBS to a final lipid concentration of 0.5 mM. (a) Liposomes alone; (b) with plasmid DNA added at the same time as HBS diluent (final [DNA] = 50 μg/ml, charge ratio (+/−) = 1.6); (c) with oligonucleotides in HBS added (final [oligo] = 50 μg/ml, charge ratio = 1.6); (d) with Na_2HPO_4 in HBS added (final[Na_2HPO_4] = 3 mM, charge ratio = 0.17). I, ice crystals; P, edge of polymer film.

ples, although this was a minority population. Irregularly shaped particles >700 nm were common for both types of polynucleotides.

Concentric lamellae were observed in complexes formed from DODAC/DOPC liposomes and oligonucleotides, similar to those of DODAC/DOPE liposome/oligonucleotide complexes (compare to Fig. 2c). Interestingly, DODAC/DOPC liposomes in the presence of Na_2HPO_4 (Fig. 4d) appeared slightly smaller than the liposomes in lactose only. These images are consistent with the QELS analysis which showed a slightly smaller vesicle mean diameter under similar conditions.

Fig. 5 illustrates the changes that occurred in DODAC/DOPC liposomes prepared in lactose and diluted in HBS, and after mixing with either plasmid, oligonucleotides or Na_2HPO_4. In salt, DODAC/DOPC liposomes alone had a tendency to form complex plurilamellar structures (Fig. 5a). Addition of plasmid DNA and HBS to the DODAC/DOPC liposomes produced loose clumps of large vesicles and structurally amorphous membrane structures, sug-

gesting that membrane fusion occurred (Fig. 5b). Addition of oligonucleotides in HBS generated very large aggregates that appeared to be denser than those formed upon addition of plasmid DNA. Similarly to DODAC/DOPE liposomes in HBS (Fig. 3c) and DODAC/DOPC liposomes in lactose (Fig. 4c), addition of oligonucleotides resulted in structurally complex regions containing concentric bilayers (Fig. 5c). Na_2HPO_4 had no apparent effect on DODAC/DOPC liposomes in HBS (Fig. 5d).

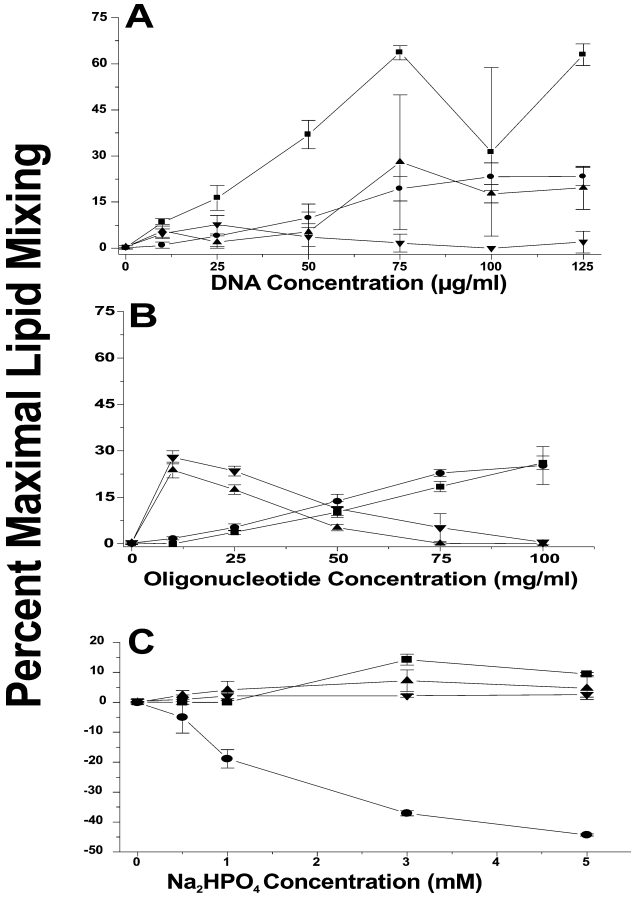

Fig. 6. Lipid mixing of liposomes as a percentage of maximum, measured by resonance energy transfer at increasing concentrations of (A) plasmid; (B) 18-mer oligonucleotides; (C) sodium phosphate. Lipid concentration was 1 mM. ■, DODAC/DOPE in lactose; ●, DODAC/DOPC in lactose; ▲, DODAC/DOPE in HBS; ▼, DODAC/DOPC in HBS. Values represent mean ± S.D., $n = 3$. Corresponding charge ratio (+/−) for A and B: 10 µg/ml = 16, 25 µg/ml = 6.4, 50 µg/ml = 3.2, 100 µg/ml = 1.6, 125 µg/ml = 0.8. Corresponding charge ratio for C: 0.5 mM = 1, 1 mM = 0.5, 3 mM = 0.17, 5 mM = 0.1.

3.3. Lipid mixing studies

To gain a further understanding of these vesicle-vesicle interactions during the process of complex formation, lipid mixing assays were performed using RET. As summarized Fig. 6, the lipid mixing data suggest that cationic liposomes behaved quite differently depending on the polyanion added. Following addition of plasmid DNA to DODAC/DOPE liposomes in lactose (Fig. 6A, squares) there was a progressive increase in lipid mixing observed up to a maximum (%maxLM) of approx. 65% at 75 µg DNA/ml (CR (+/−) 2.4). DODAC/DOPC liposomes under the same conditions (Fig. 6A, circles) showed a similar pattern; however, %maxLM only reached 20% (at CR (+/−) = 1.6). In HBS the %maxLM observed for DODAC/DOPE liposomes was comparable to DODAC/DOPC liposomes in lactose (20–25%). It should be noted that this value was significantly less then the 60% maxLM observed with DODAC/DOPE in the absence of HBS. Less lipid mixing was also evident for the DODAC/DOPC liposomes in HBS ($< 10\%$ maxLM).

Lipid mixing of the cationic liposomes after addition of 18-mer phosphorothioate oligonucleotides is presented in Fig. 6B. When the liposomes were in lactose, %maxLM corresponded to the concentration of oligonucleotides added. In lactose, DODAC/DOPE vesicles (Fig. 6B, squares) the greatest %maxLM (27%) was observed with 100 µg oligonucleotide/ml (CR (+/−) = 1.6). Oligonucleotide-induced lipid mixing was highly similar when comparing results obtained for DODAC/DOPC (Fig. 6B, circles) and DODAC/DOPE liposomes. In HBS, maximal lipid mixing was observed at oligonucleotide concentrations of 10 µg/ml (CR (+/−) = 16) but was less at concentrations > 25 µg/ml (CR (+/−) < 6) for both DODAC/DOPE (Fig. 6B, up triangles) and DODAC/DOPC liposomes (Fig. 6B, down triangles), likely due to precipitate formation in the sample. The lipid mixing assay did not provide meaningful data at high polynucleotide concentrations, illustrating a major limitation of the RET technique for these kinds of samples.

When these studies were conducted substituting Na_2HPO_4 for polynucleotides, very little lipid mixing was observed at up to 5 mM Na_2HPO_4 (Fig. 6C). The maxLM was $< 15\%$ for DODAC/DOPE lipo-

somes in lactose following addition of Na_2HPO_4 to a final concentration of 3–5 mM (CR (+/−) = 0.17–0.1) (Fig. 6C, squares). When DODAC/DOPC liposomes in lactose were mixed with Na_2HPO_4, there was a consistent decrease NBD-PE fluorescence as phosphate concentration increased These data are represented by a decrease in %maxLM from baseline (Fig. 6C, circles). This decrease is consistent with the shrinking of the liposomes observed by CTEM (Fig. 4d).

3.4. Multi-step lipid mixing assay to model in vitro transfection processes

We are primarily interested in these cationic liposomes as DNA transfer vehicles for gene therapy applications. Therefore, the relationship between the physical properties and biological behavior of cationic liposomes complexed to plasmid expression vectors is of primary interest. The remainder of this study, therefore, was focused only on the effects of plasmid DNA on liposome behavior and the effects of the liposomes on reporter gene expression in vitro. We believe that the liposomes continue to undergo major structural changes at each phase of the process of in vitro transfection. To examine this process as a continuum rather than as isolated steps, we designed a multi-step lipid mixing assay, based on the RET technique, to model in vitro transfection.

DODAC/DOPE and DODAC/DOPC lipoplexes were prepared at 10:1 lipid:DNA ratio (nmoles:μg) (CR (+/−) = 1.6), the ratio typically used for optimal in vitro transfection of the B16/BL6 melanoma cell line in our laboratory [20]. Fluorescence changes were monitored over time throughout the processes of lipoplex formation in lactose or in HBS (DNA addition at 200 s), dilution into tissue culture medium with or without 10% FBS (at 400 s) as well as following addition of DOPS/DOPC anionic liposomes (at 800 s). Then to complete the assay, at the end of the time course (at 1000 s) 0.3% Triton X-100 was added to achieve 'maximal lipid mixing'. The fluorescence values are presented as a percentage of this maximal fluorescence upon Triton X-100 addition (Fig. 7), corrected for dilutions and light scattering. There are two major points to be made concerning the results of the lipid mixing assay. First, in the absence of serum (Fig. 7A), both helper lipid composition and buffer composition affected lipid mixing. At each step processes that resulted in further dilution of the fluorescent lipids occurred. Second, serum had a measurable effect on the lipid mixing (Fig. 7B).

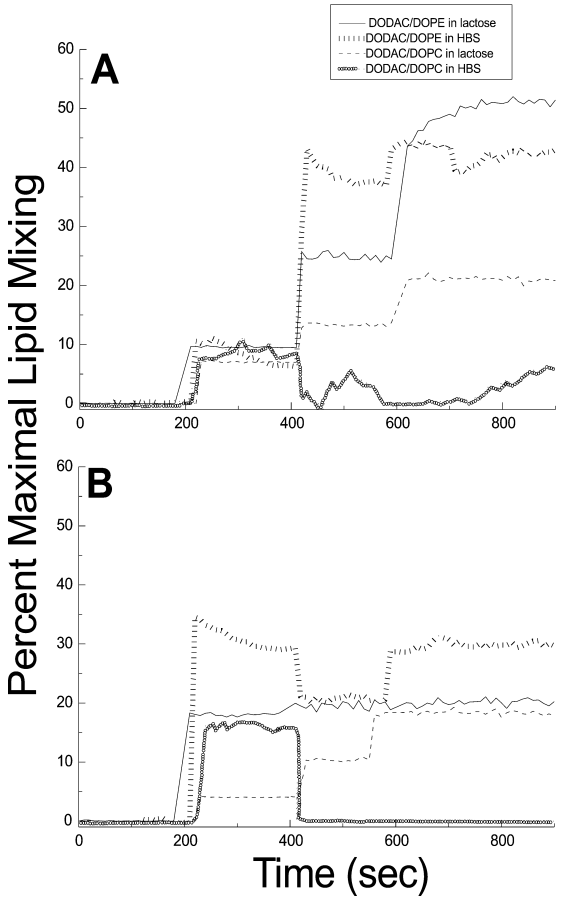

Fig. 7. Representative multi-step lipid mixing assay using RET. Sequentially, at 200 s, DNA was added to liposomes; at 400 s, tissue culture medium was added; at 600 s, anionic liposomes (DOPS/DOPE) were added. (A) Assay performed using serum-free medium; (B) medium containing 10% FBS was used. See text for explanation of calculation of percent maximal lipid mixing.

In the absence of serum (Fig. 7A) increases in %maxLM were observed in step increments for both DODAC/DOPE and DODAC/DOPC lipoplexes. All samples exhibited approx. 10% maxLM at the point of DNA addition (200 s). However, there was a disparity between samples prepared with different lipid compositions or buffers when

the tissue culture medium was added (at 400 s). DODAC/DOPC showed little or no increase in lipid mixing, but DODAC/DOPE lipoplexes reached approx. 25% when prepared in lactose and 38% when prepared in HBS. Addition of anionic liposomes (at 600 s) induced further lipid mixing except in the case of DODAC/DOPC lipoplexes prepared in HBS. Interestingly, the largest change in %maxLM at this step (reaching a maximum of 55%) occurred in DODAC/DOPE lipoplexes prepared in lactose. At all steps, DOPC-containing lipoplexes demonstrated less ability to undergo lipid mixing than those containing DOPE.

In Fig. 7B, sample conditions were the same as in Fig. 7A up to 400 s. However, when the tissue culture medium introduced at 400 s contained 10% serum (Fig. 7B), little or no lipid mixing occurred, except for DODAC/DOPC lipoplexes prepared in lactose, which went from approx. 5% to 10% maxLM at this step. The initially higher level of lipid mixing observed upon addition of DNA to the liposomes (at 200 s) in Fig. 7B compared to Fig. 7A reflects the variability inherent in lipoplex formation. It should be noted that the trend of DOPE-containing lipoplexes demonstrating greater lipid mixing ability than DOPC-containing complexes was highly consistent, as was the effect of serum. The subsequent addition of anionic membranes (at 600 s) to the lipoplexes diluted into serum-containing media showed that complexes formed were not capable of undergoing reactions at that point that would cause additional lipid mixing. DODAC/DOPC lipoplexes prepared in lactose were the exception, going from 10% to 20% maxLM at this step. For DODAC/DOPC lipoplexes prepared in HBS, lipid mixing was eliminated after addition of medium with or without serum (Fig. 7A,B).

To summarize the results of the multi-step lipid mixing assay, we found that while lipid mixing was quite similar between liposomes of differing lipid compositions or buffers at the point of DNA addition, considerable differences arose at the point of addition of tissue culture medium. DOPE-containing complexes had a greater lipid mixing ability than DOPC-containing complexes, especially at the point of adding anionic liposomes (model cell membranes). Serum in the media reduced both overall lipid mixing and the differences due to lipid composition.

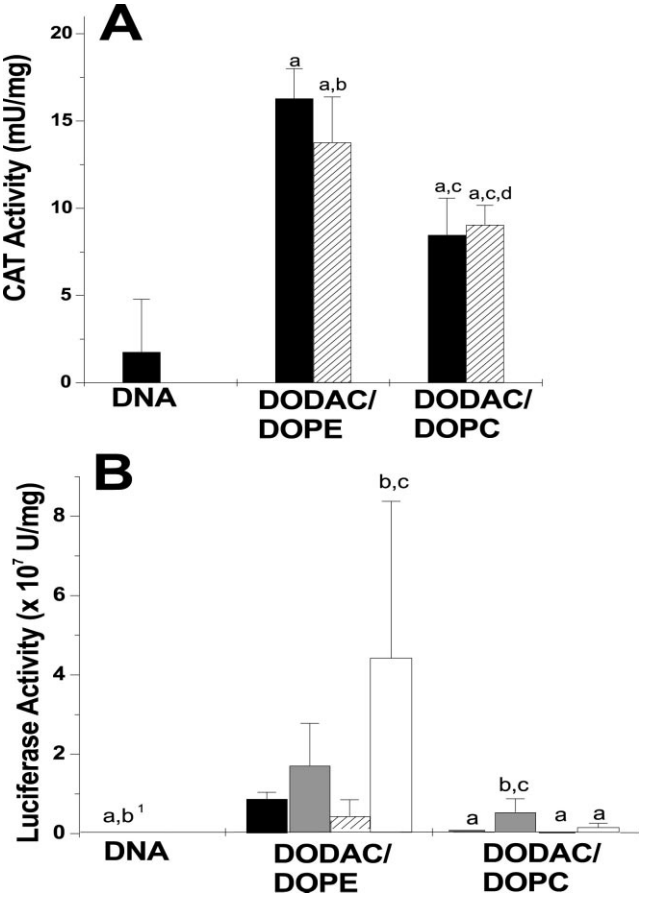

Fig. 8. (A) Expression of chloramphenicol acetyltransferase gene transfected into B16/BL6 murine melanoma cells using cationic liposome/DNA complexes prepared in either 300 mM lactose or 150 mM buffered NaCl (HBS). Black bars, samples prepared in lactose; hatched bars, samples prepared in HBS. Data represent the mean ± S.D., $n = 6$. [a]Significantly different from free DNA. [b]Significantly different from DODAC/DOPC in lactose. [c]Significantly different from DODAC/DOPE in lactose. [d]Significantly different from DODAC/DOPE in HBS ($P < 0.05$). (B) Expression of luciferase gene transfected into B16/BL6 murine melanoma cells in medium with or without 10% FBS using cationic liposome/DNA complexes prepared in either 300 mM lactose or HBS. Bars (diluent groups): black, in lactose, with serum; grey, in lactose, no serum; hatched, in HBS, with serum; open, in HBS, no serum. Data represent mean ± S.D., $n = 6$. [a]Significantly different from DODAC/DOPE for this diluent group; [b]significantly different from lactose with serum for this lipid composition; [c]significantly different from HBS with serum for this lipid composition ($P < 0.05$). [1]Significance refers to all four diluent groups for DNA alone.

3.5. In vitro transfection

To determine if there was a correlation between lipid mixing and successful transfection, we com-

pared transfection of B16/BL6 mouse melanoma cells in vitro using DODAC/DOPE and DODAC/DOPC lipoplexes, prepared in either lactose or in HBS (CR (+/−) = 1.6). The first experiment used a plasmid (pInexCATv2.0) containing a reporter gene encoding chloramphenicol acetyltransferase (CAT). Transfection with DOPE-lipoplexes prepared in lactose resulted in 16 ± 1.7 mU CAT activity/μg protein (mean ± S.D., $n = 6$) and DOPC-lipoplexes 8.5 ± 2.1 mU/μg. Lipoplexes prepared in HBS resulted in 13.8 ± 2.6 and 9.1 ± 1.1 mU/μg for DOPE- and DOPC-lipoplexes, respectively (Fig. 8A). In the second experiment, samples were prepared in the same way, as described in Section 2, but using a plasmid encoding the luciferase reporter gene (pInexL018) that had the same vector backbone and promoter as the CAT-encoding plasmid. Lipoplexes were made in either 300 mM lactose or HBS. Transfection was done for 4 h in the presence or absence of serum. Luciferase assay results, as shown in Fig. 8B, indicate there was a significant difference between cells transfected with DODAC/DOPE vs. DODAC/DOPC lipoplexes except when they were prepared in lactose and diluted into medium containing serum. All groups transfected with DODAC/DOPC lipoplexes showed a significantly lower luciferase activity than groups transfected with DODAC/DOPE lipoplexes. The luciferase activity of groups receiving DODAC/DOPC lipoplexes was not significantly different than those receiving plasmid DNA alone, except for the greater activity when they were prepared in lactose and diluted into serum-free medium. Serum reduced transfection ability of all lipoplexes. As in the first experiment, salt did not have a major effect on transfection.

In comparing the results of the multi-step lipid mixing assay (Fig. 7) and the transfection studies (Fig. 8) there is a trend toward improved transfection when using lipoplexes that undergo lipid mixing following addition of anionic membranes. However, the improvements are typically no more than 2–5-fold and may depend on the plasmid vector being used. In comparing Fig. 7A and B, for example, DOPC-lipoplexes promote transfection nearly as well as DOPE-lipoplexes when the pInexCAT plasmid was used, but not when the pInexL018 luciferase plasmid was used.

4. Discussion

The purpose of this study was to determine how certain factors (helper lipid composition, ionic environment, charge density, lipid:DNA ratio) affect the structure of lipoplexes and subsequently their in vitro transfection ability. Structure-activity relationships have been difficult to sort out for lipid-based DNA carriers due to the large number of variables involved and the inherent instability of the lipoplexes. We have demonstrated that a variety of structures form as cationic liposomes are mixed with polynucleotides, depending on the conditions, and that they also differ in their fusogenic capacity. The differences in transfection ability, however, are surprisingly small (2–3-fold) despite this heterogeneity. This implies that there may be either enough similarity in the microstructure to render the larger-scale morphological differences less important. Another possibility, not addressed here, is that plasmid-dependent factors were the most important.

We began this investigation by studying the well-known role of salt in promoting increases in particle size of the lipoplexes composed of either DODAC/DOPE or DODAC/DOPC bound to plasmid DNA. Table 1 and Fig. 1 show the extent of salt-induced aggregation measured by QELS, which is marked for DOPE-lipoplexes. This was paralleled by the lipid mixing that occurred during lipoplex formation shown in Fig. 6. Larger structures with an increased degree of lipid-lipid interactions formed as the lipid:DNA ratio approached a net charge-neutral ratio, suggesting that electrostatic interactions play the predominant role in complex formation. Net charge of the added component was also important, however. We compared by CTEM the macrostructures of liposomes interacting with double-stranded plasmid DNA (Figs. 2–5, panel b), 18-mer phosphorothioate oligonucleotides (Figs. 2–5, panel c) or sodium phosphate (Figs. 2–5, panel d). Just as the extent of disruption of liposome structure decreased (as visualized on CTEM) as the molecular weight or net charge of the added component decreases, so the extent of lipid mixing decreased (compare Fig. 6A,B,C).

Particle size growth is rapid initially (within seconds or minutes) followed by a much slower phase of

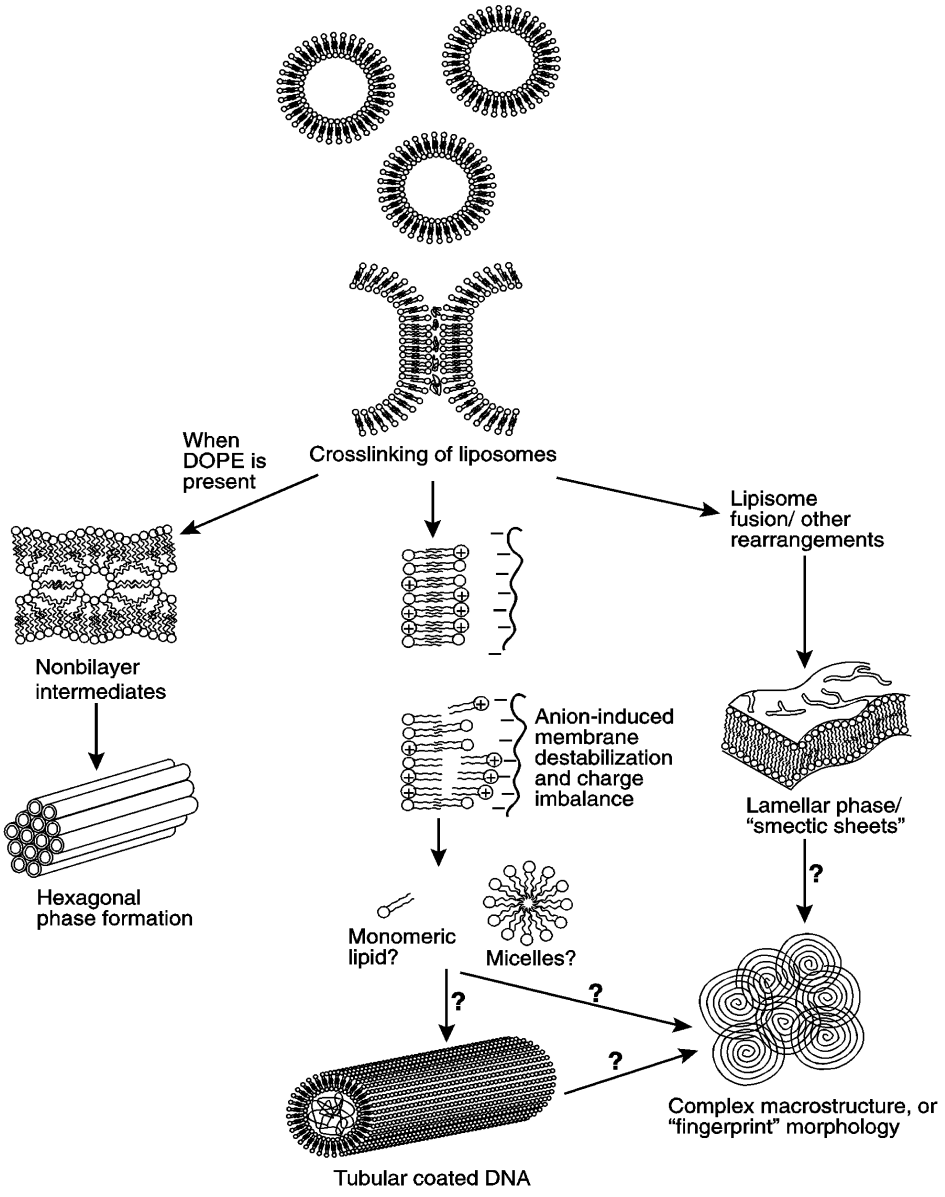

Fig. 9. Theoretical schematic of how cationic liposomes are destabilized by polynucleotides, resulting in a variety of structures. The intermediates are presently unknown, and the final structure of the lipoplexes appears to be highly dependent on lipid composition and reaction conditions.

growth after about 30–60 min, over hours or days [28], with the latter phase presumably due to the reduction in the collision rate as the total number of particles in suspension decreases. It should be noted, however, that in this study a limited timeframe was used, at 30–60 min after lipoplex preparation the assessments (particle size, lipid mixing, turbidity, CTEM) were made. This timescale is also relevant to standard in vitro transfection protocols, in which the lipoplexes are applied to cells approx. 30 min after preparation.

Cationic liposomes and lipoplexes clearly undergo significant changes in morphology and structure, changes that are partly a consequence of polyanion induced vesicle aggregation (close contact) and consequently membrane-membrane interactions that occur when two membranes come into close contact with each other. When assigning roles to the lipid

components, therefore, it is important to consider how the electrostatic interactions are affecting aggregation reactions as well as how these interactions are affected by the presence of salts and/or serum proteins. Furthermore, perturbation of membrane structure must be examined since it may result in phase separation of lipid components or formation of nonbilayer structures (e.g. micelles or H_{II}-phase lipid) and membrane fusion.

Several models have been proposed for the molecular structure of complexes formed following addition of DNA to cationic liposomes. These include smectic phase lipid and DNA sheets [28,29], rods of DNA wrapped in lipid [30], or a honeycomb-like structure [31,32]. The consensus at this point seems to be that flat lamellar structures are energetically most favorable. However, the CTEM data suggest that DODAC/DOPE liposomes can assume structures that are dramatically different depending on whether salts are present (compare Fig. 2b and 3b). The ability of DOPE to form nonbilayer structures makes these complexes highly unstable in salt-containing solutions, which was demonstrated in this study. It can be speculated that polyanion binding may cause disruption of the membrane leading to formation of monomeric lipid or micelles that are capable of binding to DNA, eventually leading to formation of lipoplexes (Fig. 9). Janoff and coworkers have shown evidence that in the transition from lamellar to hexagonal phase lipid for DOPE-containing lipid mixtures, that monomeric lipids may be released [33]. It is possible then that monomeric lipid or micelles might form in an intermediate step during such transitions, a subject of ongoing investigation. In that case, this could be the basis for the nonliposomal polymorphic structures, such as the membrane sheets, tubular lipid-coated DNA or the fingerprint-like complexes (Fig. 9), that form upon interaction of DOPE-containing cationic liposomes with DNA. DOPE is stabilized into a bilayer configuration, however, by DODAC. The interaction between cationic DODAC, DNA and zwitterionic DOPE likely influences both the availability of cationic charge for DNA binding and the phase behavior of DOPE. Both of these parameters will be influenced by the ionic strength of the solution.

Analysis of the electrostatic interactions between cationic liposomes and polynucleic acid and how they determine the lipoplex structure has recently been addressed [28,30,34,35]. The complexation of lipids and DNA fits well into existing polyelectrolyte theory, specifically Manning condensation [36,37], which is marked by the dependence of counterion condensation on linear charge density of the polymer (in this case, polynucleotides vs. phosphate anions). The fusion of anionic vesicles cross-linked by ions of opposite charge, particularly monovalent and divalent cations, has been addressed extensively [38–40].

Divalent cations cause very rapid fusion of anionic vesicles, and the percent maximal lipid mixing achieved depends on the cation/anion ratio, salt concentration, temperature, etc. The effects of multivalent synthetic cations on anionic liposome behavior have also been addressed. Oku and coworkers [17] demonstrated that polymeric cations induce maximal anionic liposome fusion (by contents release, lipid mixing assays and turbidity changes in PS/PC liposomes) when the charge ratio is approximately unity. Divalent cations such as Ca^{2+} and Mg^{2+} require much higher concentrations relative to the anionic vesicles to achieve the same degree of liposome fusion as charged polymers. This is analogous to the lipid mixing data presented here, in which sodium phosphate induced a much lower %maxLM in the cationic liposomes compared to polynucleotides, even when the phosphate was in excess. Lipid mixing was also less for most samples in the presence of salt. Thus, charge density is no less important for cationic liposome fusion by anions. Polynucleotides bind to cationic liposomes rapidly and efficiently [28]; however, we have shown by several methods that the degrees of structural change induced by double-stranded plasmid, single-stranded oligonucleotides and sodium phosphate are different. When Na_2HPO_4 was used as the anion instead of polynucleotides in this investigation, formation of dense complexes was much less pronounced. Thus it is becoming clear that the reactions between cationic liposomes and DNA can be understood based on previous work with anionic liposomes and cations, bearing in mind that both the spacing of the charges and the length of the opposing charged molecules are critical. This may explain why the cationic lipid headgroup charge is important in complex formation [41] and in transfection with lipoplexes [42].

Controlling the fusion reaction to generate defined

structures from plasmid and pre-formed liposomes, however, may prove to be considerably more problematic. Presently, the only aspect of the liposome fusion reaction that can be readily controlled is the rate. It is unlikely that lipoplexes made from pre-formed DOPE-containing liposomes and plasmid will ever be developed beyond the present stage of heterogeneous, unstable complexes with variable transfection efficiency. Alternative methods of forming lipid-based DNA carriers in a more controlled manner are currently under development in our laboratory [43].

Currently there is much discussion regarding how the 'fusogenic potential' of lipoplexes is important for transfection [19,44–46]. The fundamental assumption is that a certain minimal degree of fusogenic character is mandatory for destabilization/interaction with cellular membranes so that the lipoplex can enter the cell, be released from endosomes or lysosomes, and for the lipids of the complex to release the associated DNA. Release of the DNA has been shown to be necessary for nuclear uptake and subsequent transcription of the exogenous DNA [47]. An important factor in successful transfection is the interaction between lipoplexes and cellular membranes, which is thought to require highly fusogenic particles. However, aggregation of cationic liposomes (see Fig. 1) does not necessarily mean that complete vesicle fusion has occurred, as indicated in the CTEM images of Figs. 2–5 and the lipid mixing assays summarized in Fig. 6. Likewise, lipid mixing during lipoplex formation in simple buffer solution does not necessarily indicate the fusogenic potential of the lipoplexes when they reach the target cell membrane. In vitro transfection is a multi-step process, involving: (1) interactions with anions in the buffer solution used to prepare or dilute the liposomes; (2) formation of the complex with plasmid; (3) dilution into standard tissue culture medium which may or may not contain serum; and (4) interactions with cellular membranes as the complexes reach the cells to be transfected. Each of these steps will necessarily affect the outcome of subsequent interactions, and examining any one step in isolation gives an incomplete picture. The major advantage of the multi-step lipid mixing assay used here is the ability to monitor the sequential changes in lipid mixing that occur in the lipoplexes during these steps. The addition of anionic liposomes is designed to model contact with target cell plasma membranes. It is also similar to previous studies in which destabilization of lipoplexes was induced by anionic vesicles [10,11].

In turning the focus to the effect of lipid composition and ionic environment on lipid mixing of the liposomes and how that relates to in vitro transfection, it was observed in the multi-step lipid mixing assay that there was not a large difference between DODAC/DOPE and DODAC/DOPC lipoplexes in lipid mixing ability in the presence of serum. The addition of anionic membranes (at 600 s) to the lipoplexes diluted into serum-containing media showed that complexes formed were not capable of undergoing reactions that would cause additional lipid mixing. These data support the idea that serum is inhibitory for anionic membrane-induced destabilization of the DODAC/DOPE lipoplexes, a result that is consistent with that reported elsewhere [11]. This was also borne out in both transfection experiments (Fig. 8A,B) done in the presence of 10% FBS, where the difference between treatment groups was small. In the absence of serum there was a clear difference in the ability of PE- vs. PC-containing lipoplexes to undergo lipid mixing at the point of addition of anionic liposomes. Likewise, in the luciferase transfection experiment performed in the absence of serum, lipid composition significantly effected expression level (Fig. 8B, gray and open bars).

The presence of HBS during lipoplex formation influenced the lipid mixing ability of the lipoplexes in subsequent steps, generally decreasing it. DODAC/DOPC lipoplexes did not maintain lipid mixing ability as well as DOPE-containing lipoplexes. No significant difference in transfection was observed between lipid compositions in the first transfection experiment, however (Fig. 8A). In the transfection experiment using the luciferase reporter gene, the PE-containing lipoplexes maintained their ability to transfect cells even when prepared in salt, while DOPC-containing lipoplexes did not, showing a much better correlation to the results of the multi-step lipid mixing assay. It may be suggested therefore that compositions or conditions that favor lipid mixing at each step have a tendency to exhibit improved transfection.

What is also remarkable, however, is that the in-

ter-group differences were more pronounced when the luciferase plasmid was used rather than the CAT plasmid for transfecting the same cell line. The two plasmid preparations were purified by the same method. It is possible, however, that: (1) sequence level differences in the two plasmids affected gene expression in this cell line; (2) tertiary structure of the two plasmids was different and this had an impact on lipoplex structure – for example, the relative proportions of supercoiled vs. relaxed plasmid in the two plasmid preparations were not determined; and (3) intracellular processing of the lipoplexes (degradation, DNA release, DNA nuclear transport) differs due to plasmid factors that are influenced by lipoplex lipid composition or environment. These issues, although beyond the scope of this study, are critically important but are just beginning to be addressed in the field of DNA delivery and gene therapy.

In this report we have argued that conclusions about the roles of lipids used in the preparation of cationic liposomes, the structures adopted during lipoplex formation or during subsequent reactions are tenuous when these processes are studied in isolation. For this reason the studies described examined changes in aggregation (light scattering techniques and CTEM) and membrane structure (lipid mixing RET assay and CTEM) under conditions that mimic those used when transfecting cells in vitro. It was demonstrated that changes in particle size and membrane structure can occur at each step. It was concluded that the ability of the resulting membrane structures to undergo lipid mixing reactions influences vesicle aggregation and membrane structure. It is difficult, however, to define how these changes influence transfection. The similarity between DOPE- and DOPC-lipoplexes in the CAT transfection experiment brings up the possibility that there may be some substructure present in varying degrees in all the preparations that is mediating the gene transfer.

The CTEM studies summarized here (see Fig. 3) demonstrated that there is a great heterogeneity and subtlety of morphology that cannot be expressed by simply reporting mean diameters of the lipoplexes. The concepts of local surface charge, lipid domain formation, lipid packing constraints, DNA accessibility, and the dynamic flexibility of the supramolecular complex are likely to be just as critical as the overall charge ratio and the size of the aggregate.

Acknowledgements

Funding was provided by the Medical Research Council of Canada (MRC# UI-12761) and Inex Pharmaceuticals, Inc. (M.B.B.). Additional support was provided by the Swedish Research Council for Engineering Sciences and the Swedish Foundation for Strategical Research (K.E.). E.W. was supported by a fellowship from the Science Council of British Columbia.

References

[1] P.L. Felgner, T.R. Gadek, M. Holm, R. Roman, H.W. Chan, M. Wenz, J.P. Northrop, G.M. Ringold, M. Danielsen, Proc. Natl. Acad. Sci. USA 84 (1987) 7413–7417.
[2] A.R. Thierry, P. Rabinovich, B. Peng, L.C. Mahan, J.L. Bryant, R. Gallo, Gene Ther. 4 (1997) 226–237.
[3] D.S. Friend, D. Papahajopoulos, R.J. Debs, Biochim. Biophys. Acta 1235 (1996) 4622–4628.
[4] C.F. Bennett, D. Mirejovsky, R.M. Crooke, Y.J. Tsai, J. Felgner, C.N. Sridhar, C.J. Wheeler, P.L. Felgner, J. Drug Targeting 5 (1998) 149–162.
[5] R.P. Balasubramaniam, M.J. Bennett, A.M. Aberle, J.G. Malone, M.H. Nantz, R.W. Malone, Gene Ther. 3 (1996) 163–172.
[6] J.H. Felgner, R. Kumar, C.N. Sridhar, C.J. Wheeler, Y.J. Tsai, R. Border, P. Ramsey, M. Martin, P.L. Felgner, J. Biol. Chem. 269 (1994) 2550–2561.
[7] D.P. Siegel, Biophys. J. 49 (1986) 1171–1183.
[8] H. Farhood, N. Serbina, L. Huang, Biochim. Biophys. Acta 1235 (2) (1995) 289–295.
[9] S.W. Hui, M. Langner, Y.-L. Zhao, P. Ross, E. Hurley, K. Chan, Biophys. J. 71 (1996) 590–599.
[10] Y. Xu, F.C. Szoka, Biochemistry 35 (1996) 616–623.
[11] P. Harvie, F.M.P. Wong, M.B. Bally, Biophys. J. 75 (1998) 1040–1051.
[12] D.K. Struck, D. Hoekstra, R.E. Pagano, Biochemistry 20 (1981) 4093–4099.
[13] M. Almgren, K. Edwards, J. Gustafsson, Curr. Opin. Colloid Interface Sci. 1 (1996) 270–278.
[14] A.D. Bangham, D. Papahadjopoulos, Biochim. Biophys. Acta 126 (1966) 181–184.
[15] D. Papahadjopoulos, G. Poste, Biophys. J. 15 (1975) 945–948.
[16] P.S. Ash, A.S. Bunce, C.R. Dawson, R.C. Hider, Biochim. Biophys. Acta 510 (1978) 216–229.

[17] N. Oku, N. Yamaguchi, S. Shibamoto, F. Ito, M. Nango, J. Biochem. 100 (1986) 935–944.
[18] L. Stamatatos, R. Leventis, M.J. Zuckermann, J.R. Silvius, Biochemistry 27 (1988) 3917–3925.
[19] A.L. Bailey, P.R. Cullis, Biochemistry 36 (1997) 1628–1634.
[20] D.L. Reimer, S. Kong, M.B. Bally, J. Biol. Chem. 272 (1997) 19480–19487.
[21] M.J. Hope, M.B. Bally, G. Webb, P.R. Cullis, Biochim. Biophys. Acta 812 (1985) 55–65.
[22] J.R. Bellare, H.T. Davis, L.E. Scriven, Y. Talmon, J. Electron Microsc. Tech. 10 (1988) 87–111.
[23] J. Dubochet, M. Adrian, J.J. Chang, J.C. Homo, J. Lapault, A.W. McDowall, P. Schultz, Q. Rev. Biophys. 21 (1988) 129–228.
[24] M.J. Sleigh, Anal. Biochem. 156 (1986) 251–256.
[25] B. Seed, J.-Y. Sheen, Gene 67 (1988) 271–277.
[26] J. Gustafsson, G. Arvidson, G. Karlsson, M. Almgren, Biochim. Biophys. Acta 1235 (1995) 305–312.
[27] B.J. Batttersby, R. Grimm, S. Huebner, G. Cevc, Biochim. Biophys. Acta 1372 (1998) 379–383.
[28] N.J. Zuidam, Y. Barenholz, Biochim. Biophys. Acta 1368 (1998) 115–128.
[29] I. Koltover, T. Salditt, J.O. Rädler, C.R. Safinya, Science 281 (1998) 78–81.
[30] J.O. Rädler, I. Koltover, T. Salditt, C.R. Safinya, Science 275 (1997) 810–814.
[31] B. Sternberg, F.L. Sorgi, L. Huang, FEBS Lett. 356 (1994) 361–366.
[32] S. May, A. Ben-Shaul, Biophys. J. 73 (1997) 2427–2440.
[33] W.R. Perkins, R.B. Dause, R.A. Parente, S.R. Minchey, K.C. Neuman, S.M. Gruner, T.F. Taraschi, A.S. Janoff, Science 273 (1996) 330–332.
[34] N. Dan, Biochim. Biophys. Acta 1369 (1998) 34–38.
[35] R. Bruinsma, J. Mashl, Europhys. Lett. 41 (1998) 165–170.
[36] G.S. Manning, Q. Rev. Biophys. 11 (1978) 179–246.
[37] G.S. Manning, U. Mohanty, Physica A 247 (1997) 196–204.
[38] N. Düzgünes, J. Wilschut, R. Fraley, D. Papahadjopoulos, Biochim. Biophys. Acta 64 (1981) 182–195.
[39] N. Düzgünes, S. Nir, J. Wilschut, J. Bentz, C. Newton, A. Portis, D. Papahadjopoulos, J. Membr. Biol. 59 (1981) 115–125.
[40] J. Wilschut, J. Scholma, M. Bental, D. Hoekstra, S. Nir, Biochim. Biophys. Acta 821 (1985) 45–55.
[41] F.M.P. Wong, D.L. Reimer, M.B. Bally, Biochemistry 35 (1996) 5756–5763.
[42] G. Byk, C. Dubertret, V. Escriou, M. Frederic, G. Jaslin, R. Rangara, B. Pitard, J. Crouzet, P. Wils, B. Schwartz, D. Sherman, J. Med. Chem. 41 (1998) 224–235.
[43] Y.-P. Zhang, D.L. Reimer, G. Zhang, P.H. Lee, M.B. Bally, Pharm. Res. 14 (1997) 190–196.
[44] T. Stegmann, J.-Y. Legendre, Biochim. Biophys. Acta 1325 (1987) 71–79.
[45] K.W.C. Mok, P.R. Cullis, Biophys. J. 73 (1997) 2534–2545.
[46] L.H. Li, S.W. Hui, Biochim. Biophys. Acta 1323 (1997) 105–116.
[47] J. Zabner, A.J. Fasbender, T. Moninger, K.A. Poellinger, M.J. Welsh, J. Biol. Chem. 270 (1995) 18997–19007.