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Complexes between cationic liposomes and DNA visualized by cryo-TEM

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Abstract

The association structures formed by cationic liposomes and DNA-plasmids have been successfully employed as gene carriers in transfection assays. In the present study such complexes was studied by cryo-TEM (cryo-transmission electron microscopy). Cationic liposomes made up by DOPE (dioleoylphosphatidylethanolamine) and various amounts of three different cationic surfactants were investigated. The cryo-TEM analysis suggests that an excess of lipid in terms of charge, leads to entrapment of the DNA molecules between the lamellas in clusters of aggregated multilamellar structures. With increasing amounts of DNA free or loosely bound plasmids were found in the vicinity of the complexes. The importance of the choice of surfactant, as reported from many transfection assays, was not reflected in changes of the type of DNA-vesicle association. A tendency towards polymorphism of the lipid mixtures is reported and its possible implications are discussed.

Keywords: Liposome-DNA complex; Cationic liposome; DNA; Cryo-transmission electron microscopy; Electron microscopy

1. Introduction

The development of gene therapy and genetic engineering is depending on reliable and efficient systems for the introduction of DNA into the target cells. The possible use of cationic liposomes in transfection has been the subject of numerous studies lately [1–17] and recently promising results from a human clinical study were published where the technique was employed in vivo [15]. This simple and mild but still effective method is based on a complex formation by DNA-plasmids and vesicles composed of synthetic cationic surfactants and a phospholipid. The cationic surfactants give the vesicle a positively charged surface which promotes strong interactions between vesicles and plasmids. The formed complexes are supposed to carry a positive net charge which will facilitate their approach to the cell surface.

As evident by the number of various transfection techniques [17] the cell is capable of receiving DNA through a range of different mechanisms. In the case of cationic liposomes both fusion and endocytosis have been proposed as mechanisms for the uptake of DNA [2–4,6,7,16].

A key question to address in studies of the transfection activity of cationic liposomes is the role of lipid composition. The most promising system so far is made up of double chained quartenary ammonium surfactants in mixtures with various ratios of DOPE (dioleoylphosphatidylethanolamine) [1,2,4,7,12]. DOPE is chosen since it is generally considered to be a fusiogenic lipid and since it appears to reduce the cytotoxicity of the cationic surfactants [9]. A range of different quartenary ammonium surfactants with alkyl, ether and ester links to various backbones, was investigated in the search for a transfection agent that is easily metabolised by the cell [4,6–8]. Also the hydrophilic head groups have been varied in attempts to optimise the DNA-vesicle interactions [2,16]. Additional ammonium groups create a surfactant polycation resembling the natural spermidines which in turn are known for their supporting action on DNA.

The variability of the technique is pronounced [6,7]. A lipid formulation may fail to be efficient on one particular

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cell line; a change of surfactant or replacement of the phospholipid with another amphiphile may then be enough to regain a high transfection activity [7,8,18]. With reservations due to differences between different cell lines, it appears as if a high transfection efficiency requires certain common properties of the liposomes. In the systems using DOPE it is observed that the highest transfection efficiency of a lipid formulation is obtained at rather high ratios (not less than 50 mol%) of phospholipid to surfactant. Another important parameter is of course the ratio of DNA to lipid. The transfection activity of the lipid dispersion is reported to decrease when too high ratios of DNA to the cationic component are used [1,2,5]. A similar effect has been observed with other gene transfer techniques, as those employing polylysine-linked proteins or lipospermines [16,19,20]. The optimal transfection mixture therefore appears to require a careful choice of lipid composition, DNA/lipid ratio and total concentration. According to the suggestions by Behr et al. [16,17] are the compactness of the DNA molecule and the masking of its charges essential for its transfer through the cell membrane.

In various lipid mixing experiments attempts have been made to characterise the liposome–DNA interactions. An increase in the number of fusion events indicated by lipid mixing between cationic liposomes is registered upon addition of DNA [21], while the ability of the cationic liposomes to fuse with negatively charged liposomes is strongly reduced in the presence of DNA [6]. It has also been shown that several other anionic polymers may mediate fusion of positively charged vesicles [22,23]. Micrographs from a recent EM study [21] suggest that the cationic liposomes are attached as beads to a string gradually covering the DNA chain. At a certain ratio of the components the association leads to a complete lipid coating, as indicated by fluorescence measurements [21].

In this contribution we have employed cryo-TEM (cryo-transmission electron microscopy) to reveal the structural features of the complexes formed between DNA plasmids and cationic liposomes. In cryo-TEM the staining and drying artefacts commonly found in conventional TEM are avoided, and biomaterial is imaged close to its native state, which is extremely important when dealing with multicomponent systems where self-assembled structures governed by a combination of interactions are formed. In earlier cryo-TEM studies both the supercoiling of plasmid-DNA [24,25] and the packing of DNA into the head of bacteriophages [26] have been successfully visualised. The major obstacle for doing cryo-TEM on small dsDNA seems to be the pronounced tendency of these macromolecules to avoid thin areas of the solution film. A very thin layer (< 100 nm) of the vitreous solution is needed for a satisfactory visualisation of dsDNA due to the blurring effect from inelastical electron scattering by the vitreous water. The presence of large lipid aggregates affects the film formation and thereby also the possibilities for an optimal imaging of DNA.

2. Materials and methods

2.1. Lipids and surfactants

Cetyltrimethylammoniumchloride (CTAC) was prepared by ion exchange (Dowex 1×8) from CTAB, purchased from Sigma. Dioleoylphosphatidylethanolamine (DOPE) was obtained from Avanti and dioctadecyldimethylammonuimbromide (DODAB) was purchased from Fluka. DOTAP was synthesized and purified according to Ref. [6] as follows. 3-Dimethylamino-1,2-propanediol was acylated with oleoyl chloride. Treatment of the purified diacyl derivate with methyl iodide yielded the desired compound. Both the diacyl intermediate and the final product were purified by column chromatography on silica gel. The 3-dimethylamino-1,2-propanediol was from Tokyo Chemical Industry (TCI, Tokyo Japan). All other chemicals were of analytical grade and used as purchased.

Plasmid DNA (pTZ 18U and pUB110) was obtained from Sigma.

2.2. Cryo-transmission electron microscopy

Specimens for cryo-TEM were prepared using copper grids (200 mesh, Agar Grids) coated with a perforated film of cellulose acetate butyrate (Aldrich) and carbon according to the procedure in Ref. [27]. The majority of the grids were also hydrophilized through treatment by glow-discharge [28]. The blotting procedure and the quenching of specimens were performed using an improved version of a CEVS (controlled environment vitrification system), previously described by Bellare et al. [29] After cold stage transfer the vitrified samples were mounted and examined in a Zeiss EM 902 A electron microscope, operating at an accelerating voltage of 80 keV in filtered bright field image mode at $\Delta E = 0$ eV. The stage temperature was kept below 108 K and images was recorded at defocus settings between 1 and 3 μm. When possible, successive images were taken at different defocus values to assure that no structural information was lost due to an image formation relying on phase contrast. Micrographs from EM studies of this kind are the result of a series of subjective decisions. The selection of micrographs in the article was chosen from several hundreds of negatives to make sure that reproducible results are presented.

2.3. Preparation of liposomes and complexes

Lipids and surfactants were mixed in chloroform solution. The chloroform was then evaporated in a stream of nitrogen followed by an overnight drying under vacuum. The dried lipids were hydrated with NaCl solutions at various concentrations and sonicated using a Soniprep 150 (MSE Scientific Instruments, Crawley, UK), equipped with a titanium microprope. An ice bath was used as a cooling medium during the sonication procedure. The dispersions

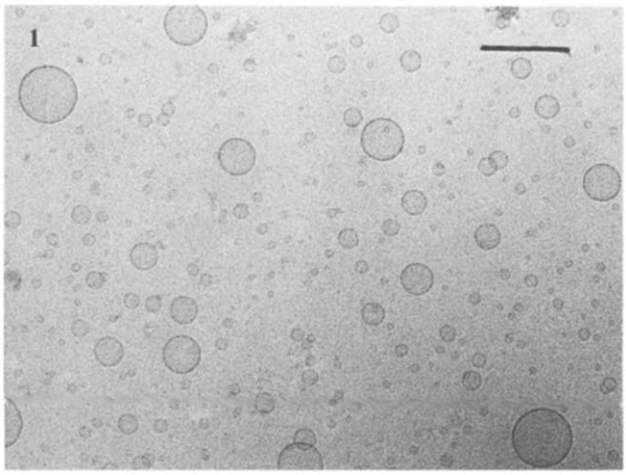


Fig. 1. Cryo-TEM micrographs of DOTAP sonicated in water. Bar 150 nm.

were sonicated until no further change in turbidity was noticed, indicative of a completed reduction of particle size. The required irradiation time varied between 5 and 40 min, depending on the lipid mixture. The resulting distribution of vesicle size was also dependent on lipid formulation. In general it can be stated that both size and polydispersity was larger than what is commonly found on sonication of egg-lecithin, which gives mean diameters about 300 Å. Fig. 1 shows a rather polydisperse vesicle population obtained by 8 min sonication of DOTAP in water. Typical vesicles of various lipid mixtures are also seen in the following micrographs. The studied complexes were obtained by direct addition of plasmids to lipid dispersion at concentration of less than 0.4 mg/ml of lipid and surfactant.

3. Results and discussion

3.1. Polymorphism of the lipid mixtures in dispersion

The cationic vesicle membrane presents a soft and oppositely charged lipid film for the interactions with DNA. The composition of the bilayer determines its preferred configurations; the planar bilayer arrangement is not the only alternative. For rationalizing the formation of curved bilayer conformations and inverted lipid structures the concept of a spontaneous curvature of the lipid monolayer is helpful [30–32]. According to this concept a phase transition, as a non-lamellar transformation, is driven by a relief in total bending and surface energy, gained when the lipid monolayer in the new geometry adopts a curvature closer to the spontaneous one.

The reason for discussing alternative lipid structures, i.e., polymorphism, in relation to the cationic liposomes is that a high proportion of DOPE is often used. DOPE is a well studied lipid [33–35]. In addition to a lamellar liquid crystalline phase it forms an inverted hexagonal phase which is the stable one at ordinary temperature and pH [33]. In dispersion, however, the lamellar arrangement is often metastable since interlamellar contact is needed for the formation of inverted structures. The vesiculation of DOPE may be facilitated by a change of pH to values which stabilizes the lamellar phase. Another more effective way to accomplish this is by adding another amphiphile which favours the bilayer arrangement. The action of the surfactant may to some extent be predicted from its geometry or value of the so called packing parameter [36]. Valuable information on the effect of stabilization and how the components are packed together would be found in the ternary phase diagram of water, DOPE and the added surfactant. Unfortunately, no phase diagrams of DOPE and cationic surfactants are available.

Many of the double chain quartenary ammonium surfactants form lamellar phases by themselves and are therefore easily vesiculated once their hydrocarbon chains are in a melted state. In the present systems the cationic surfactants act both as bilayer stabilizers and dispersion agents of DOPE. Sonication until clarity in these systems does not necessarily bring all the lipids into the vesicle state. EM studies on sonicated mixtures of DOPE and surfactants in ratios corresponding to the ones reported to be active in transfection showed additional aggregates containing inverted lipid structures among the vesicle population. Discrimination between ordinary membranes and inverted structures are not always straightforward. The aggregates presented in Fig. 2 are, however, clearly not made up by ordinary lamellas. Domains of various sizes of H_{II}-phase and other inverted lipid structures have previously been documented by cryo-TEM and can be used for comparison, see Refs. [37–40]. The found aggregates of this kind were always few in number and were not present at all in mixture of high surfactant/DOPE ratios. As expected the tendency to form inverted structures was found to increase with the ratio of DOPE in the lipid mixture. In Fig. 2 two different kinds of aggregates of inverted structures are seen. The most simple ones are seen in Fig. 2a: particles of more or less ordered inverted hexagonal phase that has been wrapped up in a bilayer. Such aggregates could result from fusing vesicles but could also represent 'unsonicated' particles and be regarded as dispersion defects. On the contrary, the aggregates seemingly formed by fusing vesicles in Fig. 2b appear to be more complex. Previously Larsson [41] has described how a reversed cubic phase may be dispersed to form particles in which the liquid crystalline structure of the phase is maintained. Fig. 2b suggests that particles of an ordered inverted phase, hexagonal or cubic, form spontaneously on sonication of a dispersion of DOPE and 30 mol% DODAB. The appearance of such structures suggests that the ternary phase diagram of the amphiphiles includes considerable regions of inverted phases, such as inverted hexagonal and bilayer cubic phases. The observation is interesting since the

presence of DNA molecules could be expected to further favour the formation of inverted structures through strong electrostatic interactions acting in a dehydrating way on the surfactant head groups.

3.2. DNA-vesicle association

Addition of DNA to the lipid dispersion leads to a spontaneous formation of discrete complexes. The typical appearance of the complexes in cryo-TEM is, as seen in Fig. 3, clusters of aggregated dense particles. The density of these aggregates was also indicated by their high sensitivity towards the electron beam. Provided there is an excess of cationic surfactant to DNA in terms of charge, the appearance of the complexes seems to be rather independent of the charge density of the vesicle surface or choice of surfactant. Thus, lipid formulations based on DOPE in mixture with CTAC, DODAB or DOTAP showed all the same type of complexes.

Low amounts of CTAC to DOPE stabilizes the vesicle structure. Fig. 3a shows DNA-complexes formed with vesicles of relatively low surface charge comprising 20 mol% CTAC. Large amounts of CTAC (> 75 mol%)

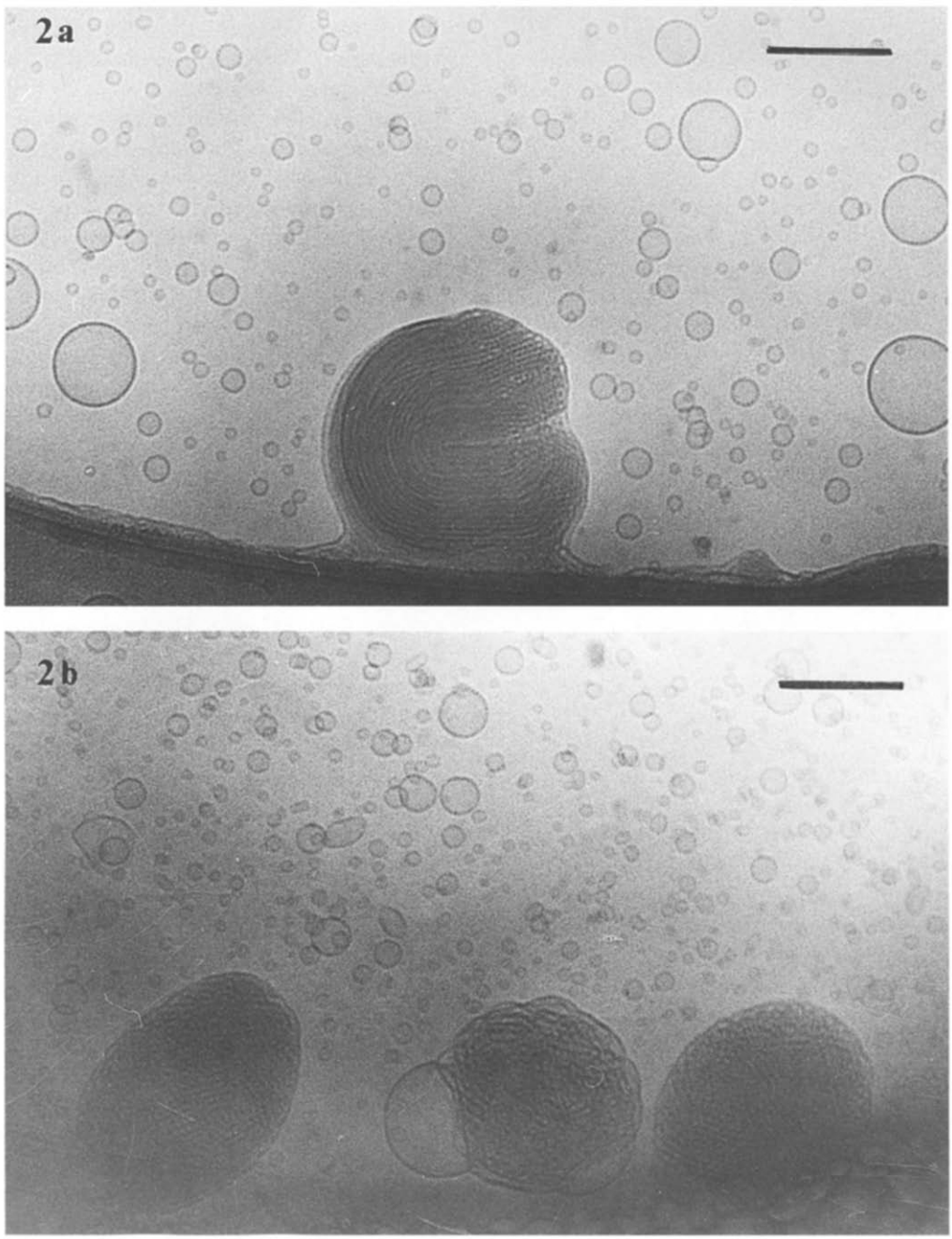


Fig. 2. (a) Dispersed particles containing H2-tubes from a sonicated mixture of 90 mol% DOPE and 10 mol% CTAC in water. Bar 150 nm. (b) DOPE and 30 mol% DODAB sonicated in water. Fusing vesicles forming aggregates of a reversed bilayer phase.

solubilizes the lipid material into micelles and can therefore not be used. The double chained surfactants DOTAP and DODAB may, thanks to their less cone shaped geometry, be used in almost all ratios to DOPE except very low ones. As mentioned earlier, low ratios of these surfactants result in lipid mixtures with pronounced inversion tendency which in turn make them difficult to disperse. Vesicles made from both of these double chained surfactants in combination with DOPE showed the same kind of complex formation as CTAC did, independently of the ratio of surfactant used. Also pure DOTAP (not known by the authors to be active in transfection vesicles), as shown in Fig. 3b, formed this particular kind of complexes with plasmid DNA. No changes occurred when pTZ18U 2880 b.p. was replaced with the larger pUB110 4500 b.p., both of closed circular form.

As the ratio of DNA to lipid approaches charge neutralisation 'free' or loosely bound plasmids begin to appear in the vicinity of the complexes, Fig. 4. For many reasons cryo-TEM does not permit a quantitative determination of the limiting DNA/lipid ratio for total entrapment or binding of DNA. Accumulation and therefore an increase in concentration of small aggregates, such as DNA-plasmids, in certain areas of the film is an often seen effect of sample preparation in cryo-TEM. It is clear, however, that the nature of the complex surface changes when the charge ratio DNA/lipid exceeds unity. It is also interesting to note that the transfection efficiency is reported to decrease when a too high ratio of DNA is used [1,2,5], which may be correlated with the appearance of protruding DNA, giving the complexes a structure that would prevent a close approach to the cell membrane.

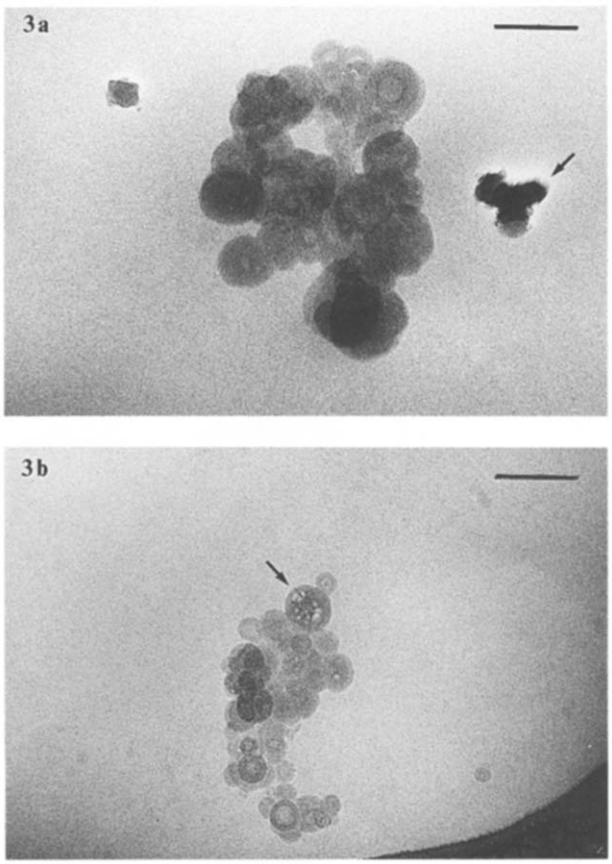
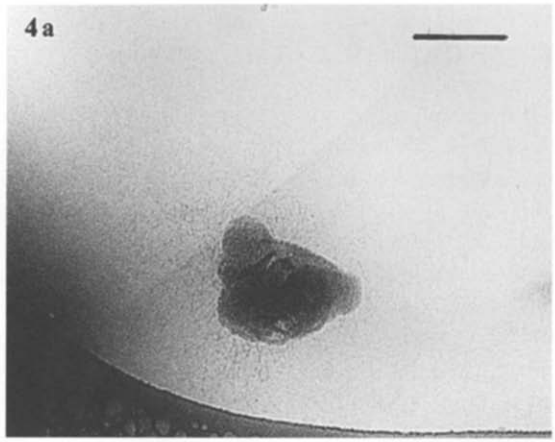


Fig. 3. Associated structures formed upon addition of a low ratio of DNA. Total entrapment of the plasmids. Bar 150 nm. (a) Charge neutralisation of 0.4 (negative DNA/positive surfactant). DNA (4500 b.p.) added to vesicles of DOPE and 30 mol% CTAC, low ionic strength. Arrow denotes frost. (b) Charge neutralisation of 0.2. DNA (2880 b.p.) and pure DOTAP vesicles, low ionic strength. Arrow denotes beam damaged material.

The location of DNA cannot be definitely established from the presented micrographs. In properly defocused micrographs of low noise, weak patterns or striations are seen on some of the aggregated particles, as in Fig. 3a. The striations may stem from an ordering of DNA when condensed on the surface of the vesicles, but more likely they represent multilamellar structures in which DNA is entrapped between the lamellas. The resolution of the lamellas of multilamellar vesicles in the cryo-TEM micrographs depends on the spacing and number of lamellas. The aggregates in Figs. 3 and 4 differ from ordinary multilamellar structures and should also do so if they contained entrapped DNA molecules. Ordinary multilamellar aggregates (for comparison see Fig. 5) usually display well-resolved lamellas since they often consist of concentric vesicles. In the DNA-mediated formation, the oligo lamellar structures may be more compact and disordered (see cartoon in Ref. [16]) and would not give well resolved projections in cryo-TEM. The formation of stacked lamellas could be regarded as a break down or collapse of vesicle structure in presence of DNA.

Results from systems based on egg-lecithin instead of DOPE, a lipid mixture not known by the authors for being active in transfection, suggest DNA induced formation of oligo lamellar structures of vesicles from such lipid formulations also. As seen in Fig. 6, it appears as if the break down of vesicle structure is less pronounced in these systems and that the complexes as a whole is much less compact. As mentioned above compactness may be a prerequisite for an optimal packing of DNA.

The reported increase in lipid mixing [21], and the fluorescence studies indicating a complete lipid coating of the plasmid [21], is in agreement with the idea of a formation of multilamellar structures mediated by DNA. It is also interesting to note that a recent paper [14] states that there are very small differences in transfection efficiency between small sonicated vesicles and larger multilamellar vesicles of the same lipid mixture. Probably, such systems also suffer a similar break down of vesicle structure in the presence of DNA and thereby result in the same kind of complexes. In general the stability of the dispersions towards precipitation is very low. Only at extreme dilution are complexes of some stability found. Precipitation occurs probably through reticulation of growing clusters and is thereby concentration dependent. The complex size was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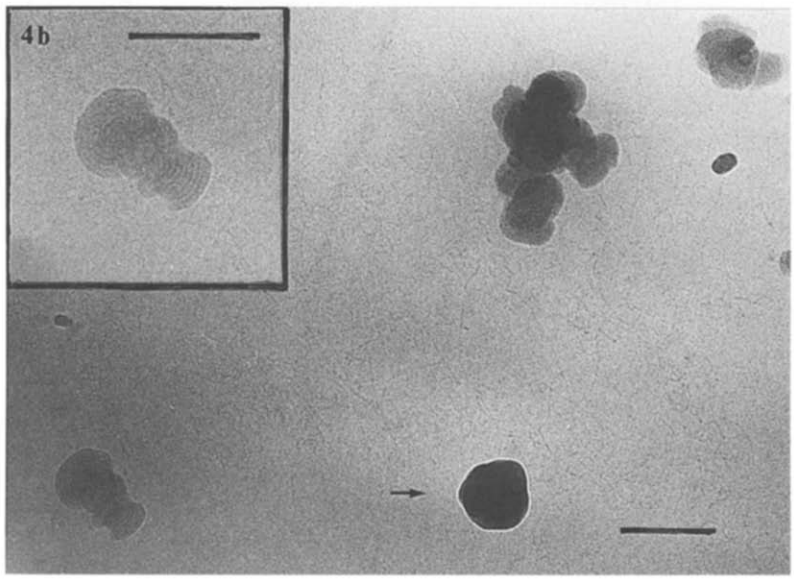
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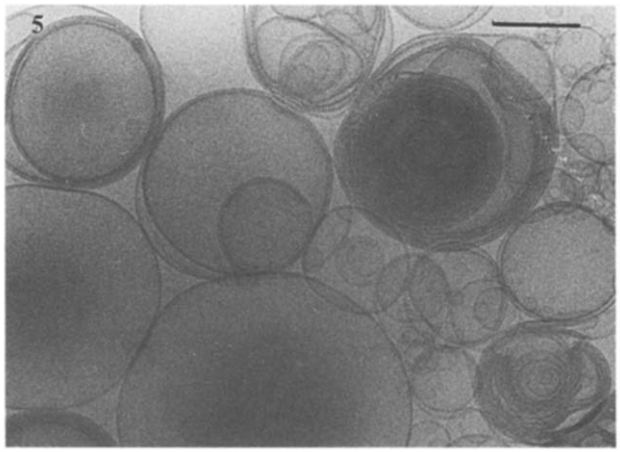
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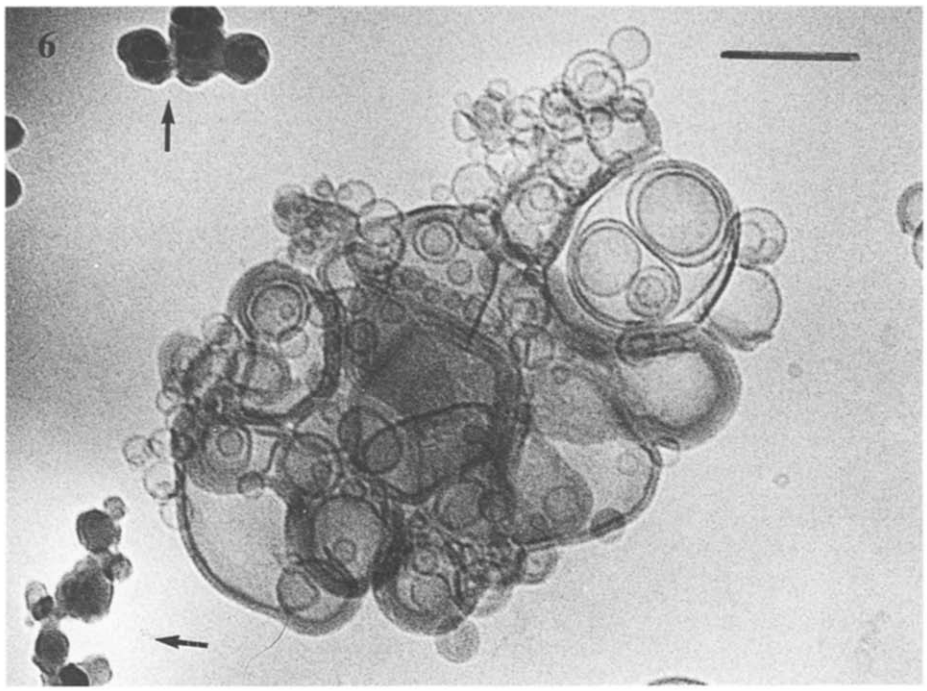

Fig. 6. Vesicles from egg-lecithin and 35 mol% CTAC with plasmids in a ratio of 0.6 in terms of charge. Arrows denote frost. Bar 150 nm.

conditions (150 mM NaCl, 37° C) were made but were prevented by an extensive formation of precipitates.

4. Summary

The main structural features of the complexes formed on interactions of DNA with cationic liposomes were very similar in several systems of different lipid composition. Free plasmids or protruding DNA-strings appeared only at high DNA/lipid ratios. At low ratios, binding and entrapment of DNA into aggregated multilamellar structures was a general finding in all studied systems. The choice of surfactant therefore, does not appear to affect the gross morphology of the DNA-lipid complexes. Concerning the choice of the often required additional lipid, our study suggests that systems based on DOPE result in more compact aggregates than similar formulations using egg-lecithin. Although the lipid mixtures (based on DOPE) themselves showed a tendency towards polymorphism, no complex formation involving inverted lipid structures was observed. It cannot still be excluded, however, that the inversion properties of the lipid mixtures is important for how the lipids pack around the DNA.

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