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Biochimica et Biophysica Acta 1372 (1998) 379–383

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Rapid report

Evidence for three-dimensional interlayer correlations in cationic lipid-DNA complexes as observed by cryo-electron microscopy

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Received 24 March 1998; accepted 1 April 1998

Abstract

A fingerprint-like pattern across multilamellar, lipid-DNA complexes is attributed to DNA condensed as parallel helices between lipid bilayers. It is argued that the patterning indicates the existence of 3-D correlation forces between DNA-covered bilayers, following the DNA-driven formation of multilamellar liposomes from unilamellar vesicles.

Keywords: Lipid-DNA complex; Cryo-transmission electron microscopy; DNA; Cationic liposome; Correlation force

Cationic lipid-DNA (CL-DNA) complexes, which are formed when DNA is combined with liposomes that contain cationic lipids (CL), are often used as gene-delivery systems. Several commercial liposome formulations are available and many transfection assays, using various cationic lipids, have been reported in the literature [1]. However, the nature of these complexes, including lipid-DNA interactions and complex morphology, has not yet been properly studied and understood. A better comprehension of these properties should lead to more efficient artificial cell transfection formulations.

To date, electron microscopy (EM), small angle X-ray scattering (SAXS) and atomic force microscopy (AFM) have been the most valuable sources of information for attempting to understand lipid-DNA interactions and the morphology of resulting associates. Studies performed so far have revealed that several types of complex can be formed and a few models have been proposed [2–4]. It appears that the differences observed between these complexes are due to some extent on which lipids are used, the preparation of complexes and the technique employed to study the complexes [4].

A popular model to emerge from the (so far) limited literature describes a multilamellar structure in which the DNA is sandwiched between lipid bilayers [5–7]. This model is based on SAXS, EM and AFM data. SAXS on several different CL-DNA systems has indicated a long periodicity of 6.5 nm and a short, weak periodicity of about 3.7 nm [4,5,7]. It has been proposed, and supported by cryo-EM measurements [4,6], that the long periodicity corresponds to a 4 nm thick lipid bilayer with one layer of attached, condensed DNA, 2.5 nm thick. Rädler et al. [7] have used SAXS to show that the DNA tends to form a two-dimensional (2-D) smectic

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0005-2736/98/$19.00
PII: S0005-2736(98)00062-5

phase, where a one-dimensional array of aligned DNA is adsorbed between 2-D lipid bilayers in multilamellar liposomes. Furthermore, the in-plane separation of parallel DNA helices adsorbed on the bilayer surfaces gives rise to the short, weak periodicity of about 3.7 nm; this has been observed by cryo-EM as a superimposition on some lamellae of CL-DNA complexes [4]. AFM studies of DNA condensed onto supported cationic lipid bilayers [8], have also indicated that the DNA is adsorbed in partly parallel helices with inter-DNA separation of about 4 nm, with no evidence of knots or crossing over of chains. This reinforces the notion that only a monolayer of DNA is adsorbed between bilayers in CL-DNA complexes and that the DNA in these monolayers is organised into parallel helices.

Although several cryo-EM studies have shown the above-mentioned long periodicity (6.5 nm), none, to our knowledge, have shown the short periodicity across whole spherical, multilamellar CL-DNA complexes. The formation of such complexes from unilamellar vesicles was also not explored in detail before.

Difficulties in applying low-dose techniques to extremely beam sensitive samples have hampered the successful imaging of the short periodicity over the complex. We have observed a clear fingerprint-like pattern across CL-DNA complexes by using a higher-than-usual magnification (55 000×) with energy filtering [9], whilst maintaining a very low dose to reduce beam damage.

Moreover, by starting out with liposome suspensions containing only unilamellar vesicles, we demonstrate that the striations result from, rather than precede, the DNA-driven formation of multilamellar complexes. We attribute this patterning to DNA organisation into parallel helices between the lipid bilayers of the spherical CL-DNA complexes.

Vitrified specimens for cryo-electron microscopy were prepared in the usual manner by rapidly plunging a thin aqueous film of the sample into liquid ethane. Cryo-transmission electron microscopy was performed using a Philips CM 120 Biofilter microscope [10] equipped with a post-column energy filter. The acceleration voltage was 120 kV and the defocus was 1 μm. Magnification of 55 000× allowed a pixel width of 0.46 nm. Images were collected under low dose conditions [11], with the dose being less than 0.1 electron per nm^2.

The neutral (zwitterionic) and cationic lipids, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC, Avanti Polar Lipids, Alabaster, AL) and 3β[*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol, Bachem Biochemica, Heidelberg, Germany), respectively, were used as purchased. Small unilamellar vesicles (SUVs) consisting of DMPC/DC-Chol in a molar ratio of 3:2 were prepared by extrusion [12]. Total lipid concentration was 1.6 mM. Dynamic light scattering revealed a very narrow size distribution around diameter 65 nm (standard deviation 18 nm).

Calf thymus DNA (Sigma, USA) was digested with the restriction enzyme *Eco*RV and purified by extraction with phenol/chloroform. Residual phenol and salts were removed by dialysis against buffer. DNA concentration was determined by measuring the absorbance at a wavelength of 260 nm.

The same buffer solution was used in all preparations and experiments. This buffer (pH = 7.4) contained 50 mM triethanolamine, 0.54 mM EDTA and water from an Elgastat UHQ water purifier (ELGA, UK).

Cationic lipid DNA complexes were formed by combining DNA and the DMPC/DC-Chol SUVs at a DNA/lipid charge ratio of 0.2. Images were taken within 24 h. After vesicle combination with DNA, a suspension that had contained previously only small unilamellar vesicles (Fig. 1) changes dramatically into one consisting of an abundance of large (100–400 nm), chiefly multilamellar, liposomes (Fig. 2). Often, a liposome has 7–10 bilayers and shows a periodicity of 7.8 ± 0.1 nm. The bilayer thickness is typically 5.1 ± 0.1 nm in the SUVs and in the multilamellar liposomes. The innermost bilayer of the liposomes frequently has a diameter (60–130 nm), which corresponds to that of an original SUV (or at most four fused SUVs).

Aggregation of the liposomes, at least in the non-stirred samples, is obvious, the resulting larger complexes having sizes in the order of 1 μm. Samples viewed more than 24 h after complexation are extremely difficult to image because the complexes aggregate over time and, therefore, grow very large. This severely reduces the probability of finding complexes to image in sufficiently thin vitreous ice.

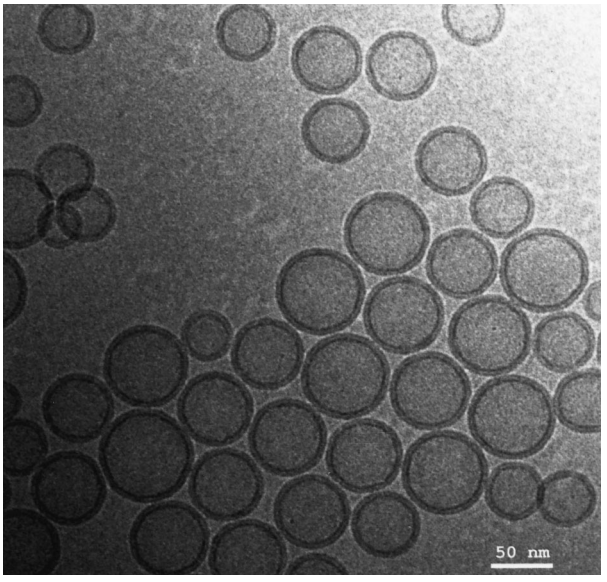

Fig. 1. Cryo-electron microscopy of extruded cationic lipid vesicles. The unilamellar vesicles (1.6 mM total lipid) consist of the zwitterionic phospholipid, DMPC, and the cationic lipid, DC-Chol, in a molar ratio of 3:2.

All complexes of the aggregated multilamellar liposomes are superimposed with a fingerprint-like pattern. This is not observed in normal multilamellar liposome suspensions, in the control DMPC/DC-Chol SUVs before mixing with DNA, or in the vitreous ice outside of the complexes. We used the technique of Fourier transform (FT) to analyse the striations observed in Fig. 2A. Applying a FT to an image breaks up the latter into its Fourier components; each component corresponding to a regular pattern of a certain direction and periodicity. The stronger a regularity is in an image, the larger is the corresponding Fourier component and the corresponding diffraction spot in the FT picture. If a vector is drawn from the origin of the FT to the spot, its direction indicates the direction of the pattern, while its length is inversely proportional to the pattern's periodicity.

The FT in Fig. 2B shows two bright spots near the horizontal axis (arrows). These spots correspond to the striations in Fig. 2A, the pattern running in a roughly horizontal direction. The distance of the spots from the origin indicates that the periodicity of the pattern is 3.8 nm, in accordance with the results of direct measurement (3.8 ± 0.1 nm).

The original samples contained no multilamellar vesicles prior to the addition of DNA. It is therefore clear that the obvious and major transition from unilamellar to multilamellar liposomes is induced by the complexation of DNA with the cationic SUVs. In the process, the DNA intercalates between the bilayers. The measured periodicity (7.8 ± 0.1 nm) in the multilamellar liposomes accounts for a bilayer of thickness 5.1 ± 0.1 nm with ample space between the lamellae for a monolayer of B-DNA chains (2.0 nm thick) and their corresponding hydration shells.

As mentioned earlier, SAXS and AFM data have both indicated that DNA can condense on cationic lipid layers, forming an ordered array of DNA chains. The distance between the parallel DNA chains in the lattice is known to change with different lipid to DNA weight ratios [7] and, more than likely, with the use of different lipids and DNA types. Thus, it is not significant to compare quantitatively the periodicity of our superimposed pattern to that found by SAXS or AFM measurements done on different systems, multilamellar vesicles or supported bilayers, respectively. We can, however, be sure that this fingerprint-like pattern across the complexes arises from DNA adsorbed in parallel helices between bilayers.

We believe that the fact that we can actually image striations across a spherical, multilayered CL-DNA complex is evidence for the existence of long range, three-dimensional (3-D) correlations between different lipid-DNA layers. Fig. 3 shows two conceivable modes of alignment, orthorhombic and tetragonal. The alignment between the complex layers could originate from the coupling between the two adjacent monolayer components of a bilayer which both have laterally non-uniform lipid distribution, as the result of DNA adsorption. Electrostatic transmembrane interactions between the charges on the cationic lipids and/or DNA chains could also contribute. We believe, however, that the later explanation is less likely. This finding is new since the previous experimentalists who have observed DNA ordering often relied on the use of multilamellar systems to begin with and, consequently, were unable to draw conclusions about the origin of 3-D order in the DNA distribution pattern.

When viewed by transmission electron microscopy, the superimposition of several unidirectional DNA lattices would give a striped pattern. The striations

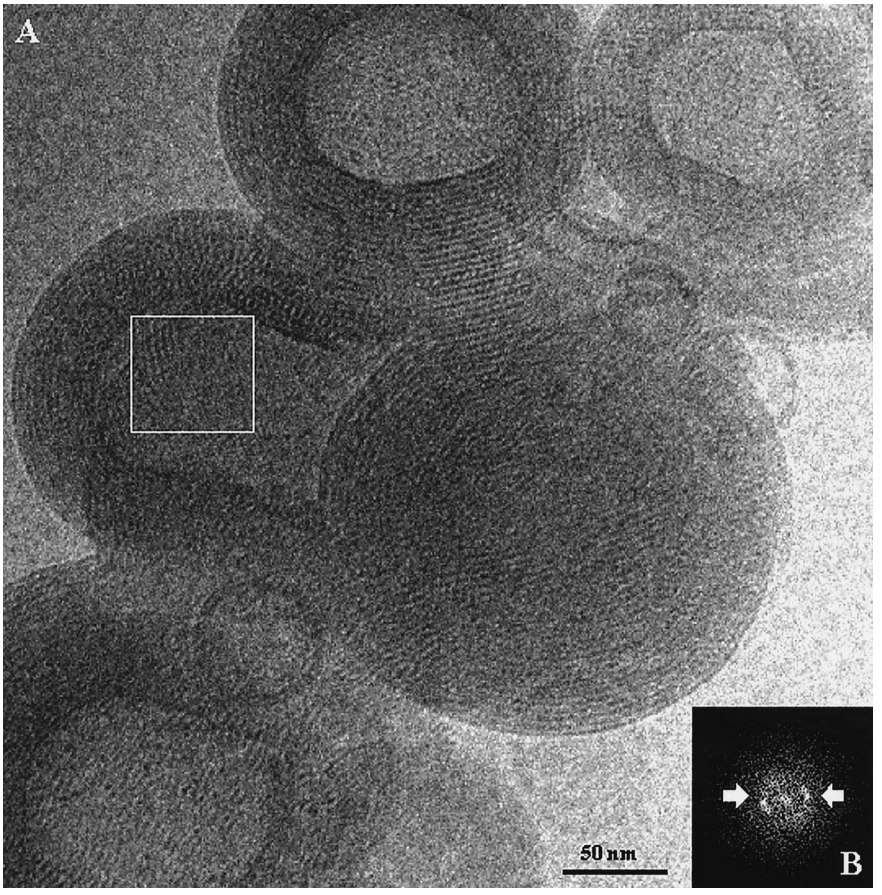

Fig. 2. Cationic lipid-DNA complexes embedded in vitreous ice and imaged by cryo-electron microscopy. (A) These complexes are formed by combining DNA with unilamellar cationic liposomes (DMPC/DC-Chol, molar ratio = 3:2). The complexes consist of aggregated, spontaneously formed, spherical multilamellar liposomes in which DNA is intercalated between the lipid bilayers. Superimposed on the complex is a fingerprint-like pattern, believed to be DNA organized into parallel helices. The latter is indicative of the existence of three-dimensional correlation forces between lipid/DNA layers. (B) Fourier transform of the detail in (A). The diffraction spots correspond to the 3.8 nm periodicity which we attribute to aligned DNA helices.

should not be observed on imaging through many layers if the directions of alignment of the DNA lattices are random. Only an increase in the grey value, when compared to the vitreous ice background, would be expected.

A mesh-like pattern indicates that not all of the DNA lattices in different layers are aligned with each other. This pattern could arise from several layers with all DNA lattices in one orientation, superimposed on a collection of other layers with DNA adsorbed in a different direction. Alternatively, the meshed pattern could just be the superimposition of the DNA lattices in the top hemisphere on those in the bottom hemisphere of layers; the curvature of the liposome would then change the apparent direction of the DNA lattices in each hemisphere.

The quality of the imaged fingerprint-like pattern was greatly enhanced by the use of energy filtering coupled with high magnification cryo-EM. Normally, high magnification imaging of complexes embedded in vitreous ice results in excessive beam damage. In order to obtain our images, we only had to subject the complexes to a very small electron dose, thereby preventing obvious beam damage. The virtues of energy filtering have been described elsewhere [9].

In conclusion, the successful imaging of a fingerprint-like pattern across cationic lipid-DNA complexes suggests that there are three-dimensional correlation forces which cause the DNA helices and/or their associated lipids in different monolayers to align. Further work needs to be done to discover whether this alignment occurs during formation of

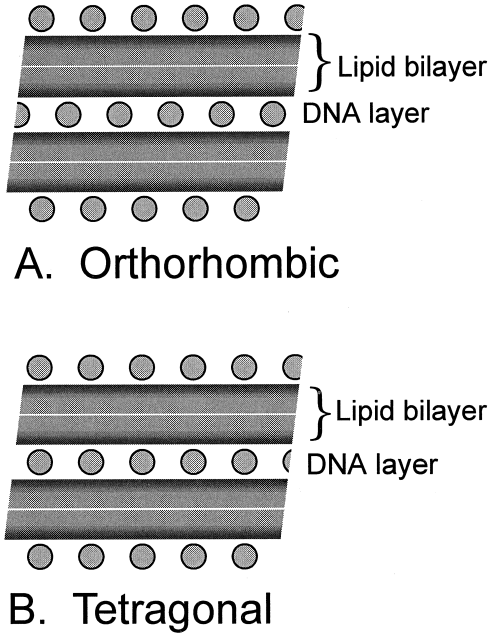

Fig. 3. Two possible modes of alignment of DNA helices between DMPC/DC-Chol lipid bilayers due to 3-D correlation forces: (A) orthorhombic packing, (B) tetragonal packing.

the complex or after the complex has been formed. Investigating the formation of CL-DNA complexes should improve our understanding of how the genetically active material interacts with cells and hopefully will lead to improved transfection formulations.

This work was supported by the DAAD (B.J.B.) and the DFG (Grant Ce. 19-13/1 to G.C.). The authors would like to thank Stephan Lambotte for the DNA purification.

References

[1] D.D. Lasic, N.S. Templeton, Liposomes in gene therapy, Adv. Drug Deliv. Rev. 20 (1996) 221–266.
[2] B. Sternberg, F. Sorgi, L. Huang, New structures in complex formation between DNA and cationic liposomes visualized by freeze-fracture electron microscopy, FEBS Lett. 365 (1994) 361–366.
[3] P.L. Felgner, Y.J. Tsai, J.H. Felgner, Advances in the design and application of cytofectin formulations, in: D.D. Lasic, Y. Barenholz (Eds.), Liposomes: From Gene Therapy to Diagnostics and Ecology, CRC Press, Boca Raton, FL, 1996, pp. 43–56.
[4] D.D. Lasic, Liposomes in Gene Delivery, CRC Press, Boca Raton, FL, 1997.
[5] D.D. Lasic, H. Strey, M.C.A. Stuart, R. Podgornik, P.M. Frederik, The structure of DNA-liposome complexes, J. Am. Chem. Soc. 119 (1997) 832–833.
[6] J. Gustaffson, G. Arvidson, G. Karlsson, M. Almgren, Complexes between cationic liposomes and DNA visualized by cryo-TEM, Biochim. Biophys. Acta 1235 (1995) 305–312.
[7] J.O. Rädler, I. Koltover, T. Sladitt, C.R. Safinya, Structure of DNA-cationic liposome complexes: DNA intercalation in multilamellar membranes in distinct interhelical packing regimes, Science 275 (1997) 810–814.
[8] Y. Fang, J. Yang, Two-dimensional condensation of DNA molecules on cationic lipid membranes, J. Phys. Chem. B 101 (1997) 441–449.
[9] O.L. Krivanek, S.L. Friedman, A.J. Gubbens, B. Kraus, An imaging filter for biological applications, Ultramicroscopy 59 (1995) 267–282.
[10] A.F. de Jong, J. Rees, W.M. Busing, U. Lücken, Integrated energy-filtering TEM – the CM120-Biofilter, Prog. Biophys. Mol. Biol. 65 (1996) H116.
[11] R. Grimm, H. Singh, R. Rachel, D. Typke, W. Zillig, W. Baumeister, Electron tomography of ice-embedded prokaryotic cells, Biophys. J. 74 (1998) 1031–1043.
[12] L.D. Mayer, M.J. Hope, P.R. Cullis, Vesicles of variable sizes produced by a rapid extrusion procedure, Biochim. Biophys. Acta 856 (1986) 161–168.