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Rapid report

EDTA-induced self-assembly of cationic lipid-DNA multilayers near a monolayer-covered air-water interface

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Abstract

The presence of EDTA in the suspending buffer can induce the formation of multilayer structures from a mixture of the cationic lipid 3β[*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl] cholesterol and the zwitterionic 'helper' lipid 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine with DNA. The resulting structures consist of stacks of alternating sheets of lipid bilayer with intercalated DNA. In the absence of EDTA, only a single layer of DNA adsorbs to the lipid membrane. The buffer composition therefore influences the morphology of the lipid-aggregate/DNA assembly, which was not known to date.

Keywords: Lipid-DNA complex; Energy dispersive X-ray reflectivity; Cationic liposome; Formation mechanism

By mixing a suspension of cationic liposomes (CL) with a solution of DNA one can initiate the spontaneous self-assembly of cationic lipid-DNA (CL-DNA) complexes [1]. The resulting structures have attracted a great deal of attention, due to their ability to carry extracellular DNA across the cell membranes. As they are inherently non-immunogenic, and also free of potentially harmful viral DNA, CL-DNA complexes are an appealing starting point for the development of effective, but relatively safe and easy to make alternatives to natural carriers, such as viruses, for gene therapy.

The transfection efficiency of conventional cationic lipid formulations is much below that of natural viruses. Evidence is accumulating that the morphology of CL-DNA complexes plays a role in this [2,3]. It is therefore important to know the precise structure of said complexes and to understand well the numerous factors that determine the complex generation and packing morphology.

Cryo-electron microscopy and small-angle X-ray scattering experiments have revealed that many CL formulations, including the formulation used in this work, yield CL-DNA multilamellae. Normally, such multilamellae adapt an onion-like structure, consisting of lipid bilayers alternating with layers of DNA [4–6]. To elucidate the detailed mechanism behind the formation of such multilayers, we devised a new experimental set-up, which makes it possible to measure the structure of the self-assembly in situ at high resolution. We achieved this by growing CL-DNA complexes near the air-water interface rather than in the bulk and by applying X-ray reflectivity as a high resolution surface sensitive technique to monitor the growth. This unveiled the surprising fact that EDTA, a divalent cationic chelator present

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in many buffers, promotes the development of multilamellar structures.

The cationic lipid 3β[*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol), purchased from Bachem Biochemica (Heidelberg, Germany), and the zwitterionic lipid 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), purchased from Avanti Polar Lipids (Alabaster, AL, USA), were mixed at a molar ratio of 2:3. Small unilamellar vesicles were prepared by repeated extrusion of a crude lipid suspension in a buffer using polycarbonate filters of decreasing pore size [7]. Quasi-elastic light scattering revealed the final vesicle diameter to be 63 (±22) nm.

Calf thymus DNA (Sigma Chemical Co., USA) was digested with the restriction endonuclease *Eco*RV (Stratagene, USA) to obtain a solution of linear DNA fragments with well defined average length of 6000 bp. The fragments were purified by repeated phenol/chloroform extraction [8] and exhaustive dialysis against the selected buffer, through a membrane with molecular weight cut off of 3 kDa.

The buffer contained 25 mM triethanolamine and water from an Elgastat UHQ water purifier (ELGA, UK). The pH of all solutions was adjusted to 7.4 with HCl. When appropriate and as stated, the buffer also contained 0.54 mM EDTA (Sigma Chemical Co., USA).

To conduct an experiment, a mixture of DNA (final concentration 6 mg/l) and vesicles (final total lipid concentration 100 mg/l) was filled into a measuring trough. Subsequently, a Langmuir film comprising the described lipid mixture was spread onto the surface of investigated DNA/vesicle suspension. The film served as a template for the self-assembly of multilayers, which was monitored with a laboratory-made X-ray reflectometer [9]. Throughout the experiment, the temperature was kept at 25°C (±0.1°C).

The reflectometer was operated in the energy dispersive mode, with incident and reflection angles fixed to $\alpha = 8.61$ mrad. A sealed 3 kW molybdenum anode served as X-ray source. A good resolution, energy sensitive germanium solid state detector (Silena, Milan, Italy) was used to analyse the specularly reflected beam. For details see [9].

In Fig. 1 the typical reflectivity of a sample prepared without EDTA (panel A) is compared with the reflectivity measured in an experiment for which

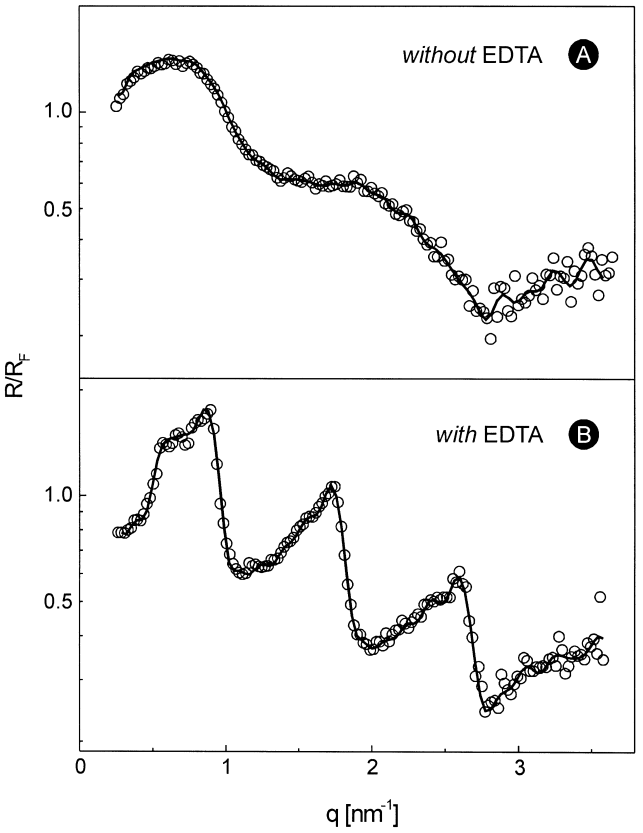

Fig. 1. Fresnel-normalised reflectivities of the complexes which self-assembled at the air water interface in the absence (A) and presence (B) of EDTA; q is the scattering vector. Only when EDTA is present, sharp sawtooth-shaped peaks appear in the reflectivity curves and thus indicate the existence of multilayers. Experimental data are shown as ○; the best fit, based on a box model, is shown as curves.

EDTA was added into the buffer (panel B). Both reflectivities were determined 100 h after the start of the experiment. The difference between the data sets is striking: while the reflectogram pertaining to the suspension containing EDTA shows a series of (at least) three peaks, the corresponding results obtained in the absence of EDTA show no comparable features.

Peaks in the reflectivity curve are indicative of repetitive structures. (They normally result from the interference of X-rays reflected from several equidistant layers.) It is therefore reasonable to assume that the presence of EDTA catalyses the generation of periodic structures in the mixed DNA, cationic liposome suspensions.

The result from a typical control experiment, where EDTA was present but DNA was absent, is

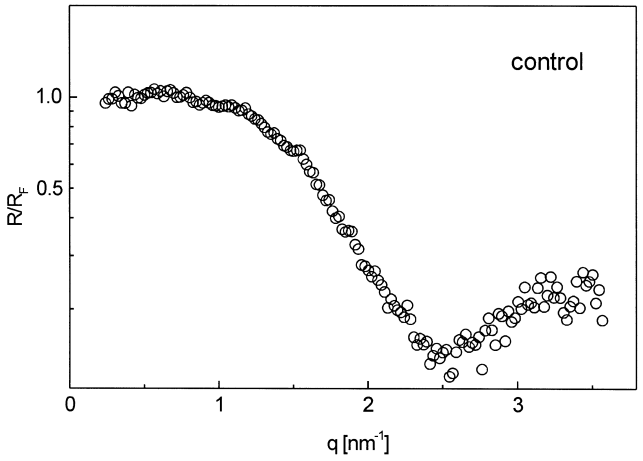

Fig. 2. Fresnel-normalised reflectivity from a control experiment in which the bulk solution contained EDTA, but no DNA. The reflectivity curve shows no signs of a multilamellar structure. It is the curve expected for a simple lipid monolayer.

shown in Fig. 2. The reflectivity curve is that of a simple lipid monolayer. Thus, since multilayers do not exist in the absence of DNA, they must involve DNA strands. To clarify this issue, we analysed the measured curves quantitatively.

Fig. 3 shows the electron density profiles obtained from the reflectivity curves illustrated in Fig. 1. (The electron density, ρ, is given as a function of depth, z, from the surface.) The profiles have been calculated within the framework of a free-form box model. (This model divides the electron density of investigated structure into slices, which run parallel to the surface and are 0.2 nm thick; in an iterative process, the electron densities of the slices are varied independently until the calculated reflectivity, stemming from the model, fits the measured reflectivity data.)

Data analysis makes clear that the first peak in the electron density profile shown in Fig. 3A (measured without EDTA) stems from the electron-dense headgroup region in the lipid film template. The adjacent less dense region confirms that the polar headgroups point towards the water whereas the hydrophobic aliphatic tails are excluded from the solvent. The second peak corresponds to adsorbed DNA, which is attracted to the surface by electrostatic interaction between the cationic lipids and the oppositely charged DNA. Behind the DNA layer, the electron density gradually drops to the value characteristic of the aqueous phase, which is here set equal to 1, for simplicity.

In the presence of EDTA, a more complex assembly emerges, as can be seen in Fig. 3B. At least three adsorbed lamellae can now be identified, consisting of lipid bilayers glued together by intercalated DNA (mono)layer. The low electron density in the middle of each bilayer corresponds to the poorly ordered lipid tail region. The high electron density regions again correspond to the relatively electron-rich lipid headgroups and to the DNA sandwiched between the lipid bilayers. The periodicity corresponds to a repeat distance of 7.1 nm.

Why does the presence of EDTA in suspension so drastically alter the morphology of CL-DNA complexes? One possible answer is the mechanism by which assemblies form. There is evidence, from cryo-electron microscopy, that the complex formation involves adsorption of vesicles from the bulk that sub-

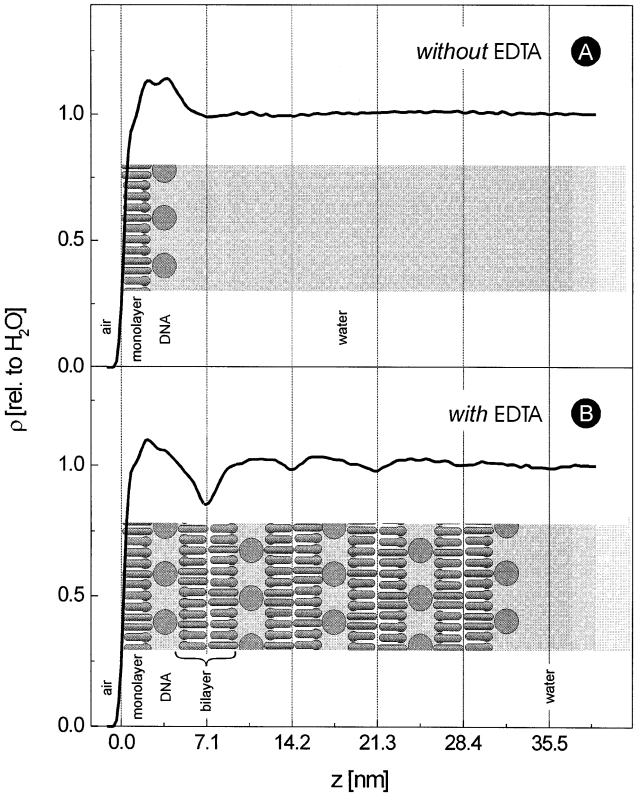

Fig. 3. Electron density profiles pertaining to the results shown in Fig. 1 and deduced from a box model. If no EDTA is present (A), only a lipid monolayer with an underlying adsorbed DNA monolayer is found. If EDTA is present in the buffer (B), multilayers consisting of lipid bilayers alternating with a monolayer of DNA emerge. The vertical grids in both panels correspond to the repeat distance of the surface adsorbed CL-DNA multibilayers.

sequently break open at the substrate surface [10]. In the process, the DNA chains function as 'molecular glue' which keeps the substrate and adsorbed vesicles close together. Under the influence of such 'glue', the ruptured vesicles roll their bilayers over the substrate to form an adsorbed bilayer, which improves over time. Underlying layers develop similarly.

As can be seen from Fig. 3A, the presence of EDTA is not a prerequisite for DNA adsorption to the lipid layer. Rather, EDTA seems to affect, in a subtle fashion, electrostatic interactions between the constituents of the complex [11]. This may rely on the formation of inter-molecular hydrogen bonds, or simply on very efficient electrostatic screening, which increases rapidly with the ion valence. Details of the mechanism remain to be elucidated, as does the capability of other (oligovalent?) salts to catalyse the process.

In conclusion, we have shown that the morphology of CL-DNA assemblies not only depends on the nature of lipids and DNA used to form the complex, but is also sensitive to the buffer composition. We found that EDTA, a common constituent of many buffers, drastically affects the morphology of CL-DNA complexes and promotes the unilamellar-to-multilamellar structure transition. It is therefore likely that oligovalent salts, such as EDTA, will influence the efficacy of transfection in vitro and in vivo. This interesting suggestion remains to be checked in dedicated biological experiments.

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