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Do DC-Chol/DOPE-DNA complexes really form an inverted hexagonal phase?

Giulio Caracciolo *, Ruggero Caminiti

Dipartimento di Chimica, Università degli Studi di Roma 'La Sapienza', P.le Aldo Moro 5, 00185 Roma, Italy

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Abstract

Using synchrotron small angle X-ray scattering and energy dispersive X-ray diffraction, we have found that cationic liposomes made of the monovalent cationic lipid, 3-[*N*-(*N*,*N*-dimethylaminoethane)-carbamoyl]cholesterol (DC-Chol) and the neutral lipid dioleoylphosphatidylethanolamine (DOPE) condense DNA molecules forming complexes (DC-Chol/DOPE-DNA) which are not assembled in an inverted hexagonal structure as recently reported, but, conversely, form a well-ordered lamellar liquid-crystalline phase with distinct regimes of DNA packing density.

1. Introduction

Cationic liposomes (CL) have found widespread application in molecular cell biology as DNA transfection agents, the main advantages with respect to viral vectors being low toxicity, easy of production, and almost no limit on the DNA size to be transferred [1–4]. Over the last years, it has been shown [5–11] that CL–DNA complexes, currently named lipoplexes, form an ordered liquid-crystalline multilamellar structure (L_{α}^{C} phase) with DNA condensed between opposing cationic lipid membranes or, alternatively, an inverted hexagonal H_{II}^{C} phase comprised of lipid-coated DNA strands arranged on a hexagonal lattice (Fig. 1).

The driving force for the lipoplexes formation is the entropic free energy gain arising from the release of the Manning condensed counterions from both the DNA and the lipids. Early experiments suggested the existence of a correlation between structure and transfection efficiency (TE) of the lipoplexes leading to the conception that the H_{II}^{C} complexes always transfected more efficiently than the lamellar ones [10 and references therein]. More recently, experimental evidence that the most efficient complexes are assembled in the L_{α}^{C} phase has been provided [12,13], suggesting that a direct correlation between structure and TE does not simply exist. However, because gene transfection by CLs requires the spontaneous in vitro self-assembling of discrete lipoplexes, the TE of CL formulations strongly depends on the structural properties of the complexes. In addition, the low TE of current CL formulations with respect to viral gene delivery methods is the result of poor understanding of the supramolecular structures of lipoplexes and their interactions with cells and of molecular mechanisms leading to the release of DNA within the nucleus [10]. Thus, structural characterization of the lipoplexes is currently an intense research area and should help toward a better understanding of CL-mediated transfection.

There are several reports in the literature that DC-Chol/DOPE CL work well in varied cell lines [14,15 and references therein]. In early freeze-fracture electron microscopy experiments, 'spaghetti form' lipoplex

* Corresponding author. Fax: +390649910631.
E-mail address: g.caracciolo@caspur.it (G. Caracciolo).

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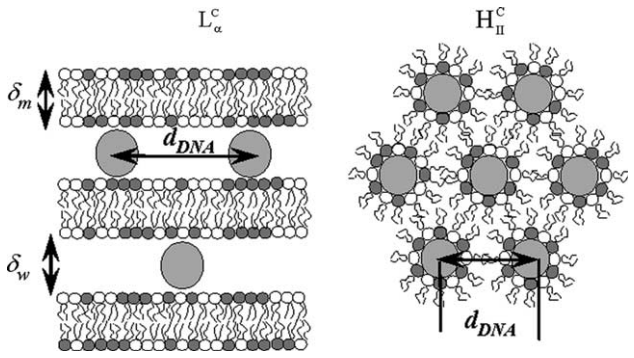

Fig. 1. Schematics of the inner structure of lamellar L_α^C and hexagonal H_{II}^C CL–DNA complexes. In the lamellar L_α^C phase, with alternative lipid bilayers and DNA monolayers, the repeat spacing $d = \delta_w + \delta_m$. The hexagonal H_{II}^C phase is composed of cylinders consisting of DNA coated with a lipid monolayer arranged on a hexagonal lattice.

morphology was observed [16] in which a cylinder of one DC-Chol/DOPE bilayer surrounds DNA molecules. More recently, Congiu Castellano et al. [17] reported, for the first time, on the structure of solid-supported DC-Chol/DOPE-DNA complexes by means of X-ray diffraction. The X-ray measurements revealed the existence of a liquid-crystalline hexagonal packing order with the DNA rods intercalated within the aqueous tubes of the hexagonal lipid matrix (Fig. 1). The relationship between the structure and TE of DC-Chol/DOPE-DNA complexes was also investigated. Nevertheless, the authors carried out the X-ray experiments on dried samples, whereas the TE experiments were performed under very different experimental conditions. Because it is widely accepted in the community that the experimental conditions play an important role on the efficiency of CL vectors [18], this discrepancy makes it hard to make significant comparisons between structural and TE data. We would, therefore, have stated the relevance of the cited study to transfection only if the effect of sample dehydration on the structure of DC-Chol/DOPE-DNA complexes had been previously investigated. To our knowledge, no study has concerned the structural characterization of unoriented DC-Chol/DOPE-DNA lipoplexes in solution so far. However, for gene delivery purposes, unoriented CL–DNA complexes in solution are the only measurement condition.

In this Letter, we report for the first time on the supramolecular structure of DC-Chol/DOPE-DNA complexes in buffer solution. We here report data from high-resolution synchrotron small angle X-ray scattering (SAXS) experiments. Lastly, to better interpret the real importance of the experimental conditions, we also performed a comprehensive low-resolution energy dispersive X-ray diffraction (EDXD) study on solid-supported DC-Chol/DOPE-DNA complexes as a function of hydration.

2. Materials and methods

2.1. Sample preparation

DC-Chol/DOPE and DOTAP/DOPC mixtures were prepared by dissolving the lipids in chloroform at two distinct weight ratios of neutral lipid in the binary mixtures Φ = neutral lipid/total lipid (w/w) ($\Phi_{DOPE} = 0.5$, 0.61 for DC-Chol/DOPE liposomes and $\Phi_{DOPC} = 0.5$, 0.61 for DOTAP/DOPC liposomes). The solvent was then evaporated at $T = 40$ °C in a rotary evaporator. After drying under a vacuum over at least 12 h to remove any residual solvent, the lipid films were added of 1 ml of a buffer solution (Hepes 10^{-2} M, pH 7.4) to obtain the desired lipid concentration (180 mg/ml) and then vortex mixed to afford an emulsion. Lipid emulsions were let to equilibrate at $T = 4$ °C for 48 h. We also sonicated a solution of calf-thymus Na-DNA (13 mg/ml) (MW(bp) = 649) for 5 min inducing a DNA fragmentation whose length distribution, detected by gel electrophoresis, is between 500 and 2000 bp. Both lipids were purchased from Avanti Polar Lipids (Alabaster, AL, USA), whereas the DNA was purchased from Sigma (St. Louis, MO). By mixing adequate amounts of the DNA solution to a fixed volume (60 μl) of the suspension of CLs, self-assembled CL–DNA complexes at seven charge ratios ($\rho = 0.3$, 0.6, 1, 1.2, 2.4, 4.8, 9.6) were obtained.

By following this procedure, the amount of lipid remained the same.

2.2. Synchrotron Solution SAXS experiments

All SAXS measurements were performed at the Austrian SAXS station of the synchrotron light source ELETTRA (Trieste, Italy). SAXS patterns were recorded on a SWAXS camera with Kratky collimation (Hecus, X-ray Systems) covering a q-range ($q = 4\pi\sin(\theta)/\lambda$) of between 0.5 and 15 nm^{-1}. The angular calibration of the detector was performed with silver-behenate [$CH_3(CH_2)_{20}$–COOAg] whose d corresponds to 5.838 nm. The unoriented samples were sealed in a 1.5-mm diameter glass X-ray capillaries. Exposure times were between 100 and 500 s. No evidence of radiation damage was observed in any sample at this exposure. The HEPES bulk solution was subtracted to the collected data. Temperature was varied in a wide temperature range ($5 < T < 65$ °C) and was controlled in the vicinity of the capillary to within ±0.1 °C.

2.3. Surface EDXD experiments

A drop of 100 μl of the DC-Chol/DOPE-DNA dispersions ($\rho = 0.6$, 1, 2.4, 9.8) were deposited onto the oriented surface of ⟨1 0 0⟩ silicon wafers (1 × 1.5 cm) and dried in a closed chamber under a gentle nitrogen

flux. The spread drop was allowed to dry for about 1 h up to a uniform dry film was obtained. After positioning the sample in the X-ray chamber, the dry film was fully hydrated by vapour up to traces of water condensation onto the support were evident. The overall hydration process lasted about 72 h. At this point, the system was in the biologically relevant excess water condition. This experimental procedure results in the formation of solid-supported lipid–DNA complexes in the biologically relevant excess water condition as recently described [19]. Reflection-mode EDXD experiments were carried out, both on dehydrated (relative humidity (RH) = 50%) and fully hydrated samples, by using an EDXD apparatus elsewhere described [20].

3. Results and discussion

At $\Phi_{DOPE} = 0.5$ (data not reported) DC-Chol/DOPE-DNA complexes show a regular multilamellar L_α^C phase in striking disagreement with the structural results previously reported by Congiu Castellano et al. [17] who found evidence of complexes assembled in the H_{II}^C phase. It is well established that, in mixed lipid systems, the shape of the molecules that controls the natural curvature of the membrane, also determines the curvature of the lipid self-assembly [10]. Accordingly, DOPE is a cone-shaped lipid with a negative natural curvature and has been found to induce the L_α^C to H_{II}^C transition in DOTAP/DOPE-DNA lipoplexes [10 and references therein]. That is why we could expect to observe the reported hexagonal phase at higher Φ_{DOPE}. This could explain the observed discrepancy in terms of different lipid composition of the samples. Thus, X-ray experiments were replicated at $\Phi_{DOPE} = 0.61$ ($T = 20$ °C) and representative SAXS patterns are shown in Fig. 2 as a function of ρ. Still, in opposition to the structural findings of Congiu Castellano et al. [17], all the investigated DC-Chol/DOPE-DNA complexes exhibit a multilamellar structure consisting of a one-dimensional (1D) arrays of DNA chains intercalated between oppositely charged membrane bilayers (Fig. 1). The set of sharp lamellar peaks at q_{001}, q_{002} and q_{003} is caused by the alternating lipid bilayer-DNA monolayer structure with periodicity $d = 2\pi/q_{001} = 6.85 \pm 0.05$ nm given from the combination of the thickness of lipid bilayer ($\delta_m = 4.35$ nm) and DNA monolayer ($\delta_w \sim 2.5$ nm) (Fig. 1). The additional broad peak, labeled as q_{DNA}, arises from the DNA–DNA spatial correlation $d_{DNA} = 2\pi/q_{DNA}$. SAXS measurements indicate that the lamellar periodicity d does not vary as a function of ρ while the 1D DNA lattice can be adjusted by varying the relative percentage of CL and DNA in the complex (Fig. 3). From a structural point of view, the phase behavior of the DC-Chol/DOPE-DNA system looks like that of lamellar complexes.

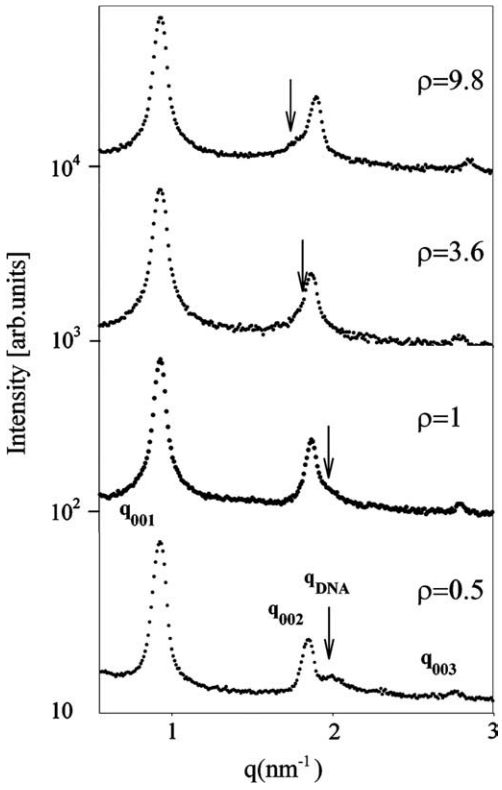

Fig. 2. SAXS patterns of DC-Chol/DOPE-DNA complexes at constant $\Phi_{DOPE} = 0.61$ ($T = 20$ °C) as a function of ρ. The mobile DNA–DNA peak (indicated by arrows) moves toward smaller values of q as ρ increases. To clarity, the q-range is restricted to between 0.5 and 3 nm^{-1}.

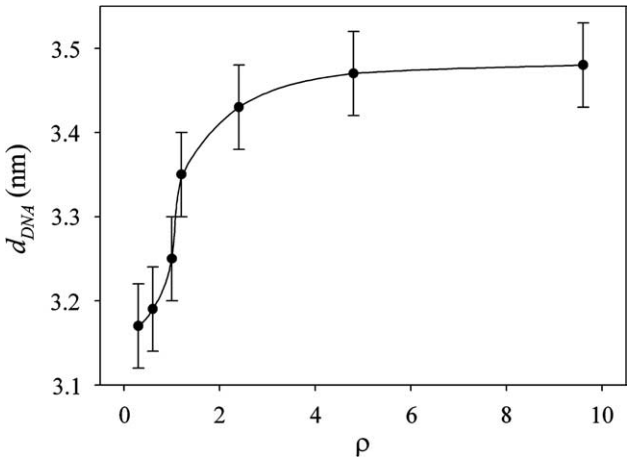

Fig. 3. Variation of the interaxial distance d_{DNA} between DNA chains in the lamellar DC-Chol/DOPE-DNA complexes as a function of ρ. Solid line is a guide to the eye.

As well established [21–23], lamellar lipoplexes adjust the interaxial spacing d_{DNA} (Fig. 3) in order to minimize the total free energy by separating the DNA strands.

Finally, electrostatic interactions set a constraint on the excess lipid or DNA the complex can accommodate and two distinct plateau regimes are reached [21–23]. At

the isoelectric point ($\rho = 1$) the interaxial distance between DNA chains is $d_{DNA} = 3.25$ nm. In Fig. 4, we report the SAXS pattern of the well-characterized complex made of the cationic lipid DOTAP and the neutral lipid DOPC (7) ($\rho = 1$, $\Phi_{DOPC} = 0.61$, $T = 20$ °C) under the same experimental conditions of temperature, pH and ionic strength. From the DNA peak position, an interaxial distance $d_{DNA} = 4.32$ nm was calculated. This finding means that, at the same percentage of neutral lipid in the complex, the interaxial distance is shorter in the case of DC-Chol/DOPE-DNA compared to the widely studied DOTAP/DOPC-DNA complex [21]. Since the interaxial DNA–DNA distance is a function of the surface charge density σ_M [21], our results show that the surface charge density of DC-Chol/DOPE liposomes is higher than that of DOTAP/DOPC liposomes at the same fraction of neutral lipid in the bilayer. In principle, it could not be a minor point. Indeed, one of the most important results of the Safinya's [13] group has been the recent identification of σ_M as the key chemical parameter which regulates the TE of lamellar complexes in vitro.

The effect of the temperature on the structure of DC-Chol/DOPE-DNA lipoplexes was also investigated. Even if a comprehensive description of the thermotropic behavior of DC-Chol/DOPE-DNA lipoplexes cannot be given here, we underline that, in the investigated temperature range ($5 < T < 65$ °C, data not reported) no temperature-induced L_α^C to H_{II}^C transition occurs and the complexes are always organized in the liquid-crystalline L_α^C lamellar phase.

In principle, one could suppose the existence of two different states: one metastable phase and an equilibrium one to be reached via relaxational phenomena. If so, two different structures could be two locally stable states and time-induced $L_\alpha^C \rightarrow H_{II}^C$ phase transition could occur. To

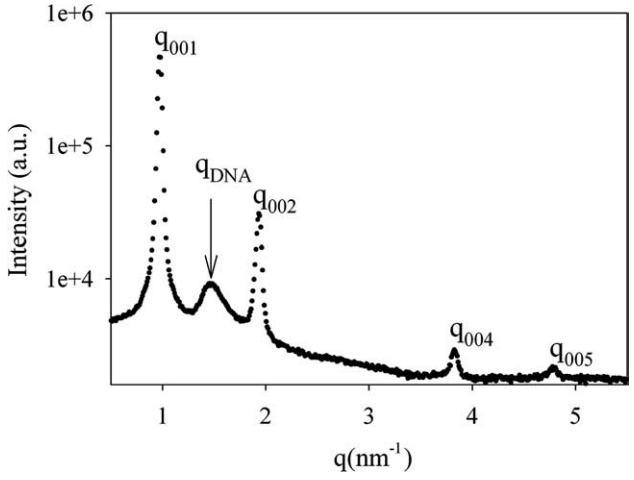

Fig. 4. SAXS pattern of DOTAP/DOPC-DNA complex ($\Phi_{DOPC} = 0.61$, $\rho = 1$, $T = 20$ °C). The (0 0 1) Bragg reflections of the lamellar phase are indicated, while the DNA peak is marked by an arrow.

shed more light on this point lipoplexes were prepared with different hydration schedules.

In the first case, lipid film was hydrated ($t = 0$), the lipid emulsions were let to equilibrate at $T = 4$ °C for 48 h and, subsequently, DNA was added. Structure of so-prepared lipoplexes was identified as a lamellar phase ($d = 6.85$ nm) and did not vary as a function of time.

In the second case, lipoplexes were prepared by injecting sonicated DNA to lipid emulsions immediately after hydration of the lipid film ($t = 0$). In this case, the lamellar structure of lipoplexes ($d = 6.5$ nm at $t = 0$) changed as a function of time in that it monotonously swelled up to the repeat distance d reached its final value ($d = 6.85$ nm, $t = 48$ h). Thus, different approaches in preparation lead to the same equilibrium state. It means that fast relaxational processes (within days) responsible for the hypothesized phase transition were excluded.

Otherwise, there is evidence in the literature of very long time scale relaxational processes [24]. To deeper investigate this point, SAXS experiments were replicated after long incubation time (three months at $T = 4$ °C) and the structure of the lamellar phase (data not reported) was found to be exactly the same, i.e. superimposable SAXS patterns were detected. All these observations allowed us to conclude that the equilibrium state of this system is likely to be unique. As a result, we could conclude that the disagreement with the structural results of Congiu Castellano et al. [17] can be explained neither in terms of fast (within days) or slow (within months) relaxation processes.

In order to give explanation for the deep discrepancy with the structural results of Congiu Castellano et al. [17], a comprehensive EDXD study was performed. CL–DNA samples were freshly prepared, deposited on the surface of silicon wafers and dehydrated under a gentle nitrogen flux. They were first characterized in this almost dry condition and, then, fully hydrated from a vapour-saturated atmosphere as reported elsewhere [25]. The total hydration process lasted 72 h and was completed when traces of water condensation appeared at the solid/air interface of the lipid/DNA film. Although a detailed hydration kinetic study is not the aim of the present work, here we emphasize the central importance of very long time scale relaxation processes in hydrating and ordering lipid/DNA complexes as recently highlighted by the outstanding work of McManus et al. [24].

The EDXD pattern of a representative fully hydrated sample ($\Phi_{DOPE} = 0.5$, $\rho = 9.8$, $T \sim 20$ °C) is shown in Fig. 5a. All over the broad compositional range investigated ($1 < \rho < 10$, $\Phi_{DOPE} = 0.5$–0.61) the system is unmistakably organized in a multilamellar structure with periodicity $d = 7.0 \pm 0.1$ nm and no inverted hexagonal phase could ever be detected. The broad DNA–DNA correlation peak was also evident ($d_{DNA} =$

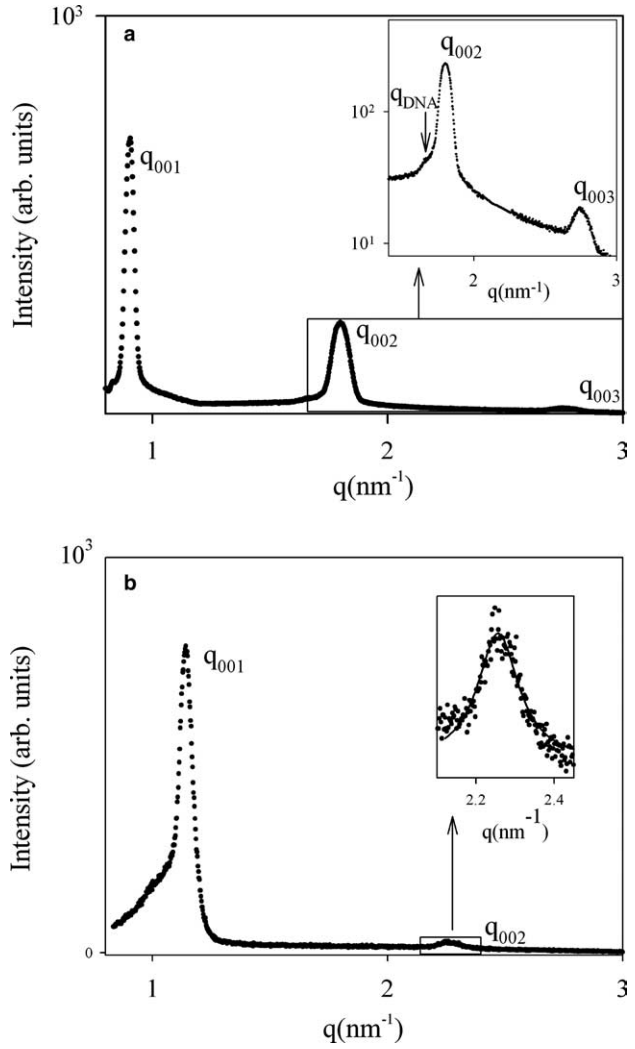

Fig. 5. (a) EDXD scan of fully hydrated DC-Chol/DOPE-DNA complex ($\Phi_{DOPE} = 0.5$, $\rho = 9.8$, $T = 20$ °C). In the inset, the second and third Bragg peaks of the lamellar phase and the DNA peak (arrow) are shown. (b) EDXD scan of dehydrated DC-Chol/DOPE-DNA complex ($\Phi_{DOPE} = 0.5$, $\rho = 2.3$, RH = 50%, $T \sim 20$ °C). In the inset the (0 0 2) Bragg reflection of the lamellar phase is shown (solid line is the best fit to the data). Dehydration reduces the lamellar *d*-spacing and forces the long-range order to be lost. The DNA–DNA peak was not observed. Upon hydration, it regularly emerged.

3.40 nm). Interestingly, the inner structure of fully hydrated solid-supported DC-Chol/DOPE-DNA complexes was found to be essentially the same as their counterpart in buffer solution.

The main effect of dehydration (Fig. 5b) was a considerable reduction of the lamellar *d*-spacing $d = 5.50 \pm 0.05$ nm ($\Phi_{DOPE} = 0.5$, $\rho = 2.3$, RH = 50%, $T \sim 20$ °C) clearly due to the reduction of the interbilayer water spacing.

In addition, we observe that, upon dehydration, the long-range order in the multilamellar lipid plus DNA system decreases with respect to the fully hydrated condition. Progressive disappearance of higher order reflections and broadening of diffraction peaks (Fig. 5b) reflects the dehydration-induced decrease of spatial coherence along the normal to the lipid bilayer according to recent results [25]. The DNA–DNA diffraction peak emerged only in the EDXD patterns of fully hydrated samples. Thus, dehydration forced the DNA chains regular packing within the interbilayer region to be lost. This observation was again interpreted in terms of the above mentioned importance of relaxation process in hydrating and ordering lipid/DNA systems [24].

In conclusion, we have shown that DC-Chol/DOPE-DNA lipoplexes in buffer solution form a well-ordered multilamellar structure L_α^C with distinct DNA packing density regimes.

The existence of fast and slow relaxation processes was excluded by preparing the lipoplexes with different hydration schedules and replicating the experiments after short (within 3 days) and long (3 months) incubation times.

When fully hydrated on a solid-support, DC-Chol/DOPE-DNA lipolexes show the same structural features and are not assembled in an inverted hexagonal one as recently reported [17]. In addition, by evaluating the DNA packing density, we have found that the surface charge density of DC-Chol/DOPE liposomes is higher with respect to that of the extensively studied DOTAP/DOPC liposomes. To what extent this latter finding could be related to TE is an open question that needs to be deeply investigated.

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