# Is the formation of cationic lipid-DNA complexes a thermodynamically driven phenomenon? Structure and phase behavior of DC-Chol/DNA complexes say not

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(Received 17 May 2006; accepted 12 June 2006; published online 27 July 2006)

The currently accepted mechanism of formation of cationic lipid-DNA complexes (lipoplexes) relies on the basic assumption that equilibrium structure of lipoplexes is regulated by thermodynamics. The main consequence is that neutral lipoplexes are one phase whereas positively (or negatively) charged ones coexist with excess lipid (or excess DNA). The authors report a small angle x-ray diffraction study on the structure of lipoplexes made of the cationic lipid 3$\beta$-[*N*-(*N*,*N*-dimethylaminoethane)-carbamoyl]cholesterol and calf thymus Na-DNA. Here the authors show that positively charged lipoplexes can coexist with unbound DNA and they claim that steric size effects are definitely important to determine the equilibrium structure of lipoplexes. © *2006 American Institute of Physics*. [DOI: 10.1063/1.2243869]

Complexes composed of cationic liposomes (CLs) and DNA, named lipoplexes, are the most promising nonviral gene delivery systems.[1] In general, CLs used for applications in gene therapy include two kinds of lipid molecules:[2,3] cationic lipids and neutral "helper" lipids. Cationic lipids serve as DNA condensing agents and allow lipoplexes to approach the anionic surface of a cell. Neutral lipids control the geometry and topology of lipoplexes and adjust the DNA packing density within them. Neutral lipid is considered even indispensable when the cationic lipid alone does not assemble into lamellar bilayers but spontaneously forms micelles in aqueous solution.[4] In that case, it is generally assumed that there is no encapsulation of the DNA by the cationic micelles but rather binding at their surface while the size and the shape of the micelle are maintained. In a recent paper, a novel structure, termed $H_1^C$, has been identified with hexagonally arranged tubular lipid micelles surrounded by DNA rods forming a three-dimensionally continuous substructure.[5] This is a point of great general interest, even though only marginally addressed so far, in view of better understanding the mechanism of DNA interacting with cationic liposomes and micelles.

In this letter, we report a small angle x-ray diffraction (SAXD) study on the structure and phase behavior of the binary complex made of the cationic lipid 3$\beta$-[*N*-(*N*,*N*-dimethylaminoethane)-carbamoyl]cholesterol (DC-Chol) and calf thymus Na-DNA. DC-Chol is a derivative of natural cholesterol and one of the most frequently used cationic lipids in gene therapy.[6] It contains a tertiary amino head group linked to a hydrophobic domain consisting of a sterol backbone (Fig. 1). At full hydration, DC-Chol lipids are truncated cone shaped molecules that favor the formation of wormlike cylindrical micelles in aqueous media.[7]

DC-Chol was purchased from Avanti Polar Lipids (Alabaster, AL) in the lyophilized form and used without further purification. Calf thymus Na-DNA was purchased from Sigma (St. Louis, MO). DC-Chol/DNA lipoplexes were formed by adding appropriate amounts of sonicated DNA solution to DC-Chol dispersions. The nominal composition of the lipoplexes is given by the charge ratio $\rho$ between the positive charge carried by DC-Chol head groups and the negative charge carried by DNA bases. The complex is stoichiometrically charge neutral when the number of DC-Chol molecules and DNA bases are equal ($\rho=1$) whereas varying $\rho$ may result in the formation of positively ($\rho>1$) or negatively charged ($\rho<1$) lipoplexes. Samples were sealed in a 1.5 mm diameter quartz x-ray capillaries. Small angle x-ray diffraction experiments were performed by using a commercial x-ray diffractometer (Bruker, AXS D8Focus) operating at 50 kV and 40 mA. The background due to bulk solution and empty capillary was subtracted from the collected data. All measurements were performed at room temperature.

Figure 2 shows the SAXD pattern of nominally charge-neutral DC-Chol/DNA complex ($\rho=1$). The set of lamellar peaks labeled as (00*l*) arises from the well-known supramolecular structure of DC-Chol/DNA complex consisting of alternating lipid bilayers and DNA monolayers ($L_\alpha^C$ phase).[8] The lamellar periodicity along the normal to lipid bilayer, $d=2\pi/q_{001}=63.5\pm0.5$ Å, is given by the thickness of lipid bilayer plus the thickness of the water region where DNA is confined $d_W$.[8] The peak marked by arrow (inset of Fig. 2) is attributed to the one-dimensional (1D) in-plane lattice of the intercalated DNA strands[8] with packing spacing $d_{\mathrm{DNA}}=2\pi/q_{\mathrm{DNA}}=24.2\pm0.5$ Å. Structural parameters of lipoplexes were deduced from the electron density profile (EDP) along the normal to the bilayers $z$ routinely calculated.[9,10] Figure 3 shows the EDP of isoelectric DC-Chol/DNA lipoplex. The central minimum corresponds to the middle of the lipid bilayer whereas the two maxima at $z=\pm19.4$ Å represent the

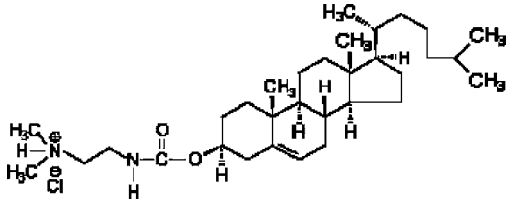

FIG. 1. Chemical structure of DC-Chol lipid.

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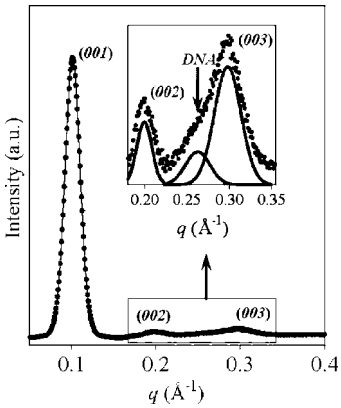


FIG. 2. SAXD pattern of DC-Chol/DNA lipoplexes ($\rho=1$). Bragg peaks labeled as (00*l*) arise from a multilayer lamellar structure with periodicity $d=2\pi/q_{001}=63.5$ Å. The inset shows a view of the higher-$q$ region on an expanded vertical scale. DNA-DNA peak is marked by an arrow. Solid lines show the fitting functions.

lipid polar head groups, their distance $d_{HH}=38.8$ Å, giving a consistent estimate of the DC-Chol bilayers thickness. The more pronounced peaks at $z=\pm 31.7$ Å are due to the DNA strands sandwiched between DC-Chol bilayers.

As DC-Chol lipids do not assemble in bilayers structure but form cylindrical micelles in aqueous solution,[7] our structural findings suggest that it was therefore the DNA that allowed the DC-Chol micelles to come into contact and fuse by reducing the repulsive barrier due to electrostatic, steric, and hydration forces.[11,12] As a consequence, DNA promoted the formation of DC-Chol bilayers and multilamellar lipoplexes even with no helper lipid added.

SAXD profiles (not reported) of overcharged DC-Chol/DNA complexes in excess of DNA ($\rho=0.5$) and in excess of cationic lipid ($\rho=2$ and 3) exhibited the same general features and all the structural parameters, as calculated by the EDPs, are listed in Table I. It is noteworthy to observe that, at $\rho=3$, the 1D DNA packing is maximum, with the DNA-DNA distance $d_{DNA}$ approaching the diameter of DNA plus a hydration shell ($\sim$25 Å). Our structural results are the first findings ever reported in the literature of positively charged lipoplexes with the highest possible DNA packing density ($d_{DNA}\sim 25$ Å). Hence, even though at lower charge ratios ($\rho<3$) the complex should be expected to accommodate further DNA,[8] DNA chains cannot come nearer and the DNA packing density (i.e., the DNA-DNA distance $d_{DNA}$) is irrespective of the charge ratio $\rho$ (Table I). Since DNA chains are so densely packed by DC-Chol molecules, further DNA

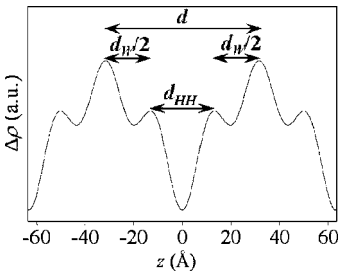


FIG. 3. Electron-density profile along the normal to the lipid bilayer $z$ of charge-neutral ($\rho=1$) DC-Chol/DNA lipoplexes. The lamellar periodicity $d$ is given by the thickness of DC-Chol bilayers $d_{HH}$ plus the thickness of the water region occupied by DNA molecules $d_W$.

TABLE I. Lamellar $d$ spacing, lipid bilayer thickness $d_{HH}$, and DNA-DNA distance $d_{DNA}$ of DC-Chol/DNA lipoplexes as a function of $\rho$, calculated from the EDPs.

| $\rho$ | $d$ (Å) | $d_{HH}$ (Å) | $d_{DNA}$ (Å) |
|---|---|---|---|
| 0.5 | 63.2 | 38.4 | 23.9 |
| 1 | 63.5 | 38.8 | 24.2 |
| 2 | 63.7 | 38.5 | 23.5 |
| 3 | 63.8 | 38.9 | 24.2 |

cannot enter the complex as a function of decreasing $\rho$, the complex coexists with unbound DNA, and the actual composition becomes more and more different from the nominal one.

The currently accepted mechanism of formation of cationic lipid (CL)-DNA complexes relies on the basic assumption that structure and phase behavior of lipoplexes as a function of $\rho$ are driven by thermodynamics: the lipoplex is one phase (with no excess material) close to the isoelectric point whereas it is expected to separate into complex plus excess lipid for $\rho>1$ and complex plus excess DNA for $\rho<1$.[8,13] In this scenario, electrostatic repulsions play a key role to adjust DNA packing density in that they set an upper limit on the amount of excess material that a complex can accommodate.

We emphasize that our structural findings demonstrate, for the first time, that positively charged lipoplexes can coexist not only with excess lipid, as assumed so far, but also with unbound DNA. It means that steric size effects and packing properties dominate over thermodynamics and are even more important than electrostatic interactions to determine the equilibrium structure of overcharged lipoplexes.

In conclusion we have shown that, upon complexation, DC-Chol micelles transform into multilamellar structure with DNA intercalated between DC-Chol bilayers. Even more, we have found unambiguous evidence that the nominal charge ratio $\rho$ does not govern universally the structure of lipoplexes. Thus, we claim that the universally accepted mechanism of formation of overcharged lipoplexes may be different from which hypothesized so far and needs to be further investigated.

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