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We have carried out a systematic structural study of novel liquid-crystal phases of self-assembled neutral lipid-DNA-metal cation complexes in water solution. Small-angle X-ray scattering experiments have given new insight into the structures of these ternary complexes. We have shown that different divalent metal cations are equally active in promoting the DNA condensation into the ternary complex when different lipid and DNA species are used. In addition, strong indication has been found that the phase of the complexes reflects the structure and symmetry of the parent lipid phase. These achievements provide an important structure-composition correlation that may drive the best design of these materials for future applications as non-viral DNA carriers in gene therapy.

Keywords: complexes; DLPC; DNA; DOPC; DOPE; x-ray diffraction

INTRODUCTION

Gene therapy is a technique for correcting defective genes responsible for disease development. The goal is the transfer of extracellular genetic material into appropriate cells of a patient providing therapeutic

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effects. Recent completion of the working draft of the human genome has convinced researchers about the possibility of using gene medicines to fight genetic diseases. Because of its aim to eliminate causes rather than symptoms of diseases, gene therapy is believed by many scientists to be the therapy of the 21st century.

It is now well-established that the development of gene therapy is strictly depending on safe and efficient gene delivery vectors. The entire field of non-viral gene therapy has known a great development after the pioneering studies of Felgner [1] on *lipoplexes*, i.e., the condensed complexes between cationic liposome (CLs) and DNA [2–4]. CL-DNA complexes have been tested as efficient vehicles for the delivery of DNA, both *in vitro* and *in vivo*. The plain advantages shown by lipoplexes include simple manufacture, ease in handling and preparation techniques, low toxicity, lack of immune response, ability to link large pieces of DNA and act as carriers of extracellular DNA across outer cell membranes and nuclear membranes (transfection). In spite of this, the transfer efficiency is still relatively low compared to that of viral vectors. Some findings suggest that this could be related to a non specific immunogenic response [6,7]. The biological fate of lipid-DNA complexes inside the cell involves complex mechanisms. Therefore, it is important to understand the correlations between the lipid-DNA structures and their biological activity. Indeed, the transfection efficiency of a given complex depends highly on its structural and physicochemical properties. For these reasons, extensive effort is being addressed worldwide to understanding the various possible structures of L-DNA complexes.

Within this general outline, we have recently started an extended project focused on the study of the self-assembled association of neutral lipid (L), DNA and divalent metal cations (Me^{2+}) in ternary L-DNA-Me^{2+} complexes. The structural and morphological studies of mixtures of dioleoyl-phosphatidylcholine (DOPC), DNA and Me^{2+} ($Me^{2+} = Mn^{2+}$, Mg^{2+}, Co^{2+}, Fe^{2+}) in water solutions have revealed that such complexes exhibit the lamellar symmetry of the liquid-crystalline L_α phase [8,9]. Their structure consists of smectic-like arrays of stacked lipid bilayers with monolayers of DNA molecules intercalated within the intervening water gaps. More recently, we carried out an extended study at higher resolution on a variety of L-DNA-Me^{2+} complexes starting from different neutral liposomes (L), namely dioleoyl-phosphatidylcholine (DOPC), dilinoleoyl-phosphatidylcholine (DLPC) and dioleoyl-phosphatidylethanolamine (DOPE), different divalent metal cations, i.e., Mn^{2+}, Mg^{2+}, Co^{2+}, Fe^{2+} and different varieties of DNA (from calf thymus and salmon sperm). The goal of this study is twofold: (*i*) to test whether the fusogenic action of the

metal cations is equally active in promoting and stabilizing triple complexes L-DNA-Me^{2+} when different neutral lipids and DNA molecules are used and (*ii*) to test to which extent the structure and phase-symmetry of the pure lipids in water solution reflect into the structure of the self-assembled complexes. The latter is a particularly important aspect connected with structure-transfection efficiency relationships. For example, experimental evidence exists [10] that inverted hexagonal lipid phases (H_{II}) exhibit higher transfection efficiency than lamellar phases (L_α). On the other hand, the formation of a L_α or a H_{II} phase in pure lipid water-solutions depends primarily on the intrinsic shape of the molecule [11]: lipids with small and/or less hydrated headgroups (e.g., phosphatidylethanolamine) often form H_{II} phase whereas lipids with large and/or well hydrated headgroups (e.g., phosphatidylcholine) preferably form L_α phase. Within this scheme, understanding whether the tendency of a lipid molecule to form a given phase in maintained in the formation of the complex is an important aspect of investigation.

EXPERIMENTAL

Dioleoylphosphatidylcholine (DOPC), dioleoyl-phosphatidylethanolamine (DOPE) and dilinoleoyl-phosphatidylcholine (DLPC) were purchased from Avanti Polar Lipids. DNA from calf thymus (DNA_{ct}) and DNA from salmon sperm (DNA_{ss}) and metal ions Mn^{2+}, Mg^{2+}, Co^{2+}, Fe^{2+} as chloride were purchased from Sigma Chemical Co. The ternary complexes were prepared in HEPES buffer (20 mM, pH = 7.2). DOPC or DLPC were dissolved in chloroform solution and then the solvent was removed first in a stream of nitrogen, then under vacuum for two hours in order to remove any trace of solvent. The multilamellar vesicles (MLVs) were prepared vortexing the lipid suspension several times during the hydration period (4 hours), and leaving then this suspension to equilibrate for 1 day. The same procedure was utilized for DOPE; in this case the thin lipid film, obtained after evaporation of the solvent, was hydrated in excess of HEPES solution. The triple complexes were obtained upon mixing equal volume of the solutions (with appropriate concentration of the free components) previously prepared. The samples were prepared at different relative molar ratio of the three components (L:DNA:Me^{2+}) varying the metal cations. Initial concentration were as follows: DOPC, DLPC, DOPE, 1.8 mM; DNA, 2.4 mM; Me^{2+}, 0.6–14 mM. SAXS and WAXS measurements were carried out at the ID02 beamline of the European Synchrotron Radiation Facility (ESRF, Grenoble, France). A monochromatic beam of 12.5 KeV($\lambda = 0.995$ Å) was used. We investigated

the q range between $0.04\,\text{Å}^{-1}$ and $0.5\,\text{Å}^{-1}$ with a resolution of $5{\cdot}10^{-3}\text{Å}^{-1}$ (FWHM). Exposure time of 10 s/frame was used to avoid the radiation damage.

RESULTS AND DISCUSSION

As concerns the DOPC-DNA_{ct}-Mn^{2+} complexes with DNA from calf thymus, the present higher resolution experiments mainly confirmed previous results [8]. Figure 1A shows the SAXS pattern of the ternary complex DOPC:DNA_{ct}:Mg^{2+} at 3:4:24 molar ratio. This pattern exhibits two sets of peaks (each one including the fundamental and higher-order harmonics) related to distinct lamellar structures, L_α^c (where c stands for complex) and L_α (with layer spacings $d_1 = 75.2\,\text{Å}$ and $d_2 = 59\,\text{Å}$, respectively), corresponding to the DOPC-DNA_{ct}-Mg^{2+} complex together with the coexisting uncomplexed pure lipid. The electron density profile of the L_α^c phase in the unit cell, obtained by Fourier inversion of the SAXS data following the procedure described in ref, [8], is shown in Figure 1B. The two peaks of electron density correspond to phospholipid headgroups, while the minimum at the centre of the membrane correlates with terminal hydrocarbon chain region. The distance between the centers of the density maxima gives the phosphate-phosphate group separation $d_{PP} \approx 45.1\,\text{Å}$, it follows the water-layer thickness $d_w = d_1 - d_{PP} \approx 75.2\,\text{Å} - 45.1\,\text{Å} \approx 30.1\,\text{Å}$,

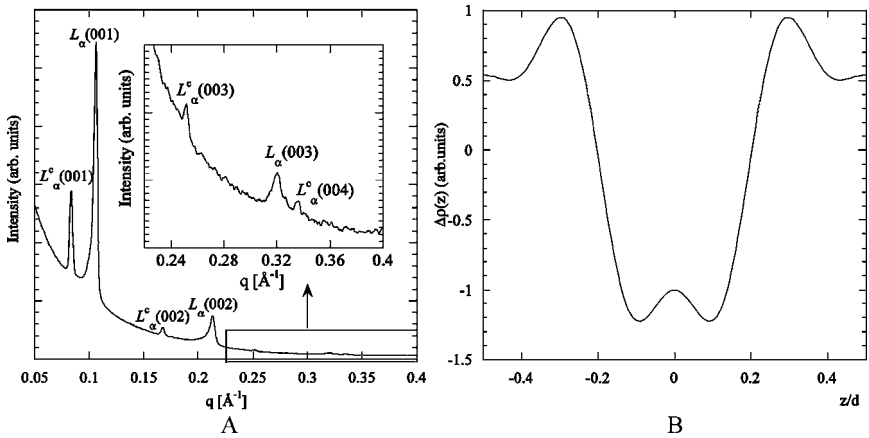

FIGURE 1 A) Synchrotron SAXS pattern of DOPC:DNA_{ct}:Mg^{2+} at 3:4:24 molar ratio. B) Electron density profile along the normal to the bilayers in the L_α^c phase of the triple complex DOPC:DNA_{ct}:Mg^{2+}.

sufficient to accommodate a double-stranded DNA helix surrounded by a one-water hydration layer plus two thin layers of hydrated metal ions.

Figure 2 shows the SAXS pattern of the water mixture of DOPC:DNA_{ss}:Mn^{2+} (3:4:18). X-ray data show the same two coexisting lamellar structures observed in the previous sample, with only a small reduction of the unit cell of the L_α^c phase, i.e. $d_1 = 74.1$ Å.

A systematic series of SAXS measurements on DOPC-DNA_{ct}-Me^{2+} (3:4:n) complexes prepared from different metal cations (Me^{2+} = Mg^{2+}, Mn^{2+}, Co^{2+}, Fe^{2+}) was also performed as a function of the metal ion moles (n). All these measurements showed the formation of the triple complex and its coexistence in equilibrium with the pure DOPC multilamellar-vesicle (liposome) structure. The relevant structural data obtained from these spectra are summarized in Figure 3. A remarkable constancy of the lamellar spacing of the L_α^c phase (d_1) accompanied by a slight decreases of the lamellar repeat distance of the uncomplexed lipid L_α phase (d_2) is apparent, which agrees with the model of complex formation proposed in ref. [8,9].

Similar results were observed for the complexes prepared with the neutral lipid DLPC. In Figure 4 a representative example of the diffraction pattern of DLPC:DNA_{ct}:Mn^{2+} (3:4:12) is reported (A), together with the electron density profile along the normal to the

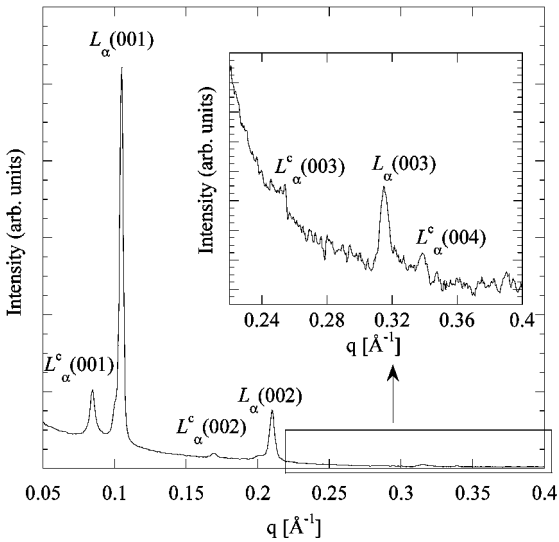

FIGURE 2 Synchrotron SAXS pattern of DOPC:DNA_{ss}:Mn^{2+} at 3:4:18 molar ratio.

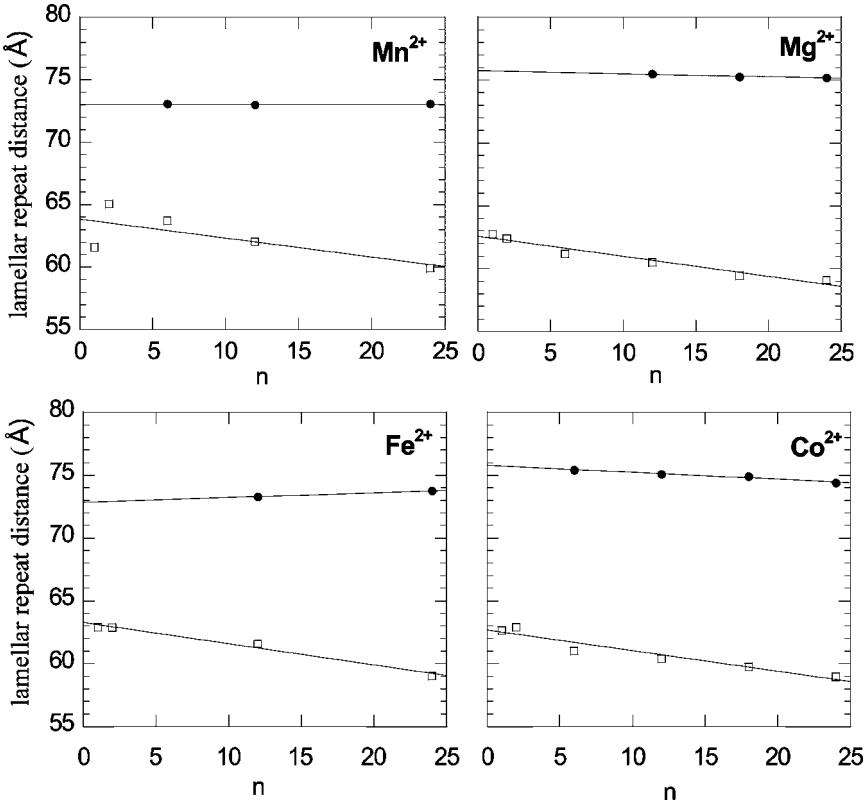

FIGURE 3 Lamellar d-spacing of the L_α^c of the triple complex (●, d_1) and the L_α phase of DOPC (□, d_2) as a function of the metal mole number, n, in DOPC:DNA_{ct}:Me^{2+} complexes ($Me^{2+} = Mn^{2+}$, Mg^{2+}, Fe^{2+}, Co^{2+}) at molar ratio 3:4:n.

bilayers (B). Table I collects the relevant XRD data of the complexes obtained from different metal cations (at the same 3:4:12 molar ratio L:DNA: Me^{2+}). From the layer spacing d_1 and the bilayer thickness determined form the density profile, d_{PP}, we could calculate the water-layer thickness $d_w = d_1 - d_{PP}$ to be in the range between 28 Å and 30 Å, depending on the metal cation. These values are comparable to those obtained for the previous ternary complexes DOPC:DNA_{ct}:Me^{2+}.

All the above investigated complexes exhibit the same structure and symmetry of the pure lipid parent phase (i.e. the L_α phase) irrespective of the nature of the cation and DNA. The L_α^c phase of these complexes consists of an ordered multilamellar assembly similar to that found in CL-DNA complexes [5], where the hydrated DNA helices are

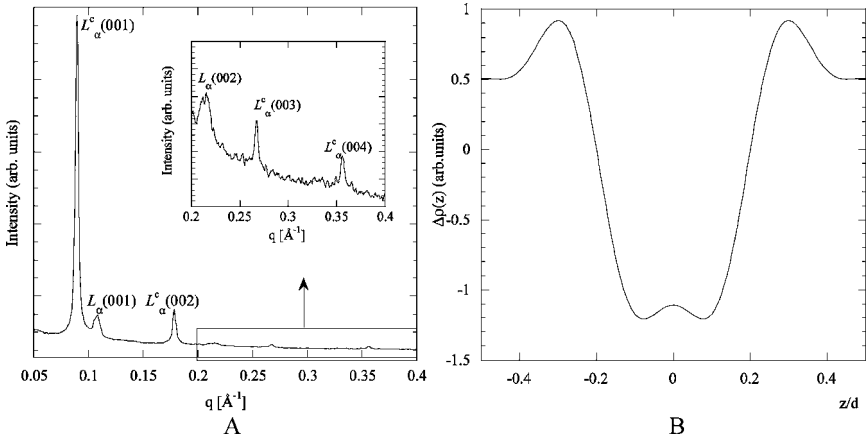

FIGURE 4 A) Synchrotron SAXS pattern of DLPC:DNA_{ct}:Mn^{2+} at 3:4:12 molar ratio. B) Electron density profile, along the normal to the bilayers in the L^c_α phase of the triple complex DLPC:DNA_{ct}:Mn^{2+}, calculated from data of Figure 2A.

sandwiched between the liposome bilayers (Fig. 5B). Previous experimental data [8] have shown that globules of the triple complex in the L^c_α phase coexist in equilibrium with globules of multilamellar vesicles of the neutral lipid in the L_α phase, the volume ratio of the two structures depending on the molar ratio of the components. The results of the present study suggest that an increase of the incubation time after sample preparation changes the situation in favor of the ternary complex.

Changing the basic geometrical shape of the lipid molecule from cylindrical (DOPC) to wedge-like (DOPE) is expected to determine structural transformations of the corresponding L-DNA-Me^{2+} complexes. In fact, it is known that the equilibrium phase of pure DOPE in excess water is the inverted hexagonal, H_{II}, whose structure elements are infinitely-long rigid rods, all identical and cristallographically equivalent, regularly packed in a 2D hexagonal lattice. As recently demonstrated [12], DOPE and divalent metal cations (Fe^{2+}, Co^{2+}, Mn^{2+}, Mg^{2+}) in water solution can condense DNA into a novel inverted-hexagonal phase, H^c_{II}, in which DNA strands fill the water gap inside the cylinders of pure DOPE. The structure, derived from synchrotron XRD, consists of cylindrical DNA strands coated by neutral lipid monolayers and arranged on the 2D hexagonal lattice. As a representative example, Figure 6(A) shows the SAXS pattern of DOPE-DNA_{ct}-Fe^{2+} complex at 3:4:12 molar ratio. Two distinct sets of peaks are

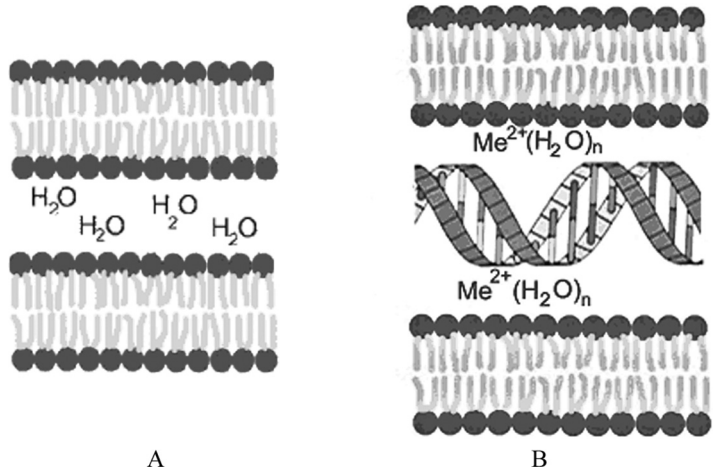

FIGURE 5 Schematic pictures of the structures of the L_α phase of the DOPC (A) and the L_α^c phase of the triple complex L-DNA_{ct}-Me^{2+}(B) (after Francescangeli *et al*. [8]).

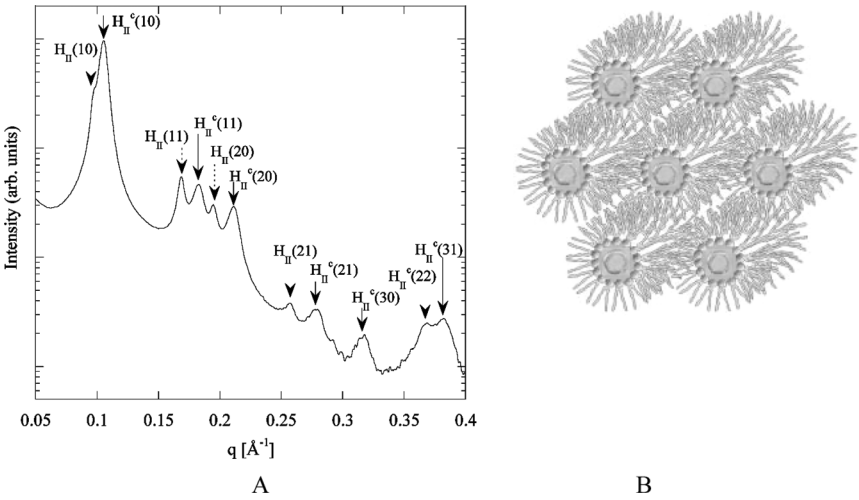

FIGURE 6 A) SAXS pattern of the DOPE-DNA_{ct}-Fe^{2+} complex at 3:4:12 molar. B) Schematic picture of structure of the H_{II}^c phase of DOPE-DNA_{ct}-Me^{2+} complexes. The DNA strands are surrounded by a lipid monolayer, with the DNA-lipid inverted cylindrical micelles arranged on a hexagonal lattice.

identified, labelled as H_{II} and H_{II}^c, which can be indexed on 2D hexagonal lattice with different unit cell spacings, namely $a = 74.5\,\text{Å}$ and $a_c = 68.7\,\text{Å}$, respectively. The first set of peaks corresponds to the phase H_{II} of pure DOPE, whereas the second set is consistent with the 2D columnar inverted hexagonal phase, H_{II}^c, of the DOPE-DNA_{ct}-Fe^{2+} complex shown in Figure 6(B).

In conclusion, this study has shown the self-assembled formation of ternary L-DNA-Me^{2+} complexes in a variety of systems differing for the chemical nature of the components (lipid, DNA or metal cations). Data on structures confirm that in all these systems the fusogenic action of divalent metal cations (between the polar headgroups of the lipids and the negatively-charged phosphate groups of DNA) is equally active in promoting and stabilizing the formation of the complex. In addition, the results of this study, even though still limited to a small number of different lipids, seem to confirm that the structure of each self-assembled complex reflects the structure and symmetry of the parent phase of the pure lipid. These achievements provide an important structure-composition correlation that may drive the best design of these materials for future applications as non-viral DNA carriers in gene therapy.

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