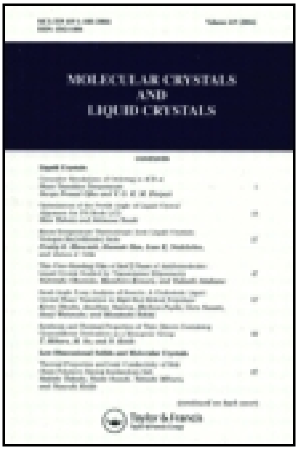

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X-RAY Diffraction Study of Dioleylphosphatidylcholine-DNA-Mn 2+ Complexes

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X-RAY DIFFRACTION STUDY OF DIOLEYLPHOSPHATIDYLCHOLINE-DNA-MN^{2+} COMPLEXES

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We have studied the structural properties of the triple complex dioleoylphosphatidylcholine-DNA-Mn^{2+} by means of synchrotron x-ray diffraction. The data revel a novel multilamellar structure similar to that recently found in cationic liposomes-DNA complexes. The study of the bilayer undulations suggests that condensation of the DNA within the lipid bilayers reduces the flexibility of the lamellae in the complex and suppresses almost completely the undulatory fluctuations. These complexes are of grate interest for potential applications as synthetically-based nonviral carriers of DNA vectors for gene therapy.

Keywords: DOPC; DNA; metal; complexes

INTRODUCTION

Extremely rapid developments in molecular biology are making gene therapy a promising new therapeutical modality. Somatic gene therapy depends on the successful transfer of extracellular DNA to the cell nucleus, with the aim of replacing a defective or adding a missing gene [1]. While appropriate plasmids (genes) can be prepared in large quantities, their efficient and safe delivery into appropriate cells in vivo has been the main obstacle so far in successful medical applications. Cationic liposomes (CL) complexed with DNA have shown to be promising non-viral gene delivery systems [2–5]. The solution structure of CL-DNA complexes was probed on length scale from subnanometer to micrometer by synchrotron x-ray diffraction (XRD) and optical microscopy [3,4,7]. In particularly, XRD revealed a novel multilamellar structure with alternating lipid bilayers and DNA monolayers [3–7].

Within the general scope of providing new biological materials of potential interest for gene delivery systems, we have recently undertaken a systematic investigation of water solutions of neutral liposomes (L), DNA and metal cations (Me^{n+}). Evidence of the formation of L-DNA-Me^{2+} triple complexes was previously given by different techniques such as turbidimetry and FT-IR spectroscopy [8]. L-DNA-Me^{2+} complexes are expected to be more stable than binary DNA-neutral liposome complexes [9,10] and to exhibit lower cytotoxicity than the complexes with CLs [11]. The structural characterization of these ternary complexes is essential in order to understand the role played by the metal cations in stabilizing the L-DNA interactions and therefore in the formation of supramolecular aggregates. To this purpose, complexes of neutral multilamellar liposome of dioleoylphosphatidylcholine (DOPC) and DNA, with different bivalent cations (Mn^{2+}, Co^{2+}, Fe^{2+}, Mg^{2+}) were recently prepared and studied by means of synchrotron XRD and freeze-fracture transmission electron microscopy [12]. The results clearly evidence the formation of triple L-DNA-Me^{2+} complexes that self-assemble when water solutions of DOPC, DNA and metal ions are mixed, which represents a striking example of supramolecular chemistry. In this paper we review the main results of the XRD structural characterization of DOPC-DNA-Mn^{2+} complexes and we report an original quantitative study of the bilayer undulations involved in the multilamellar vesicle (MLV) structure. This study gives information on the elastic properties of MLVs of the complex, which may be useful in view of future studies aimed at a deeper comprehension of the microscopic mechanism underlying the complexation and transfection processes.

EXPERIMENTAL AND RESULTS

DNA from calf thymus (bp = 2675, contour length $\cong 0.9\,\mu m$), dioleoylphosphatidilcholine (DOPC) and metal ions Mn^{2+} as chloride were purchased from Fluka. HEPES buffered solutions of DOPC, DNA and metal ions were prepared following the procedures previously reported [8]. In all cases, the triple DOPC-DNA-Me^{n+} complexes were prepared by adding the metal ion solution to the DOPC-DNA solution. Simultaneous small- and wide-angle x-ray scattering (SAXS and WAXS) measurements were carried out using the 8 keV branch ($\lambda = 1.54$ Å) of the SAXS beamline at the synchrotron radiation source ELETTRA, Trieste, Italy. Experimental details of the XRD measurements have been reported in ref. [12]. Triple complexes

DOPC-DNA-Me^{2+} were prepared using different component ratios obtained by mixing equal volumes of solutions of appropriate concentration of the free components. Measurements were performed at room temperature after two minutes of incubation of the samples.

As a representative examples, Figure 1 shows the XRD pattern of DNA-DOPC-Mn^{2+} at 4:3:12 molar ratio. Two sets of sharp peaks are identified, the first one corresponding to the fundamental and the first harmonic of the reflection at $d_1 = 73.2$ Å, the second one corresponding to the fundamental and first harmonic of the reflection at $d_2 = 60.0$ Å. The WAXS region shows only a wide diffuse peak centered about $q \approx 1.43\,\text{Å}^{-1}$ which reflects the short-range liquid-like positional ordering of the lipids within the layers, with an average distance $\approx 2\pi/q = 4.4$ Å. A similar pattern was observed for all the samples investigated, that ranged from 4:3:1 to 4:3:24 molar ratios. The above data were interpreted [12] as indicative of the coexistence of two distinct liquid-crystalline (LC) multilamellar structures with incommensurate periodicities: the d_2 spacing is associated with the $L_\alpha$phase of the DOPC whereas the higher spacing d_1 reflects the presence of the lamellar L_α^c phase of the triple lipid-DNA-metal complex ("c" stands for "condensed" or "complex"). This latter consists of a structure similar to that found in DNA-cationic liposome complexes [3,5], which is made of stacks of alternating lipid bilayers and DNA monolayers. A schematic picture of the local structural arrangement of the triple complex in the phase is shown in Figure 2. From the XRD data, it was concluded that the

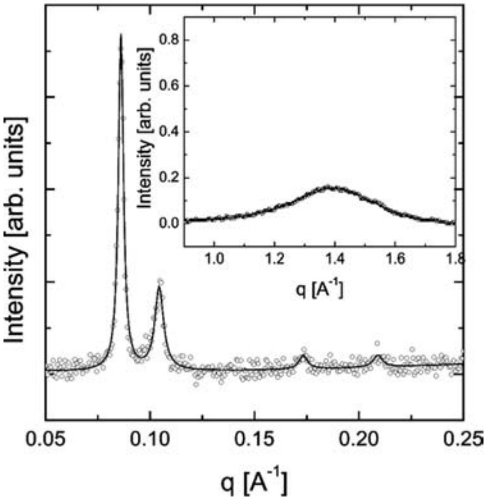

FIGURE 1 XRD pattern of DOPC-DNA-Mn^{2+} at 3:4:12 molar ratio. The continuous line is the best fit to the experimental data (o). The inset show the WAXS region.

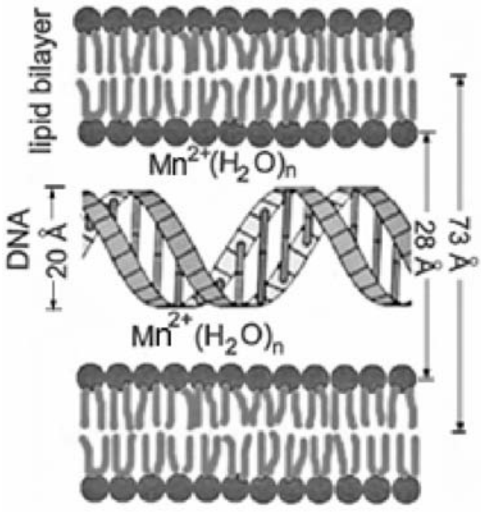

FIGURE 2 Schematic picture of the structure of the L_α^c phase of the triple complex DOPC-DNA-Mn^{2+} (after Francescangeli *et al.* [12]).

formation of the triple complex is promoted by the positively charged metal ions that bind the polar heads of DOPC with the negatively charged phosphate groups of DNA. Accordingly, neutral liposomes in the presence of metal cations behave similarly to cationic lipids: in the examined sample the Mn^{2+} ions tend to fully neutralize the phosphate groups on the DNA, thus replacing and releasing in the solution the original counterions [12].

BILAYER FLUCTUATIONS

Accurate evaluation of the entity of the bilayer fluctuations in liposomes imposes an accurate determination of the bilayer absolute form factors, which in turn requires high resolution diffraction data [13]. This was not possible in our case because of the lack of high-resolution and absolute XRD data. Therefore, an estimate of the thermal fluctuation was attempted following the procedure first reported by Leadbetter [14,15].

The positional distribution function of the lipid bilayers, $f(z)$, is expanded in terms of a Fourier series

$$f(z) = \frac{1}{d}\left[1 + \sum_l 2\tau_l \cos(2\pi l z/d)\right], \qquad (1)$$

where d is the lamellar repeat distance and $\tau = \langle\cos(2\pi lz/d)\rangle$ is the l-th layer order parameter. The scattering factor F_l corresponding to the scattering vector q along the normal z to the layers can be written as

$$F_l = \sum_m \langle f_m \exp(2\pi i l z'_m/d)\rangle, \tag{2}$$

where m denotes the atom (or molecular fragment) in the molecule, f_m is the atomic form factor of the m-th atom (or fragment), and the average is taken over all molecular configurations. If we denote by z_{m0} the z coordinates for a perfectly ordered lamellar structure (i.e. $\tau_l = 1$), then $z'_m = z_{m0} + z_m$, where z_m gives the fluctuation of the m-th atomic position around z_{m0}, and Eq.(2) can be rewritten

$$F_l = \sum_m \langle\exp(2\pi i l z_m/d)\rangle f_m \exp(2\pi i l z_{m0}/d). \tag{3}$$

If we suppose z_m to be the same for all atoms, then the intensity I_l of the $00l$ reflection can be written as

$$I_l = C|F_l|^2 = \tau_l^2 I_l^0 \tag{4}$$

where C is a proportionality constant, and I_l^0 is the intensity for a perfectly ordered lamellar structure. Indeed, more accurate investigations [16] have shown that the thermal spreadings of the positional distribution functions of the different molecular fragments in the L_α phase of DOPC differ by less than 30% from their average value. Even though this is not a negligible difference, we believe it is not so large to make unreliable an estimate of the average thermal fluctuations based upon the above approximation.

When higher orders of reflection are observed, then the assumption of a simple model for I_l^0 and a form for $f(z)$ enables one to obtain τ_l. By assuming a Gaussian distribution for $f(z)$, i.e. $f(z) = \left[2\pi\langle z^2\rangle\right]^{-1/2} \exp\left(-z^2/2\langle z^2\rangle\right)$, the layer order parameters take the form

$$\tau_l = \exp\left(-2\pi^2 l^2 \langle z^2\rangle/d^2\right), \tag{5}$$

where $\langle z^2\rangle$ is the mean-square displacement of the atoms along the normal to the layers, i.e. the mean-square longitudinal fluctuation of the layers. From Eq.(4) we obtain

$$\frac{I_{l+1}}{I_l} = \frac{\tau_{l+1}^2}{\tau_l^2}\frac{I_{l+1}^0}{I_l^0}. \tag{6}$$

The intensity ratios I_{l+1}^0/I_l^0 can be calculated for a simple model where the different units constituting the periodic bilayer are perfectly ordered. Within this model each unit is characterized by its own length, area and total number of electrons. In our case, we considered the distinct quasi-molecular fragments identified in the joint-refinement structural determination of ref. [16] and shown in Figure 3. The structural data (fragment lengths, thicknesses, area/chain and area/headgroup) were obtained from combined standard atomic bond-lengths and previous structural determinations available in the literature [17,18]. These data allowed us to calculate the volume of each unit. Then, given the number of electrons of each unit, the following values of average volume electron density of the distinct quasimolecular fragments were obtained: $\rho_1 = 0.290\,\mathrm{e}^-/\text{Å}^3$, $\rho_2 = 0.258\,\mathrm{e}^-/\text{Å}^3$, $\rho_3 = 0.299\,\mathrm{e}^-/\text{Å}^3$, $\rho_4 = 0.453\,\mathrm{e}^-/\text{Å}^3$, $\rho_5 = 0.328\,\mathrm{e}^-/\text{Å}^3$, $\rho_6 = 0.441\,\mathrm{e}^-/\text{Å}^3$, and $\rho_7 = 0.422\,\mathrm{e}^-/\text{Å}^3$. In addition, $\rho_8 = \rho_{\mathrm{H_2O}} = 0.333\,\mathrm{e}^-/\text{Å}^3$ or $\rho_8 = \rho_{\mathrm{H_2O+DNA}} \approx 0.395\,\mathrm{e}^-/\text{Å}^3$, respectively for DOPC and the triple complex. The form factors F_l for the perfectly ordered structure was then calculated as the transform of the corresponding one-dimensional electron density profile $A\rho(z)$, $F_l = \int_{-d/2}^{d/2} A\rho(z) \exp(2\pi i l z/d)\,dz$, where $\rho(z)$ is the volume electron density profile of the single isolated repeat unit and A is the fully hydrated area/lipid measured parallel to the layers ($A = 72.2$ Å^2 from ref. [17]). The values obtained are not very sensitive to the details of the model, especially for the lower values of l. For the original DOPC vesicles, taking $l = 1$ we obtained $I_2^0/I_1^0 = 1.56$. The ratio I_2/I_1, determined from the XRD data, was found to be 0.84. With these values, Eq.(6) gives $\tau_2/\tau_1 = 0.73$. Finally, expressing

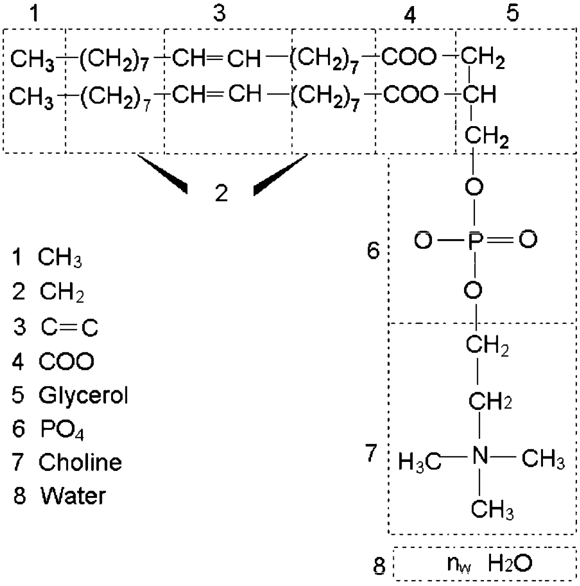

FIGURE 3 The parsing of DOPC into the quasimolecular fragments used for the calculation of the form factors of the perfectly ordered structure.

the ratio τ_2/τ_1 by means of Eq.(5) we obtained the mean-square amplitude of the layer fluctuations $\langle z^2\rangle = 21.4$ Å^2 or equivalently $z_{rms} = \langle z^2\rangle^{1/2} = 4.6$ Å. With the same calculations for the triple complex, for which the model density gave $I_2^0/I_1^0 = 0.64$, while the experimental intensity ratio was $I_2/I_1 = 0.42$, we obtained $\tau_2/\tau_1 = 0.81$ and then $\langle z_2\rangle = 19.0$ Å^2 or $z_{rms} = \langle z^2\rangle^{1/2} = 4.3$ Å. Even though the reduction of z_{rms} in the triple complex is rather small, we believe that it has physical meaning and we propose the following arguments to explain it. So far we have tacitly assumed that the spreading of the positional distribution function is entirely due to thermal fluctuations relative to the bilayer's mean position. This is true only if there are no whole-body fluctuations of the individual bilayers. This assumption is not valid for liquid-crystalline systems with very flexible lamellae, as shown by Smith *et al.* [19], Sirota *et al.*, [21] and Safinya [20]. Depending upon their elastic properties, the lamellae may undergo undulations which result in fluctuations around the mean positions, with mean square amplitude $\langle z^2\rangle_U$. Therefore, besides the thermal motions, it is important to consider in our systems the possibility of contributions to the layer fluctuations coming from these undulatory motions. The mean-square amplitude of the undulatory fluctuations is given by [19]

$$\langle z^2\rangle_U = d^2\eta_1 \ln(L/a)/2\pi^2, \tag{7}$$

where d is the bilayer repeat, L is the characteristic thickness of the sample, and a is the in-plane interparticle distance. The parameter η_1 describes the stiffness of the membrane and is defined as $\eta = q^2kT/(8\pi\sqrt{KB})$, where K is the bending modulus of lipid bilayers, B is the bulk modulus for compression, $q = 2\pi/d$ and kT is the thermal energy. If we assume a Gaussian distribution of the undulatory fluctuations then, as far as the thermal (T) and undulatory (U) motions can be considered to be independent, we can separate their contributions to the meansquare displacement as

$$\langle z^2\rangle = \langle z^2\rangle_T + \langle z^2\rangle_U. \tag{8}$$

Since very likely the thermal effects play the same role in DOPC and in the triple complex, the different values of $\langle z^2\rangle$ obtained for the two MLV structures should be ascribed to a different contribution of the undulatory term. The parameter η_1 may be determined from the lineshape of the diffraction peaks in high-resolution measurements. Smith *et al.* [19] have analyzed the lineshapes of diffraction peaks from L_α-phase of dimyristoylphosphatidylcholine (DMPC) and determined that $\eta_1 \approx 0$, but they could not rule out a value as large as 1.6×10^{-3} because of the experimental uncertainty. Using the same value for DOPC, we can establish an upper limit for $\langle z^2\rangle_U$ in our experiment: taking $d = 63.2$ Å, $L = 4$ μm, $a = 9.6$

Å (estimated from the known value of the area/lipid, i.e. $\pi(a/2)^2 = 72.2$ Å^2), Eq.(7) yields $\langle z^2 \rangle_U = 2.7$ A^2 *or* $z_U^{rms} = 1.6$ A. In the DOPC-DNA-Mn^{2+} triple complex, the presence of the DNA between the lipid bilayers should reflect into a significant increase of the stiffness of the membrane, with consequent further lowering of η_1. Accordingly, η_1 in the triple complex is likely to be considerably smaller than its estimated upper limit and the motions observed should be due almost entirely to thermal motions relative to the mean position of the bilayer. Therefore, we may assume for the triple complex $\langle z^2 \rangle \approx \langle z^2 \rangle_T = 19.0$ A^2 and use this result to estimate the undulatory contribution in DOPC as $\langle z^2 \rangle_U \approx \langle z^2 \rangle - \langle z^2 \rangle_T =$ (21.4–19.0) A^2 = 2.4 A^2 *or* $z_U^{rms} = 1.5$ Å This value is lower than the estimated upper limit and seems to be quite reasonable. The above arguments suggest that insertion of the DNA helices in the water gap between the lipid bilayers reduces the flexibility of the lamellae of the DOPC vesicles, thus suppressing almost completely the undulatory fluctuations and allowing only for thermal fluctuations. A deeper insight into these aspects deserves further investigation using higher resolution x-ray diffraction experiments. To test how promising these complexes are as synthetically-based nonviral carriers of DNA vectors for gene therapy, we have planned specific experiments to measure the transfection efficiency and to determine the structural parameters relevant to it including bending and elastic properties.

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