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Report:

HEXAGONAL DNA ORGANIZATION IN A NEUTRAL LAMELLAR PHASE:

A SUPPORTED FILM EXPERIMENT

Aims of the experiment and scientific background

Gene therapy is a very promising technique which consists in introducing a nucleotide sequence (DNA) into the cell nuclei that will control the expression of a given gene[1]. A strategy must be envisioned to deliver the genetic material into the nucleus, since the DNA does not do it naturally. During the last decade, different approaches to the elaboration of synthetic vectors have been tried, e.g. cationic phospholipids (CL). Some of them have been successful for *in vitro* applications; however the transposition to *in vivo* applications has not yielded the same performance. It has been shown that such DNA-CL systems have an ordered liquid crystal structure due to electrostatic interactions between the negatively charged DNA and the cationic membrane[2]. For such formulations, although the encapsulation ratio is high, the main problem is toxicity due to the cationic behavior.

Recently, we have demonstrated that a large amount of DNA can be incorporated in lamellar phase of neutral lipids, with the DNA intercalated between the lipid lamellae[3]. In particular, it was shown that multilamellar

vesicles in a close-packed structure can be obtained as a result of a hydrodynamic instability[4] specific to L_α phases. The vesicles, obtained by shear, are called onions or spherulites, and consist of regularly stacked membranes. In a second step, biological molecules can be attached to the neutral surfaces of the onions to interact with a specific protein in the cell surface. Using the neutral lipid system seems to be a very promising strategy in order to obtain **non-cationic and non-viral DNA vector.** A theoretical model, based on the Flory model, has been proposed to explain the ternary phase diagram observed for the DNA-neutral lipid-water system[5] considering mainly steric (excluded volume) interactions.
The aim of the present experiment was to obtain new information about the structure of the DNA/neutral lipid/water system. In particular, we planned to monitor the DNA-DNA correlation peak as a function of the water concentration. **Using well oriented samples** of freely suspended films, we expected to obtain—for the first time—the complete structure of our system including layer-to-layer correlations, 2D or 3D organization, and evidence DNA phase transition.

Experimental setup.

X-ray scattering experiments were performed on the BM32 beam line at the European Synchrotron Radiation Facility (ESRF). We worked with the selected energy of 12 keV, corresponding to a wavelength of λ=1.0332Å. The final beam size was 50 × 500 μm2 (V × H) at sample position, hence fixing the vertical resolution in reciprocal space to 2.6 x 10^{-3} $Å^{-1}$. The scattered X-rays were recorded either on a low background scintillation detector (NaI) located at about 600 mm from the sample, or on a 2D CCD array detector (Princeton 1242*1152 pixels). In order to optimize the accessible domain of wave vectors, the sample-to-detector distance has been fixed to 1138 mm when using the CCD detector. Only 1/4 of the surface of the detector was irradiated. In order to check the film orientation, we made reflectivity scans with the NaI detector. A special humidity control chamber designed at Centre de Recherche Paul-Pascal for free standing films [relative humidity (rH) +/- 1%] was mounted on the goniometer. Two different geometries were used: freely suspended films and open supported films. The size of the film was 10 x 2 mm^2 and 10 x 30 mm^2 respectively. For both geometries, the chamber was oriented with the bilayer plane parallel to the x-ray beam, along the y axis.

The various samples were prepared and controlled at Centre de Recherche Paul-Pascal before the run. We drew films using samples at different compositions. We finally extensively studied 4 suspended films with a lamellar phase without DNA (45% of water or more) and 3 open supported films with weight % lipid / weight % DNA = 2.

Results and discussion.

The first step of the experimental procedure was to calibrate the new chamber. It was crucial to validate the possibility to obtain very well oriented films with a constant and identified lamellar periodicity versus rH. We choose the DNA-free sample in order to focus our study on the lamellar structure. The swelling kinetics at different values for rH has been studied for 4 different films. With the NaI detector, we obtained the classical diffraction pattern, showing 4 orders of the lamellar phase. With the reflectivity geometry and the 2D detector, the signal is extremely anisotropic, proof of a very well oriented sample, as expected from a freely suspended film with the normal of the smectic layers perpendicular to the x-ray beam ("homeotropic" configuration). The experimental procedure consisted in studying the evolution of the smectic Bragg peak (converted into a smectic period d) as a function of rH. Figure 1a shows the evolution of d when rH is—almost instantaneously—increased from 89 and 91%. The layer spacing increases continuously from 46 to 46.3 Å with a characteristic response time of approximately 20 minutes. Once the asymptotic value is reached, it is remarkable that the periodicity can remain constant and homogeneous throughout the film during several hours. This result indicates a great stability of the device for such an operating range. Unfortunately, the device proved to be unsuited to stabilize rH values higher than 93%, unless an additional water supply was added. The diluted lamellar phase (figure 1b) was therefore only obtained after having added a liquid water tank (a piece of sponge soaked with water) inside the device. Then, significant periodicity gradients could be measured along the film according to the distance sample - water tank (figure 1c).

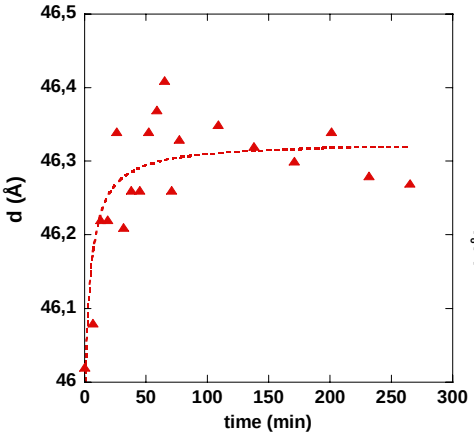

Figure 1a: Evolution of the lamellar periodicity (d) when the relative humidity (rH) is increased from 89 to 91%

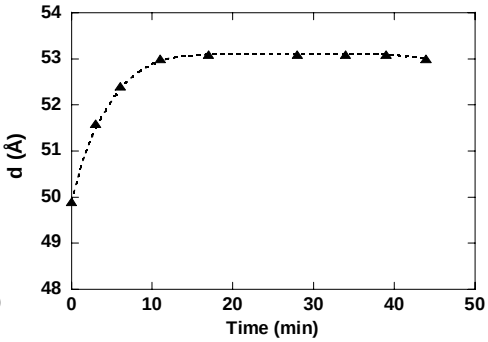

Figure 1b: Evolution of the lamellar periodicity (d) when the relative humidity (rH) is increased after having added a liquid water tank inside the device.

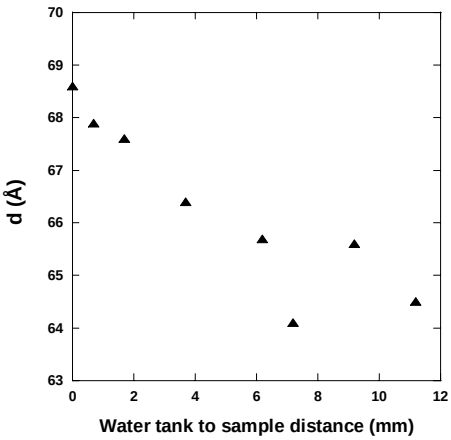

Figure 1c : Evolution of the lamellar periodicity (d) along the film according to the distance sample – water tank.

The second step of the experimental procedure was to draw a film with the neutral lipid / DNA / Water system. Figure 2-a is an example of the images obtained in the reflectivity geometry, while figure 2-b displays the azimuthally-averaged intensity of the same image as a function of the scattering wave vector. We clearly distinguish the second and third order for the lamellar phase. The signal is clearly anisotropic with the Bragg peak reflections on the vertical axis, proof of a very well oriented sample, as expected from a supported film.

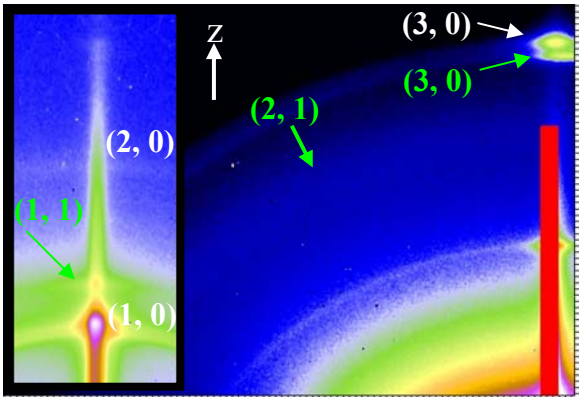

Figure 2a: image recorded on a CCD detector for a supported film of a very concentrated DNA-neutral lipid- water system.

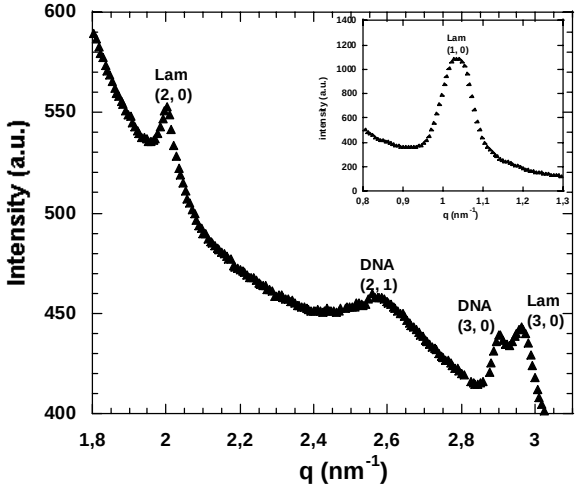

Figure 2b: azimuthally-averaged intensity for figure 2a as a function of the scattering wave vector.

The data shows the two harmonics of the lamellar scattering at q_{20} = 2 nm^{-1} and q_{30} = 2.96 nm^{-1}, which corresponds to a first order peak (inset of figure 2b) of q_{10} = 1.04 nm^{-1}, very close to q_{20} / 2. The associated spacing is d = 6.28 nm^{-1}, consistent with the thickness of the lipid bilayer (δ = 3.7 nm^{-1}) plus a hydrated DNA diameter (2 nm). After a beam stop translation (inset of figure 2a and 2b) we were indeed able to visualise the first order peak (q_{10} = 1.04 nm^{-1}) of the lamellar structure. In a more spectacular way, three additional peaks are observed on these images and cannot be attributed to the lamellar structure. These additional reflections cannot be explained either by an in-plane positional correlation of an intercalated DNA rod lattice, a centered rectangular columnar DNA lattice or an inverted hexagonal phase as described in detail in the literature for cationic systems.

The peak positions can be indexed to a 2D, hexagonal DNA lattice with $q_{hk} = \sqrt{h^2 + k^2 + hk}$ inside the lamellar neutral lipid phase as shown in figure 3.

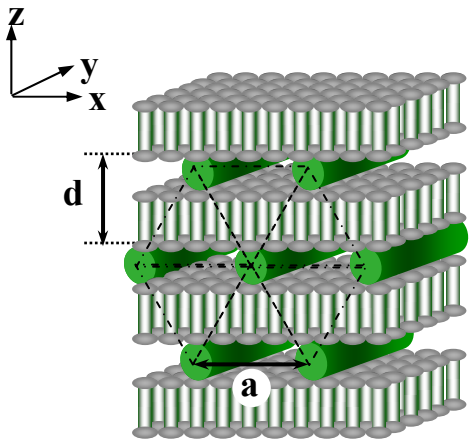

Figure 3: schematic representation of the hexagonal DNA organization between the neutral bilayers of a 3D smectic phase. The x-ray beam is parallel to the y axis

The three DNA scattering peaks are indexed (1,1), (2,1) and (3,0) respectively. As proposed by Durand *et al.*[6] for the 2D hexagonal phase of the DNA/water system, the systematic absence of the (2,0) reflection, which is normally observed for such a lattice, could be due to the form factor.

As expected for an oriented hexagonal lattice with the DNA long axes parallel to y, the (1,1) and (2,1) reflections are off the z axis. For a perfectly oriented sample (as shown in figure 3) the (1,1) reflection (6 positions allowed) is expected to be +/-30 deg with respect to the z axis and the (2,1) reflection (more intense with 12 positions allowed) will appear in the reciprocal space for +/- 11 deg with respect to the z axis modulo 60 deg. In the other case, an oriented sample with the DNA long axis perfectly oriented along **x** will only show the (1,0) and (3,0) reflections along z. Our data seems to indicate a DNA orientation degeneracy in the xy plane since the (1,1) and (2,1) reflections appear at 18 and 36 deg with respect to the z axis respectively.

Conclusion :

With this experiment SI-1304, we got new information about the structure of the DNA/neutral lipid/water system. Most importantly, we obtain for the first time a hexagonal DNA organization in a neutral lamellar phase. This new phase has been found in a very concentrated system—that is to say with a small amount of water. To our knowledge, this new phase has not yet been described in the literature for any cationic system. Indeed, the cationic vectors are obtained by mixing together the constituents in a one pot process. In contrast, we obtain directly a DNA condensation by controlling the concentration and the mixing protocol in a lamellar phase of phospholipids. With this new kind of DNA organization, the neutral (i.e. non-toxic) lipid system shows once again all its originality and seems to be an excellent alternative to the cationic systems for non-viral vectors.

Using a home-made special chamber designed for humidity control, we obtained oriented samples with supported films. This geometry allows the accurate description of the DNA orientation with respect to the lipid bilayer. Our data demonstrate degeneracy for the DNA orientation in the (xy) plane. High resolution is crucial in order to allow an accurate measurement of the scattering wave vectors: the reflections on the z axis for the DNA and for the lipids signals are very close. The use of the BM32 beam line was crucial for this experiment in order to obtain sufficiently high instrumental resolution, maximum intensity and 2D detection. The relatively long experiment time of 18 shifts was essential to be able to prepare the films, to change slowly and continuously the relative humidity and to wait for the equilibrium. Following the success of the experiment, a publication is currently in preparation.

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