Eur. Phys. J. Special Topics **141**, 217–221 (2007)

DOI: 10.1140/epjst/e2007-00043-9

THE EUROPEAN PHYSICAL JOURNAL
SPECIAL TOPICS

In situ X-ray and neutron diffraction study of lipid membrane swelling

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Abstract. Liposome suspensions comprising the synthetic cationic lipid DOTAP, the zwitterionic DPPC and the anti-cancer agent Paclitaxel were deposited on solid substrates and investigated by X-ray and neutron reflectivity measurements at ESRF and ILL. Well ordered multilamellar drug/lipid membranes were obtained, such as indicated by the several orders of Bragg reflections in the diffraction pattern. With an excess of Paclitaxel, additional Bragg peaks from the drug crystalline phase could be observed. Changes of the molecular organization in the drug-lipid system were investigated as a function of relative humidity using a custom-built controlled humidity chamber. For DOTAP multilayers the effects of bilayer swelling were much more pronounced than for DPPC under similar conditions, while the crystalline peaks of the drug were not affected. The pronounced swelling of DOTAP multilayers might be related to the electrostatic repulsion between the charged lipid headgroups.

1 Introduction

Lipid multilayers loaded with anti-cancer drugs as produced by deposition of drug/lipid liposome suspensions on solid substrates have been used to study drug insertion into the membranes [1, 2]. In the present work, effects of hydration on membranes made by the synthetic cationic lipid dioleoyl trimethylammonium propane chloride (DOTAP), and the zwitterionic dipalmitoyl phosphatidylcholine (DPPC), including the anti-cancer agent Paclitaxel (PXL) were investigated. Such compounds are useful to make up cationic liposomes, which display enhanced binding and uptake at the activated (angiogenic) tumor vasculature. Thus, in a new approach for tumor therapy, cationic nanoparticulate carriers (EndoTAG®, Medigene AG) are used to deliver cytotoxic agents to the tumor vasculature in order to destroy the blood vessels and therefore also the tumor itself [3]. EndoTAG®-1, a cationic liposome preparation comprising paclitaxel as an active agent, is currently tested in clinical trials phase II. In order to improve the shelf life, the product is lyophilized for reconstitution with water before application to a patient. It is essential, that the molecular organization of the sample be not affected by lyophilization in an uncontrolled way. Investigation of the molecular organization as a function of humidity can give fundamental insight into the processes occurring during hydration and dehydration on the molecular level. In order to enable such experiments, a controlled humidity chamber was used for experiments with neutrons and with X rays on the swelling of PXL loaded multilamellar thin films made by DOTAP and DPPC.

2 Materials and methods

Synthetic L-α-DPPC was purchased from Sigma, Paclitaxel (PXL) was from Natural Pharmaceuticals, Berverly, MA, USA; DOTAP was from Merck Eprova, Schaffhausen, Switzerland. The lipid was first dissolved in chloroform and PXL was dissolved in methanol (pa grade, Merck). The two solutions were mixed together in appropriate proportions to achieve the desired molar fractions. The solvent of this mixture was evaporated under N_2 stream and the drug/lipid film was reconstituted with pure water and sonicated following the method described by Lasch et al [4]. The resulting liposome suspensions were deposited on glass slides for X-ray experiments and on Si wafers for neutron experiments. The samples were dried in an environment of controlled relative humidity = 84%.

Neutron reflectivity measurements were performed in the Institut Laue Langevin (ILL), France at D16 beamline using a wavelength of 4.54 Å. The sample was kept at 25°C in a controlled temperature/humidity chamber specially built for neutron measurements at D16 described by Perino-Gallice et al [5]. The sample is hydrated by contact with water vapor produced in the chamber by controlling the temperature of an internal water container. To acquire data we used a pseudo theta/two-theta configuration made by fixing the position of a two-dimensional detector and scanning the sample by 0.2 degrees around the first Bragg peak of the multilayer films. The central part of the 2D image was integrated and plotted versus q_z, the scattering vector perpendicular to the membrane stacks ($q = 4\pi \sin\theta/\lambda$). The resolution in q_z was about $0.0025\,\text{Å}^{-1}$. The scan was repeated 5 times for each value of relative humidity previously set. As the diffraction patterns were identical, the 5 curves were summed up to increase signal/noise ratio. The typical exposure time was 220 seconds per scan, resulting in about 2 hours for each sample passing through the 6 chosen relative humidity values.

X-Ray Reflectivity (XRR) technique was performed in the European Synchrotron Radiation Facility (ESRF), France at Troika II (ID10-B) beamline. The X-ray beam energy at the sample was 8.015 keV selected by a double crystal diamond monochromator from the first harmonic of three-undulator source at ID10B (Troika-II) beamline. The sample was kept at 25°C in a controlled temperature/humidity chamber specially built for X ray measurements at Troika II based on that one used for neutrons. Here the relative humidity was controlled through the vapor saturation of H_2O put in the reservoir. To acquire data we used a pseudo theta/two-theta configuration made by fixing the position of a one-dimensional detector and scanning the sample around the six first Bragg peaks of the multilayer films. The images on the linear detector were integrated and plotted versus q, the scattering vector, for each value of relative humidity set. Subsequent measurements were done after 45 min waiting time for humidity stabilization. The typical exposure time was 20 min per each relative humidity value chosen. In order to prevent X ray radiation damage on the sample, the XRR spectrum for each relative humidity was taken from a fresh sample area after a lateral displacement of the chamber larger than the horizontal size of the beam, which was typically 0.5 mm. For these measurements the resolution in q_z was about $0.001\,\text{Å}^{-1}$.

3 Results and discussion

We first studied the swelling of DPPC system with neutron reflectivity using the controlled humidity cell putting D_2O on the reservoir used for the vapor saturation of the chamber. The vapor of D_2O diffused between the lipid bilayers in the swelling processes replacing H_2O molecules and increasing the neutron diffraction signal from the lipid multilayer structure. Figure 1 shows the neutron reflectivity of DPPC_10%PXL presenting the two first orders of diffraction from the lamellar structure of the multilayer thin film. The peak position shifted to lower q when the relative humidity (RH) increased. The net increase of the bilayer period was about 1.2 Å from 68% to 91% RH giving an average swelling rate of 0.05 Å per unit of RH, roughly linear for this range of hydration. For pure DPPC system (data not shown) this value was about the same, see Table 1. The peak widths do not change with the hydration ($\sim 0.005\,\text{Å}^{-1}$ for the first order and $\sim 0.007\,\text{Å}^{-1}$ for the second one) indicating that the coherence between

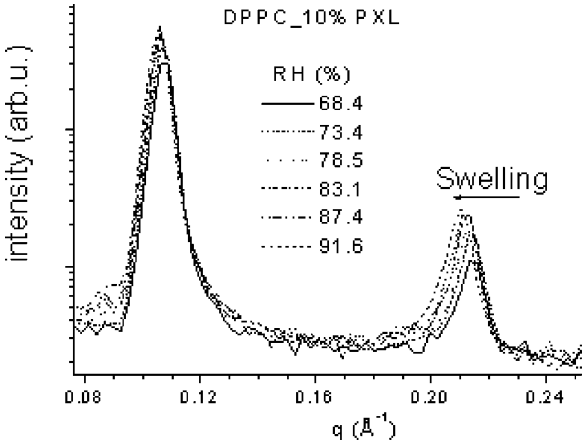

Fig. 1. Neutron Reflectivity pattern from the lamellar structure of DPPC_10%PXL multilayer thin film. The peak positions shifted to lower q when the relative humidity (RH) increased.

Table 1. Compared results for swelling of DPPC and DOTAP multilayer thin films.

	Net swelling (Å)	Studied range (% RH)	Swelling rate (Å/%RH)	
DPPC	0.9	76 to 92	0.06 ± 0.01	Neutrons
DPPC_10%PXL	1.2	68 to 91	0.05 ± 0.01	
DOTAP	6.8	52 to 88	0.19 ± 0.01	X-Rays
DOTAP_10%PXL	4.8	52 to 84	0.15 ± 0.01	

layers was not lost by the swelling. The effects of swelling as observed for the present system were small in comparison with the instrumental resolution. Further measurements would be necessary for an accurate study of the curve parameters.

The same kind of controlled humidity chamber was built at ID10-B beamline, ESRF, to study multilayer thin films using XRR. Figure 2 shows the XRR pattern for a multilayer thin film prepared with pure DOTAP. At 52% RH we observe five diffraction orders from the bilayer period of 42 Å. The period increased with the RH until 46 Å at 68%. At this point a 6th diffraction order becomes visible. The periodicity remained constant until 84% forming a plateau with the in the graph of figure 4. The net increase of the bilayer period was about 6.8 Å from 52% to 88% RH giving an average rate of 0.19 Å per unit of RH. The peak width was $\sim 0.004\,\text{Å}^{-1}$ and it did not change until 84% RH, however it doubled at 88% RH indicating loss of coherence between layers above this humidity value. A satellite peak, visible around the first order diffraction and also around $0.45\,\text{Å}^{-1}$, might indicate a second phase of different lamellar spacing like the tilt domains observed by Asmussen and Riegler [6] from similar

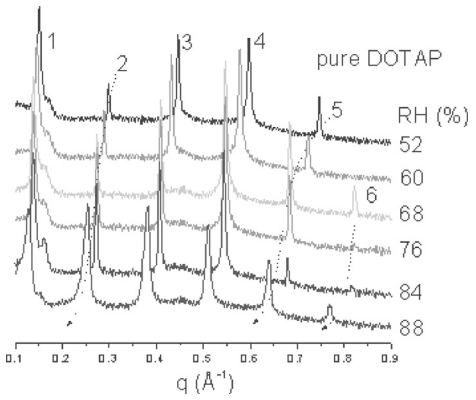

Fig. 2. X Ray Reflectivity pattern from the lamellar structure of pure DOTAP multilayer thin film. A sixth diffraction order appears after 68% of relative humidity (RH). The y-axis gives the scattered intensity in arbitrary units. For comparison, the curves were stacked vertically.

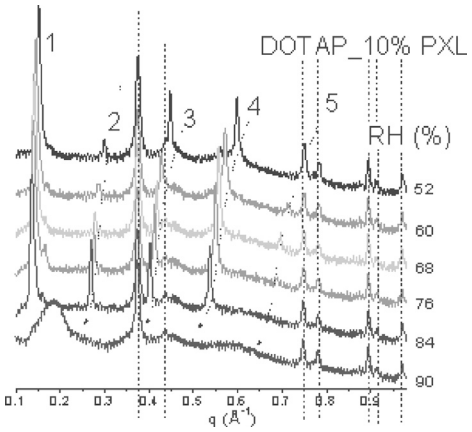

Fig. 3. X Ray Reflectivity pattern from the lamellar structure of the DOTAP_10%PXL multilayer thin film. Peaks indicated by vertical dashed lines are from the PXL crystalline structure.

thin films. Annealing of the sample could be a method to apply for producing more homogeneous structure in such samples. Figure 3 shows the XRR results for DOTAP_10%PXL. The bilayer period as a function of humidity was similar to that of the pure system, however it did not present the plateau that we observed before. The net increase of the bilayer period was about 4.8 Å from 52% to 84% RH giving an average rate of 0.15 Å per unit of RH. The peak widths for each RH did not change until 84% like in the pure system, with an average value of 0.005 Å^{-1}. At 90% RH the system lost completely the lamellar structure, such as indicated by peak broadening and absence of higher order diffraction peaks, which were recovered when the system came back to the starting point at 52% RH. In figure 3, in addition to the diffraction pattern of the lamellar structure, Bragg peaks from the crystalline phase of the drug PXL can be observed. The concentration of 10% mol/mol for this sample is above the critical concentration for incorporation of the drug in molecular form and the excess of material can crystallize as found in previous work [7] with Ellipticine, another hydrophobic drug [8]. The Bragg peaks from the crystalline phase of the PXL in figure 3 did not change with the increase of the humidity, and also the peak width was constant, indicating that the crystalline domains were not growing or agglomerating. Figure 4 shows a comparison of the bilayer period of DPPC and DOTAP, loaded and unloaded systems as a function of RH. The swelling rate was roughly linear for all the samples except for the system of pure DOTAP, which displayed the plateau between 68% and 84% RH. In table 1, it can be seen that the slope for DOTAP systems is at least three times more than the swelling rate of DPPC systems for the studied range of RH. The much more pronounced effects of swelling for DOTAP in comparison to DPPC can be explained by the electrostatic repulsion between the cationic lipid layers, which could facilitate the swelling. As for DOTAP/10%PXL the swelling rate was a smaller than the pure system

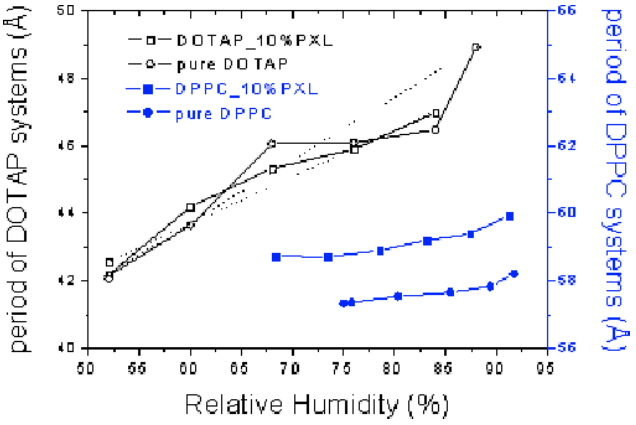

Fig. 4. Periodicity of DPPC and DOTAP pure systems and with 10% of PXL mol/mol. Dotted lines are indicating the deswelling made in the end of the experiment.

we can suppose that the crystalline phase of PXL in the aqueous media might neutralize the repulsion between layers.

The data demonstrate that with the present experimental setup, effects of hydration on lipid multilayers can be investigated in a controlled way. Precise information on the swelling behavior of different lipids can be obtained and the findings can be correlated with molecular properties of the multilayer-forming lipids. Further measurements are foreseen for combining the main advantages of contrast variation for neutrons and large dynamic range for X rays.

We would like to thank Thomas Weiss, Muriel Mattenet and Henry Gleyzolle that assisted the construction and commissioning of the humidity chamber at Troika II; all the staff of Troika II, ESRF and D16, ILL for technical support during the experiments. **Note:** LPC present address, LLS Institute Laue Langevin, France; HNB present address, SF6 - Hahn-Meitner Institut Berlin, Germany.

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