# X-ray and neutron reflectivity study of solid-supported lipid membranes prepared by spin coating

Johanna Generosi, Carlo Castellano, Daniela Pozzi, and Agostina Congiu Castellano[a)]
*Dipartimento di Fisica and Istituto Nazionale per la Fisica della Materia, Università "La Sapienza," P.le Aldo Moro 2, 00185 Rome, Italy*

Roberto Felici and Francesca Natali
*Operative Group in Grenoble-Istituto Nazionale per la Fisica della Materia, 6 rue J. Horowitz, 38043 Grenoble Cedex 9, France*

Giovanna Fragneto
*Institute Laue-Langevin, 6 rue J. Horowitz, 38042 Grenoble Cedex 9, France*

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We present a study of x-ray synchrotron radiation and neutron reflectivity on solid-supported lipid membranes prepared by spin coating. This technique has the advantage of allowing the control of the number of lipid layers by varying the deposition parameters. The experiments were performed on the cationic lipid 1,2-dioleoyl-3-trimethylammonium-propane chloride salt (DOTAP), the neutral lipid 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), the lipid mixture (DOTAP-DOPC), and the complex (DOTAP-DOPC/DNA) deposited on wafers. Only single neutral lipids or lipid-peptide mixtures were deposited on solid substrate using the spin coating technique and characterized. Results on the structure of the deposited lipid layers indicate that DNA contributes to the order in the lipoplexes. © *2004 American Institute of Physics*. [DOI: 10.1063/1.1814412]

## I. INTRODUCTION

Solid-supported membranes have aroused the interest of researchers mostly because of their possible applications in the medical and biotechnological fields.[1,2] Lipid bilayers can be considered good model systems for biological membranes. For years, the deposition of organic molecules on insulating or semiconductive substrates was carried out by the Langmuir-Blodgett technique or simply by spreading a lipid solution on wafers, obtaining monolayers or thick multibilayers. It has been reported[3] that spin coating offers the possibility to have a limited number of lipid layers and to control this number by varying the parameters of deposition (amount and concentration of lipid solution and rotational speed). Only single neutral lipids were deposited on a solid substrate using the spin coating technique.

This paper is about the structural characterization of lipid films obtained using spin coating. This technique was used to spread over silicon wafers, the neutral lipid 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), the cationic lipid 1,2-dioleoyl-3-trimethylammonium-propane chloride salt (DOTAP), the lipid mixture (DOTAP-DOPC), and the complex (DOTAP-DOPC/DNA) at the isoelectric point. Neutral lipid DOPC was also deposited on gallium arsenide wafer to compare the lipid structure in the case of different solid supports.

We have used x-ray and neutron reflectivity to study these systems, which present a well-defined structure with a small distribution of the bilayer normal vector, typically of the order of 0.01° or less.[4] Specular reflectivity was used to determine the static structural properties of the film in terms of its scattering length density profile which provides information on the number of bilayers composing the film, thickness, and density of the repeating unit.

We also carried out studies of the lipid film stability as a function of hydration, which is a very important parameter in the case of the lipids interaction with the biological serum.

## II. EXPERIMENTAL DETAILS

*Sample preparation*. DOPC and DOTAP were bought from Avanti Lipids, and double-stranded calf thymus ($MW_{bp}=649$) from Sigma. The single lipids and the mixture were dissolved in chloroform at a concentration of 5 mg/mL. The preparation of the complex lipid-DNA was a multistep procedure: Firstly, the two lipids were dissolved in chloroform, which was removed afterwards via rotary evaporation at 35 °C, leaving a thin film of lipid that was placed under vacuum over night to ensure that all traces of solvent had been removed. The lipids were then suspended in water at 45 °C until the films were hydrated and vortex mixed to afford an emulsion. Finally, the lipid mixture was sonicated for 30 min and blended in the DNA water solution (2.2 mg of DNA in 1 ml of de-ionized water, sonicated for 1 min; this induces a DNA fragmentation whose length distribution, detected by gel electrophoresis, is between 200 and 1000 bp).[5] The complex lipid-DNA at isoelectric point is neutral, the number of DNA bases and DOTAP molecules being equal.

Neutral and cationic lipids, their mixture, and complex lipid-DNA were spread on silicon and gallium arsenide wafers by spin coating: about 10 $\mu$L of solution per mm$^2$ was pipetted onto the surface; immediately after, the sample was

[a)]Author to whom correspondence should be addressed; Electronic mail: a.congiu@caspur.it

 

accelerated to rotation (3000 rpm). The solvent evaporates very fast and a well-defined number of bilayers nucleates at the substrate surface. The silicon wafers were hydrogen terminated[6] since the surfaces were prepared by wet chemical treatment in basic HF solutions and $HNO_3$. This procedure should ensure that the surfaces are free from oxide. The samples were put in vacuum overnight.

X-ray reflectivity measurements were carried out using the 4+2 circles diffractometer of the ID1 beamline at the European Synchrotron Radiation Facility (Grenoble). The incident-beam energy was chosen to be 16 keV in order to limit the radiation damage.[7] During the x-ray experiments, the films were kept inside a cone of kapton under a flux of helium. Despite the highly brilliant beam, we did not observe any variation on the measured signal, implying that no or negligible radiation damage was induced in the sample even after exposure times of several hours. The incident beam was collimated to a dimension of 0.1 mm vertical and 0.03 mm horizontal. The sample was mounted vertically and had surface dimensions of $\sim 4 \times 4$ mm$^2$. The reflected intensity was then measured as a function of $\alpha_I$ under specular conditions (exit angle $\alpha_R = \alpha_I$). Thus, the momentum transfer of the elastic scattering $q$ was always along $q_z$, with the $z$ axis parallel to the sample normal.

Neutrons are uncharged and therefore they are highly penetrating. This can give them some advantages with respect to x rays in the case of reflectometry: neutrons can easily penetrate into the bulk of samples allowing us to measure reflectivity from buried interfaces (solid–liquid). Moreover, the scattering length of neutrons varies in a random fashion between elements as well as between different isotopes of the same element. In our case, this is particularly important because of the large scattering length of deuterium which makes neutrons highly sensitive to the hydrogenated tail of lipid in deuterated species. On the other hand, x-ray scattering length varies monotonically with increasing atomic number. These differences in scattering between x rays and neutrons allow us to obtain complementary information in biological systems. In our case, x rays are more sensitive to the head part of the lipids while neutrons are more sensitive to the tails. The combination of both x-ray and neutron reflectometry measurements helps in solving complex surface and interfacial problems.

Neutron reflectivity experiments were carried out at the D17 reflectometer at Institute Laue-Langevin (Grenoble) in time of flight configuration.[8] The samples ($\sim 75 \times 75$ mm$^2$) were kept in a controlled humidity chamber[9] to avoid structural changes induced by an uncontrolled water exchange with the atmosphere. The chamber consisted of two concentric aluminum cylinders; the top and the bottom of the inner cylinder were connected to a water bath; the temperature was fixed at 20 °C. When the hydrated films were analyzed, $D_2O$ was poured in the chamber with saturated water vapor atmosphere to allow the full hydration (nearly 100% relative humidity) during the measurements. Measurements were done using a wavelength spread of 2–20 Å at two incident angles (0.7° and 4°).

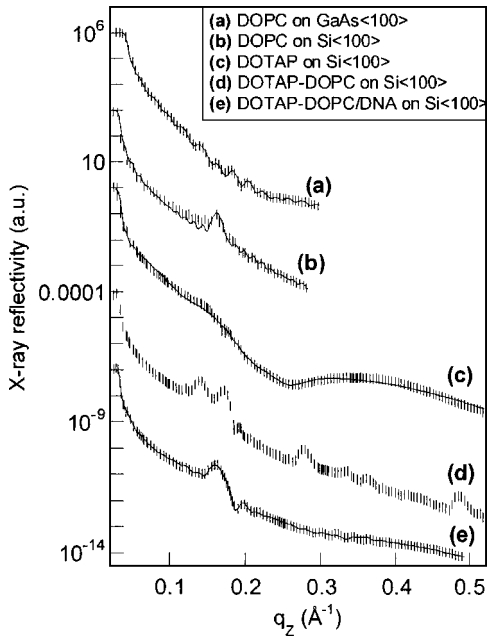


FIG. 1. X-ray reflectivity measurements of the spin-coated samples, DOPC on GaAs (a), DOPC on Si (b), DOTAP (c), DOTAP-DOPC (d), and the complex DOTAP-DOPC/DNA (e), along with the simulations based on Parratt's dynamical theory. The experimental points are illustrated with vertical lines, which length indicates the error.

## III. RESULTS AND DISCUSSION

Figure 1 shows x-ray reflectivity measurements performed on five samples: DOPC in the $L_\alpha$ phase deposited on silicon and gallium arsenide wafers, DOTAP in the $L_\alpha$ phase on silicon, the mixture DOTAP-DOPC with molecular weight fraction $\Phi = \text{DOPC}/(\text{DOTAP-DOPC}) = 0.5$, and the complex DOTAP-DOPC/DNA at the isoelectric point (zero total charge), both deposited on silicon. The figure also includes simulations based on Parratt's dynamical theory. The curves, measured at ID1, have been corrected for illumination, background, and storage ring current and were shifted in figure for clarity.

The analysis of a reflectivity curve provides several informations: the position of the Bragg peak determines the periodicity of the bilayers $d = 2\pi/q_z$. From the periodicity and intensity of the oscillations between the total reflection region and the Bragg peaks (Kiessig fringes), one can determine the total thickness ($L$) of the film, $L = 2\pi/\Delta q_z$ with $\Delta q_z$ being the distance between two minima. The observation of Kiessig fringes indicates a well-defined film thickness and the number of oscillations determines the number of bilayers.[10] The errors on $d$ are estimated[11] to be on the order of 10%. The total thickness determined in this way can then be compared with the Bragg-peak width, which is related to the thickness of the "ordered" layer. This comparison allows us to understand if the whole film has a well-ordered layered structure.

In the curve (a) of Fig. 1, from DOPC on GaAs, no Bragg peaks can be observed, but only a few damped oscillations. The simulation and the corresponding electron-density profile, shown in Fig. 2 [curve (a)], lead to the individuation of only five lipid bilayers, each one with a thickness of 32 Å and a sample thickness of $\sim 333$ Å. The

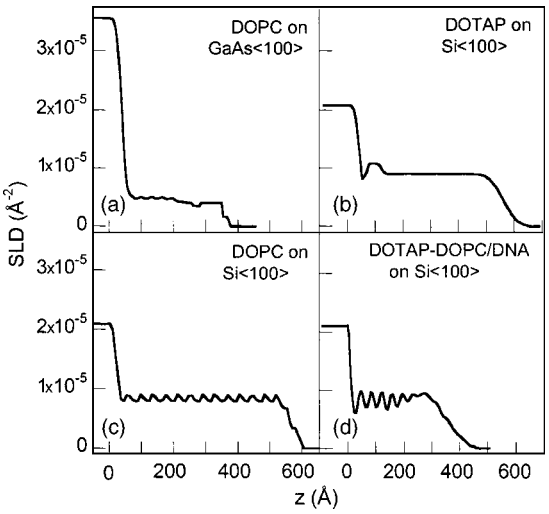


FIG. 2. Electron-density profile model corresponding to the x-ray reflectivity simulations. The letters (a), (b), (c), and (d) correspond, respectively, to DOPC on GaAs (a), DOPC on Si (b), DOTAP (c), and the complex DOTAP-DOPC/DNA (d).

scattering length densities (SLDs) of the lipid heads and tails are similar, so there is a small contrast in the density profile, as seen in figure. The total thickness (333 Å) is much bigger than the coherence thickness ($32\times5$ Å$=160$ Å) showing that there is some disorder during the formation of the layer.

In the curve (b) (DOPC on Si), one Bragg peak is observed, indicating a periodicity of $d=39$ Å in good agreement with literature values.[12] From the fit of the reflectivity, which shows very well-defined Kiessig fringes, a total thickness of 581 Å is obtained for the film. This, together with the bilayer periodicity obtained by the Bragg-peak position and with the coherence length connected with the Bragg-peak width, indicates that the film is made of 13 highly ordered bilayers. The DOPC head can be approximated to a sphere, with an area of 77 Å$^2$, the tails to cylinders with a total length of 24 Å and volume of 961 Å$^3$. The density profile in Fig. 2 [curve (b)] shows that the first layer on the substrate (corresponding to the heads) interacts with the bulk. The three external layers are not completely occupied, indicating the presence of some surface roughness. However, even in this region, the layers maintain a well-defined layered structure.

By comparing curves (a) and (b) in Fig. 1, we note that silicon is much more useful for lipid film deposition than gallium arsenide. The first substrate seems to induce in the thin film an order of deposition, while the second does not allow the lipid to create lamellar bilayers.

Curve (c) represents the DOTAP (on Si) reflectivity profile. Few oscillations appear only in the first part of the curve. The fringes permit us to estimate the total film thickness ($\sim$520 Å) and to elaborate a model in which only a thick monolayer ($\sim$14 Å) adheres to the substrate, while the remaining cationic lipid has not formed any bilayer and exhibit a high roughness. Some homogeneity tests have been performed to verify that the disorder was spread all over the sample. This can be explained by the chemical properties of the DOTAP itself (charge and chemical structure) that could require a different spin coating procedure.

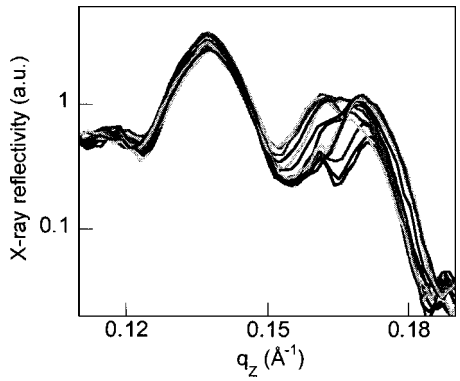


FIG. 3. X-ray reflectivity curves of the mixture DOTAP-DOPC in different regions of the sample.

The reflectivity profile of DOTAP-DOPC on silicon [curve (d)] shows Bragg peaks up to the second order. The first two Bragg peaks indicate a periodicity $d_1=45$ Å and $d_2=37$ Å. Simulation for this curve has so far not been successful, which may be due to the fact that the sample consists of different domains; in some of them, the two lipids form an independent bilayer, while in the others they mix with different concentrations. So the first Bragg peak can be attributed to the neutral lipid DOPC that seems to be very stable, while the second one is attributed to the mixture of DOPC and DOTAP. The position of this peak has some dependence on the illuminated sample region, probably due to different concentrations in different regions (Fig. 3).

When DOTAP-DOPC is mixed with the DNA, the two lipids appear to blend and form highly oriented bilayers. In fact, curve (e) shows a single Bragg peak, with periodicity $d=36$ Å. From the analysis of the simulation, four regular bilayers can be observed. Contrary to the case reported in the literature, we find that the DNA molecules are dispersed in the lipid structure. In fact, if they were distributed between the heads, we should observe an increase in the head region thickness; on the other hand, if they were only dispersed in the tail region, we should observe a variation in the tail SLD. In our case we observe that the SLDs have an average value between the SLDs of the heads (tails) and the SLD of the DNA. This could be explained by two different hypotheses: (i) DNA is mixed in a disordered way in the film and (ii) DNA molecules are localized in the domain boundaries caused by defects in the bilayer structures without changing the bilayer periodicity. The latter explanation seems to be the most physically plausible by considering the lower energetic cost; moreover, this picture has been confirmed by experimental data obtained on our samples by atomic force microscopy to be published in a forthcoming paper. Some homogeneity tests have been performed on different zones of the sample to verify that the positions of the peak and oscillations do not change.

During the neutron reflectivity experiment, we were able to study four samples of highly oriented lamellar lipids: the cationic DOTAP and the neutral DOPC; a mixture of DOTAP and DOPC with molecular weight fraction $\Phi=\mathrm{DOPC}/(\mathrm{DOTAP\text{-}DOPC})=0.5$; a DOTAP-DOPC/DNA

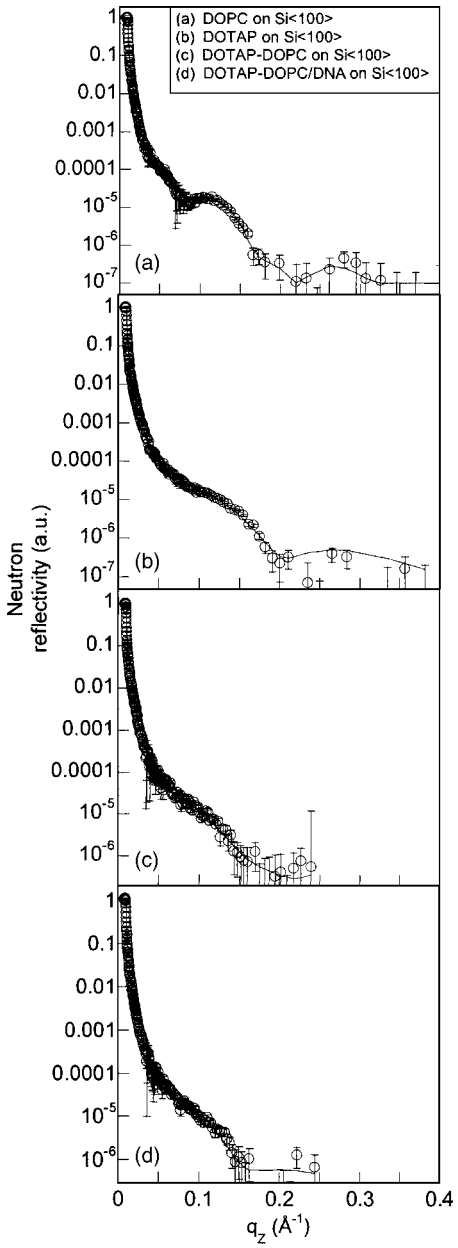


FIG. 4. Neutron reflectivity measurements of the spin-coated samples DOPC (a), DOTAP (b), DOTAP-DOPC (c), and the complex DOTAP-DOPC/DNA (d), along with the simulations based on Parratt's dynamical theory. The measurements were carried out at 20 °C.

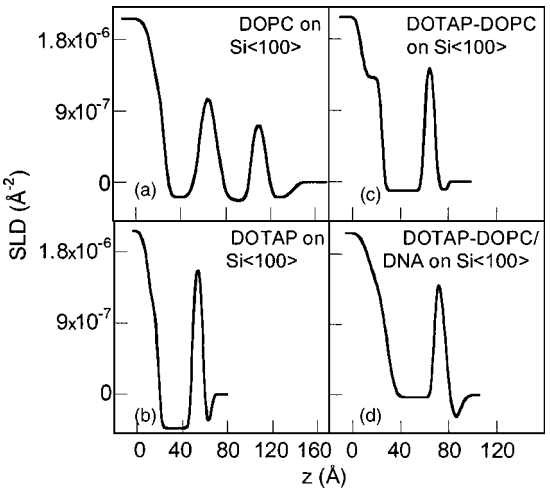


FIG. 5. Electron-density profile model corresponding to the neutron reflectivity simulations on the samples DOPC (a), DOTAP (b), DOTAP-DOPC (c), and the complex DOTAP-DOPC/DNA (d).

complex at the isoelectric point (zero total charge). All of them were deposited by spin coating on Si⟨100⟩.

The neutron reflectivity profiles of the samples and their best simulations are plotted in Fig. 4, the corresponding density profiles in Fig. 5. The model used to fit the data indicates the presence of two bilayers in the film of DOPC [curve (a)] and only one on the one of DOTAP [curve (b)]. Each bilayer of the neutral lipid has an average thickness of 43 Å; we also deduce that 71 $Å^2$ is the area of the head, 24 Å is the length, and ~877 $Å^3$ is the volume of the tail region. The total thickness of the single bilayer of the cationic lipid instead, is thinner, 40Å; the head group region is smaller and the tails are longer (~16 Å). These results are in agreement with the lipid chemical structure and with the x-ray reflectivity measurements (Table I).

Curve (c) shows the reflectivity profile of the two lipids mixed together. The fit provides us the values of the SLD of the heads and tails; if the two lipids form a stable bilayer, the SLDs should correspond to an average made between the SLD of the two different heads (or tails); but in our case, the layer is disordered and we suppose the presence of different domains (the two components seem to segregate as reported in literature).[13,14]

Curve (d) shows the reflectivity profile of the DOTAP-DOPC/DNA complex. The fit provides information about the way the lipids behave in the presence of the DNA: the complex forms a single bilayer; the SLD of the heads remains the same as in the mixture, and this means that the DNA is not inserted there; while the density and the length of the tails changes: this could be explained considering the DNA inserted as revealed by the x-ray reflectivity experiments, not forming a regular array such as detected by x-ray diffraction.[15]

Samples were hydrated for 6–12 h with $D_2O$ in water vapor atmosphere and the measurements were carried out in the humidity chamber at 20 °C. We observed an increase in the dimensions of the bilayers: during the hydration process the heads exchange their water molecules with the $D_2O$ present in the chamber, so their areas and densities should change, while the tails should remain unchanged.

It has been known for some time that it is experimentally very difficult to swell membranes to their equilibrium periodicity $d_0$ if the membranes are exposed to water vapor of (nominally) 100% relative humidity.[12] In theory, the chemi-

TABLE I. Comparison of neutron and x-ray reflectivity measurements on DOPC.

| DOPC | Bilayer thickness $d \pm \Delta d$ (Å) | Head's section $S \pm \Delta S$ ($Å^2$) | Tail's volume $V \pm \Delta V$ ($Å^3$) |
|---|---|---|---|
| x-ray reflectivity | 39±4 | 77±10 | 961±96 |
| Neutron reflectivity | 43±4 | 71±9 | 877±88 |

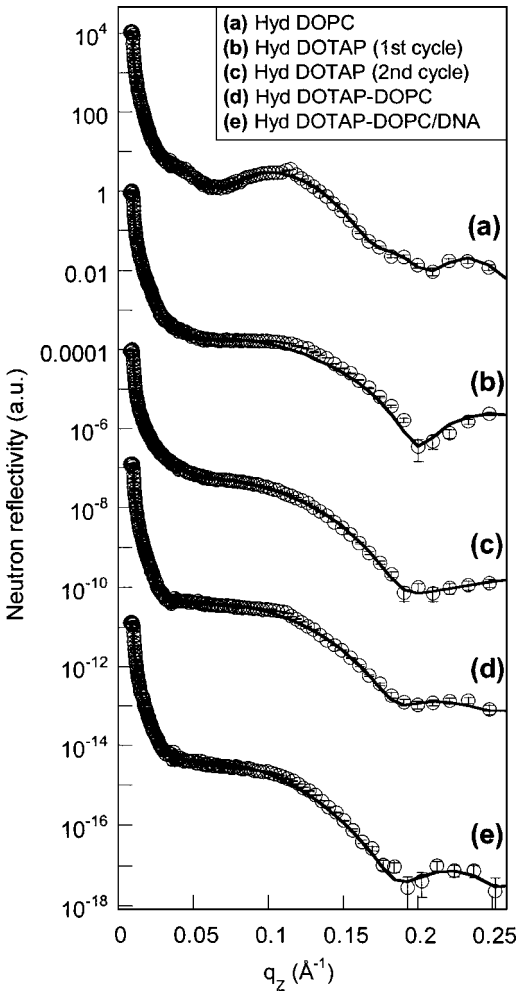


FIG. 6. Neutron reflectivity measurements of the hydrated samples DOPC (a), DOTAP [(b): first cycle; (c): second cycle], DOTAP-DOPC (d), and the complex DOTAP-DOPC/DNA (e), along with the simulations based on Parratt's dynamical theory. The measurements were carried out at 20 °C and nominally 100% relative humidity.

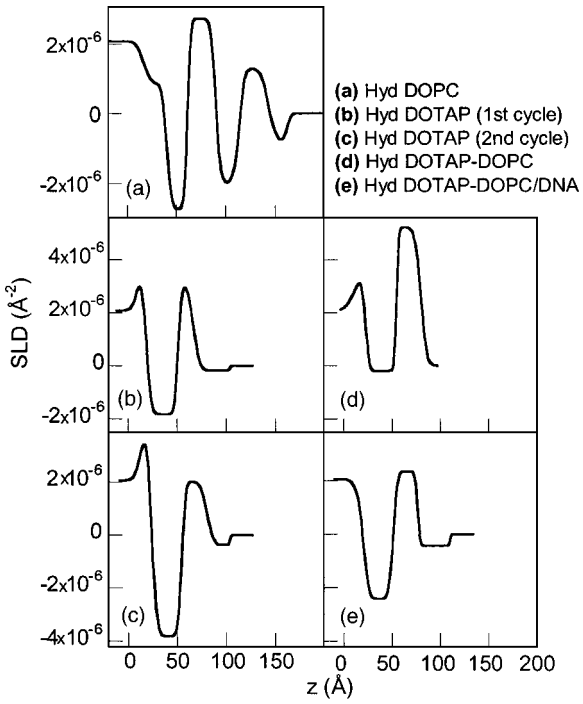


FIG. 7. Electron density profile model corresponding to the neutron reflectivity simulations. The letters (a), (b), (c), (d), and (e) correspond, respectively, to DOPC (a), DOTAP [(b): first cycle, (c): second cycle], DOTAP-DOPC (d), and the complex DOTAP-DOPC/DNA (e).

cal potentials of water and saturated water vapor should be the same and warrant the so-called full hydration of the lipid bilayers. The observation that this result was never achieved was called vapor pressure paradox (VPP), but Katsaras experimentally proved[16] that there is no paradox if the sample chamber is appropriately constructed. It should be noted that the VPP was studied in neutral bilayers, while it has been undisputed that charged bilayers can be easily swollen up to a few hundred ångströms in a humidity cell.[17]

Figure 6 shows the reflectivity curves of the four hydrated different samples. Curve (a) (the hydrated DOPC) presents a Bragg peak at $q_z = 0.116$ Å$^{-1}$, which was neglected during the simulation. Its presence indicates that there are zones of the samples where there is a building up of new bilayers. This underlines an instability of the neutral lipid during the hydration process. In the other parts of the sample, the film is swollen (~145 Å) but the number of layers remains unchanged; the head group has an average thickness of 15 Å and the SLD is higher. The tail region is thinner and the scattering length density is more negative.

Curves (b) and (c) show two different cycles of hydration of the cationic lipid; the first was acquired after 2 h of stay in the humidity chamber, while the second was acquired after8 h. The two curves are different; the cationic lipid continues swelling and does not seem to saturate even after 8 h. In the model of the dry DOTAP, the bilayer does not form, while the model of the hydrated indicates the presence of an ordered bilayer; the hydration process probably induces more stability in the cationic lipid than in the neutral one.

Curve (d) does not bring additional information to the already discussed dry mixture. The simulation shows a deviation from the measured curve in the region $0.05 < q < 0.12$ Å$^{-1}$.

The number of layers in the lipid/DNA film remains the same after the hydration, as seen in the density profile in Fig. 7 [curve (e)], while the thickness is 25 Å higher than in the dry complex. The tail region is reduced by 25% and its SLD is higher. The head group has swollen, while its density is unchanged. The DNA remains in the domain boundaries, as in the dry sample.

## IV. CONCLUSIONS

The focus of this work is the x-ray and neutron specular reflectivity study on thin films of membranes prepared by spin coating. This kind of deposition allows us to control the number of layers $N$ in the range of 1–30. Another advantage of this technique is that it requires only a very few micrograms of materials.

Single lipids or lipid-peptide mixtures have already been deposited on solid substrates by spin coating.[3] In this study, we use single cationic lipid, lipid mixture and a complex lipid-DNA.

The DOPC samples in the $L_\alpha$ phase are ordered and have a well-defined number of layers. The results reported in Table I give the thickness of each bilayer, the head's section, and the volume of the tails, and show that neutron reflectivity data simulations are in agreement with x-ray results. Only a qualitative comparison between x-ray and neutron reflectivity measurements is possible for the other samples because of the too different number of layers among the systems. Neutron specular reflectivity on DOTAP shows the presence of a thin film with a single bilayer, characterized by a 40 Å total thickness, while by x-ray measurements on the same lipid no trace of well-defined bilayers is revealed. Lipids in the DOTAP-DOPC mixture, with molecular weight fraction $\Phi = 0.5$, seem to segregate in both measurements. However, the presence of the DNA brings an order in the lipid mixture and probably induces the formation of bilayers with both lipids, as indicated by the presence of a single Bragg peak in Fig. 1 [curve (e)].

When the membranes are exposed to water vapor of 100% relative humidity, the heads of the lipids exchange their water molecules with the $D_2O$ present in the humidity chamber; consequently, their area changes and so do the densities, while the tails remain unchanged. During this process, we notice the building up of new bilayers in some zones of the DOPC sample which emphasize an instability of the neutral lipid. DOTAP, the DOTAP-DOPC mixture, and the complex lipid-DNA, instead, appear more stable, even though after 8 h the hydration of the cationic lipid does not reach saturation but continues swelling.

Experimentally, a reflectivity curve with a Bragg peak and Kiessig fringes indicates an ordered film and a well-defined number of bilayers, but the sample may still exhibit a defect structure on various length scales. These inhomogeneities can be traced by x-ray diffuse scattering, which gives access to the lateral film structure on the mesoscale between a few nanometers and several micrometers. In-plane diffuse x-ray measurements are in progress.

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