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Lipid Rearrangement in DSPC/DMPC Bilayers: A Neutron Reflectometry Study

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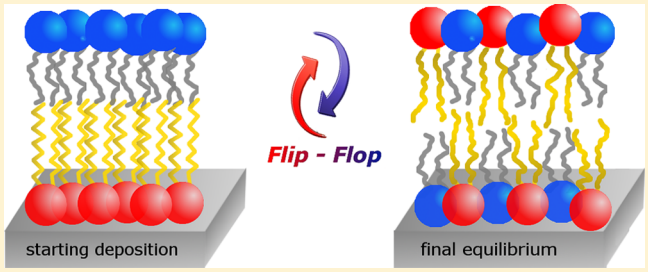
Supporting Information

ABSTRACT: Lipid translocation in membranes is still far from being understood and well characterized for natural cell membranes as well as for simpler bilayer model systems. Several discrepancies with respect to its occurrence and its characteristic time scale are present in the literature. In the current work, the structural changes induced by lipid rearrangement in a distearoyl-/dimyristoyl-phosphocholine binary lipid system have been addressed by means of neutron reflectivity. It has been shown that a fast, spontaneous compositional reorganization with lipid transfer between the two leaflets of the bilayer takes place only when the lipid species are both in the fluid phase. This process has been identified as the so-called lipid flip-flop. Moreover, the influence of the preparation protocol on the structural properties of the system has been investigated.



INTRODUCTION

The study of the structural properties of lipid membranes and their modifications induced by interaction with peptides,[1−5] drugs, and other interfacially active macromolecules[6,7] are key points in understanding biological processes occurring on the membrane level as well as for the development of new drugs and drug carriers. Among all of the available techniques, neutron reflectivity (NR) is a unique tool for the investigation of the absolute composition and the relative location of molecules within the bilayer and at its interfaces.[8] Reflectivity techniques are not suitable for membranes in their native environment (i.e., with a 3D organization as in vesicles, liposomes, and organelles). In fact, single bilayers have to be deposited on a substrate, usually silicon, quartz, mica, or sapphire, for both neutron and X-ray reflectivity measurements. Adsorbed bilayers are widely studied because it is possible to achieve, with the same probe, a more detailed structural characterization with respect to bulk techniques (small-angle scattering) because the sample is not affected by non-well-controllable effects such as polydispersity, aggregation, and, in some cases, phase separation.[9] Furthermore, by means of Langmuir−Blodgett/Schaefer deposition methods, it is possible to prepare successive adsorbed layers resulting in a final asymmetric composition. In spite of that, the lost curvature, the proximity to the substrate, and the absence of distinct inner and outer media can limit the number of cases in which a solid-supported bilayer can be used as model system. For example it is known that in nature the lipid distribution across the inner and outer leaflets of cell membranes is asymmetric[10−12] and that there is interplay among the compositional asymmetry, curvature, and functionality of the cell membrane.[13] Recently, it has been proposed that the asymmetric lipid distribution within biological membranes is not likely to be driven only by the spontaneous curvature of the lipids[14] but is a consequence of multiple factors, including the biophysical properties that dictate the ability of a lipid to cross the bilayer spontaneously, retentive mechanisms that trap lipids in one leaflet of the bilayer, and the presence of transporters that assist lipid translocation.[12] The latter are usually proteins that transfer (by flipping) phospholipids across the membrane.[13] Lipid asymmetry is important for certain cellular processes: interactions between phosphatidylserine (PS) lipids located in the inner leaflet and skeletal proteins such as spectrin improve the mechanical stability of the membranes of red blood cells.[15,16] Because of all of these factors, the study of asymmetric bilayers is a key point in developing mimetic model membranes. As mentioned above, the presence of the substrate and its consequent interaction with the adsorbed molecules might influence the self-organization of the lipids and their diffusion[17] with respect to the properties of the same system studied in solution. Highly hydrated polymeric cushions,[18] well-characterized and stable membranes,[19,20] and grafted lipids[21−23] have been placed between the deposited membrane and the solid substrate to overcome this limitation. Although in nature lipid translocation is a slow process and is usually controlled by proteins, in a pure lipid system this mechanism can occur spontaneously under certain conditions. The exchange of lipid molecules between inner and outer leaflets of a bilayer can be investigated from both dynamical and structural points of view. Nevertheless, for different lipid systems and different

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techniques, disagreement exists about the occurrence of the flip-flop and its characteristic time scale. Small-angle neutron scattering (SANS) has been, and it is still, widely used to study this mechanism in vesicles; an analysis of partially deuterated DMPC and DSPC/DMPC vesicles led to a characteristic flip-flop half-time ($t_{1/2}$) of about 9 h at 30 °C and 1 h at higher temperatures (40−50 °C).[24,25] (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine is DMPC, and 1,2-distearoyl-*sn*-glycero-3-phosphocholine is DSPC.) A dynamical investigation of the process can be performed using sum-frequency vibrational spectroscopy (SFVS) on solid-supported lipid bilayers.[26−28] This technique studies the kinetics of the flip-flop process by a direct measure of the $t_{1/2}$ time. For solid-supported DMPC bilayers in the gel phase, the flip-flop half time measured by this technique suggested much faster kinetics with respect to that observed in lipid vesicles, namely, $t_{1/2} \approx 3$ min at 16 °C.[26] Moreover, small changes in temperature have a strong effect on the half-time value. For DSPC bilayers, the dependence not only on temperature but also on the lateral pressure applied during the deposition was investigated, resulting in a wide range of kinetics. For example, a characteristic half-time going from 26 min (18 mN/m) to 2.5 h (40 mN/m) at relatively high temperature (46.8 °C) was found. In all of the reported cases, the sample was studied in the gel phase; no results arising from a fluid bilayer were reported in the literature. The molecular packing of Langmuir monolayers strongly depends on the lateral pressure applied. It is well known[29] that large differences in pressure (according to the phase diagram of each lipid) usually correspond to different phases of the monolayer at the water−air interface, resulting in different characteristics of the deposited layer. One of them is the larger number of defects (lower degree of coverage) usually present for depositions at lower pressure. This could induce a higher mobility in the lipid molecules for deposition at low lateral pressure. The presence of defects in adsorbed bilayers might explain the large variability in the half-time constant measured by SFVS.

It is worth mentioning that examples of spontaneously self-assembling asymmetric and stable lipid bilayers are reported in the literature.[30,31] This is the case of supported bilayers formed by the adsorption of small unilamellar vesicles made of a binary lipid mixture; asymmetric bilayers are formed spontaneously with preferential adsorption of the higher-melting-temperature lipid facing the solid support. In the present work, a binary lipid mixture was used to produce asymmetric (in terms of composition) solid-supported lipid bilayers deposited with the higher-melting-temperature (T_m) lipid facing the silicon surface; their structural reorganization induced by flip-flop translocation and the associated kinetics was investigated by means of neutron reflectivity. The influence of the sample preparation protocol (thermodynamic parameters used during the deposition) was addressed by comparing two DSPC/DMPC asymmetric bilayers prepared at 25 and 14 °C (i.e., above and below the T_m of DMPC). Using different schemes of deuteration in combination with three different buffer scattering-length densities, structural changes in DMPC/DSPC bilayers were followed in a time window of 10 h and in a temperature range of 14 to 60 °C.

MATERIALS AND METHODS

1,2-distearoyl(d_{70})-*sn*-glycero-3-phosphocholine-1,1,2,2-d_4-*N*,*N*,*N*-trimethyl-d_9 (DSPC-d_{83}), 1,2-distearoyl-*sn*-glycero-3-phosphocholine-1,1,2,2-d_4-*N*,*N*,*N*-trimethyl-d_9 (DSPC-d_{13}), and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) were purchased from Avanti Polar Lipids (Alabaster, AL). Phospholipid solutions were prepared in chloroform at a 1 mg/mL concentration. Solid-supported lipid bilayers were deposited using the Langmuir−Blodgett (LB) and Langmuir−Schaefer (LS) techniques. Silicon single crystals (5 × 5 × 1 cm^3) cut along the (111) plane were cleaned by rinsing, under sonication, in chloroform, acetone, and ethanol sequentially. Thirty minutes of UV/ozone treatment was used to make the polished surface more hydrophilic. Lipid bilayers were deposited using a Nima 1212D Langmuir trough filled with Milli-Q water (Millipore Corporation, Bedford, MA). The lateral pressure applied to the Langmuir film during both the LB and LS stages was 40 mN/m. Solid-supported asymmetric depositions were obtained by depositing via LB DSPC-d_{83} in the inner leaflet (facing the silicon block) and via LS DMPC on the outer side. To compare the influence of the deposition conditions, two samples were prepared at room and trough temperature of 25 and 14 °C, respectively; they are labeled in the present work as the *RT* sample and the cold sample, respectively. A symmetric 1:1 DSPC-d_{83}/DMPC bilayer was characterized and used as a reference for the complete mixed structure (with equal numbers of DMPC and DSPC molecules in every leaflet).

Sample holders were laminar flow cells that allowed the solvent exchange to apply the well-known contrast variation method.[32] For this purpose, high-purity D_2O (ILL) and Milli-Q water were used in ratios of 1:0, 0.66:0.34, and 0:1 in order to obtain media with scattering-length densities (SLDs or ρ values) of 6.35, 4.00, and −0.56 ($\times 10^{-6}$ Å^{-2}), respectively. Neutron reflectivity experiments were performed at the silicon/water interface on D17[33] and FIGARO[34] reflectometers at the Institut Laue-Langevin (Grenoble, France). Time-of-flight measurements were performed using wavelengths in the range of 2 to 18 Å (D17) and 2 to 30 Å (FIGARO) together with two different angular configurations for each instrument, resulting in a covered Q range from 0.005 to 0.3 Å^{-1}. It is worth remembering that for a given incident wavelength λ and angle α_i the wave-vector transfer in the direction perpendicular to the layer interface is $Q_z = {}^{4\pi}/_{\lambda} \sin \alpha_i$.

Structural modifications induced by temperature changes were studied by measuring the NR signal during a heating−cooling cycle at temperatures of $T = \{14 \rightarrow 30 \rightarrow 60 \rightarrow 30 \rightarrow 14\}$° for the cold sample. These temperatures were chosen on the basis of the difference in the chain melting temperature between the two lipid species used: $T_m = 23.5$ °C for DMPC and $50 < T_m < 51$ °C for DSPC-d_{83}.[35] In this way, bilayers coexisting with molecules in fluid and gel phases as well as with both of the components in the same state were obtained. The *RT* sample was measured at different temperatures, but for comparison purposes, only the data taken at $T = 30$ °C are shown and discussed.

Kinetics Measurements. During the heating part of the thermal cycle, kinetics measurements were made in the higher Q range. In fact, possible changes such as thinning or thickening of the bilayer as well as lipid molecule translocation within the bilayer are detectable, for the current sample composition, for Q_z values higher than ∼0.04 Å^{-1}. To have valid statistics, the acquisition time was 2.5 min for each run, and the measurements were performed in D_2O.

Preliminary Measurements. Newly polished silicon blocks were characterized before the experiment by ellipsometry. They turned out to be of the same quality with very similar features. They were characterized by the presence of a silicon oxide layer with a thickness and roughness of about 13.5 Å and 4.5 Å respectively. Three blocks were used in total, and for two of them, it was possible to perform a preliminary characterization by NR measurements that confirmed the ellipsometry results. These parameters were kept fixed during the modeling of the NR data of the bilayer system.

Data Analysis. The reflected wave from an interface between two compounds having different scattering-length densities, ρ, is[36]

$$R(Q_z) = \frac{16\pi^2}{Q_z^4} \left| \int \frac{d\rho(z)}{dz} e^{iQ_z z}\, dz \right|^2 \quad (1)$$

In the case of a multi-interface system, the total reflected intensity arises from the sum of the neutrons reflected by each interface. In fact, a fraction of the incident beam is reflected from the first interface, and the other portion is transmitted to the second layer. At this stage, it

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can be reflected or transmitted again and so on. Parratt's recursion relation[37] is an iteratively exact method of calculating the reflectivity for such a system; the sample is divided into several layers, and the portions of the reflected and transmitted beams are calculated. If still inside the sample, these neutrons can be reflected by or transmitted to another layer. Because of this dynamical aspect of the method, multiple scattering effects as well as self-adsorption are taken into account. Each layer is described by a triplet of parameters (thickness t, ρ, and roughness σ). The interface roughness reduces the specularly reflected intensity, and here it was modeled using an error function profile. The effect of the instrumental resolution was introduced in the model by a convolution between the theoretical reflectivity curves and the computed instrumental resolution for both instruments, resulting in a smearing of the theoretical curves. The software for the data analysis, written in Matlab, allowed the simultaneous analysis of all data taken in the three different contrast configurations, thus reducing the number of free parameters. This number was also reduced by imposing constraints on the scattering-length densities of the components used (Table 1). The minimization routine used was

Table 1. Calculated Scattering-Length Densities of the Dry Components of the Studied System[a]

material		ρ^{dry} ($\times 10^{-6}$ Å^{-2})
Si		2.07
SiO_2		3.41
heads−PC	$C_{10}H_{18}O_8PN$	1.74
	$C_{10}H_5D_{13}O_8PN$	5.70
	1:1 mixture	3.72
H-chains	gel phase	−0.39
	liquid phase	−0.34
D-chains	gel phase	7.45
	liquid phase	6.47
mixed chains	1:1 H-gel/H-liquid	−0.36
	1:1 H-liquid/H-liquid	−0.34
	1:1 D-gel/H-liquid	3.55
	1:1 D-liquid/H-liquid	3.07

[a]The SLD values for mixed-phase chains were evaluated assuming simply the perfect miscibility of the components. Reference values were taken from the literature.[1,39,40]

Fminuit, a χ^2 fitting program for Matlab based on the MINUIT minimization engine.[38] In the case of the adsorbed depositions, the Q_z profile of the scattering-length density was divided into five layers as reported in the following list:

1. silicon oxide (t_{ox}, f_{ox}, and σ_{ox})
2. inner headgroup layer (t_{h1}, f_{h1}, and σ_{h1})
3. inner hydrophobic region (t_{t1}, f_{t1}, and σ_{t1})
4. outer hydrophobic region (t_{t2}, f_{t2}, and σ_{t2})
5. outer headgroup layer (t_{h2}, f_{h2}, and σ_{h2}).

The SLD of the ith layer was determined from

$$\rho_i = f_i\rho_s + (1 - f_i)\rho_i^{dry} \quad (2)$$

where ρ_s and f_i are the SLD of the medium and its volume fraction within the ith layer, respectively. The SLDs of the components used are reported in Table 1. For symmetric bilayers, the model could be simplified by forcing the third and fourth layers to be described by exactly the same parameters, with the hydrophobic region being homogeneous in its overall extension $t_t = t_{t1} + t_{t2}$. Also, the thickness of the second and fifth layers could be set to the same value while f_{h1}, σ_{h1} f_{h2}, σ_{h2} were decoupled because of possible differences due to the fact that the inner leaflet is facing the solid substrate and the outer leaflet is facing the external medium.

RESULTS AND INTERPRETATION

From the specular reflection, the reflectivity curves were extracted as a function of the wave-vector transfer. A higher accuracy in determining the occurrence of flip-flop can be achieved at a high scattering contrast between the components (i.e., using DMPC and DSPC-d_{83}). An analysis of the DSPC-d_{13}/DMPC bilayer (data not shown) led to the same interpretation of the results despite the lower scattering contrast between the two lipid species.

Cold Sample. The cold sample was prepared by keeping room and trough temperatures in the range of 14 °C−16 °C (i.e., below the phase-transition temperature of both DMPC and DSPC). During all of the necessary steps to perform NR experiments, the sample temperature was continuously cooled with an external circulating thermal bath keeping the nominal temperature fixed at 12 °C, corresponding to a sample temperature (at the silicon interface) of approximately 14 °C as monitored with an external thermocouple sensor. Data taken in pure D_2O, pure H_2O, and 4MW (i.e., four match water) (ρ_s = {6.35, −0.56, 4.00} × 10^{-6} Å^{-2}, respectively) were analyzed using the five-layer model for all of the temperatures. Figure 1 shows the 14 °C data and their fits. The agreement between model and experimental data is remarkably good. The SLD profiles derived from the data modeling are reported in Figure 2 together with a pictorial sketch of the investigated system.

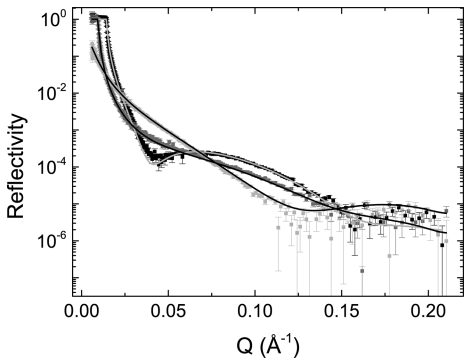

Figure 1. Reflectivity curves for the DSPC-d_{83}/DMPC asymmetric depositions measured at 14 °C in three different media: D_2O (black), 4MW (gray), and H_2O (light gray). The solid lines show the model fits obtained with the common set of parameters listed in the first column of Table 2. The SLD profiles derived from the fits are shown in Figure 2.

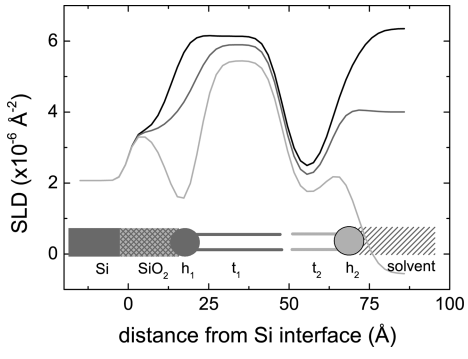

Figure 2. SLD profiles for the cold deposition measured at 14 °C in D_2O (black), 4MW (gray), and H_2O (light gray).

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The structural parameters derived from the model fits are reported in Table 2. To our knowledge, structural information about asymmetric bilayers made of DMPC and DSPC is not available in the literature. Nevertheless, it was possible to compare individually the inner and outer leaflets of the current system to the values found for pure DMPC and DSPC bilayers.[41,42] The good agreement indicates that DMPC molecules were still mostly located in the outer leaflet of the bilayer. The asymmetry is quite evident from the SLD profiles. Moreover, it was possible to determine that there was mixing between the two lipid species of less than 10% of the material. This quantity was not sufficient to affect the overall out-of-plane structure of each leaflet appreciably. As will be shown later, the mixing could be induced by mechanical shocks caused by the LS stage during sample preparation. The sample stability at 14 °C was monitored by measuring the reflectivity in H_2O at the beginning and end of the contrast variation series; the two curves were identical, indicating that no changes took place within the 4 h dividing the repetitions of the measurement. During the heating cycle, going from 14 to 30 °C, kinetics measurements were recorded. NR data sets are shown in Figure 3. The crossing of the DMPC chain-melting transition temperature is detectable in these NR curves. In fact, the chain melting took place between 20 and 25 °C in a time window of 25 min. It is clear that the structure of the bilayer changed as it went through the DMPC phase transition; as the temperature increased, the reflectivity smoothly decreased in the mid-Q range, and the first minimum was shifted toward higher Q. It is worth noticing that every curve describes the NR signal in a time window of 2.5 min; therefore, the transition was quite broad both in time and temperature. After the transition, the NR signal was stable, and it was possible to perform complete analysis at T = 30 °C using both the instrumental setup to cover the full Q range and applying the contrast variation method. The new stable structure could be described by taking into account the change in parameters induced by the melting of the shorter lipid molecules (DMPC) without introducing any lipid mixing and therefore any decreased asymmetry of the structure. As shown in Table 1, the chain SLD of a lipid molecule is different in the gel and liquid phases, and this changed the reflectivity curves. The derived parameters are summarized in the second column of Table 2. The DMPC/DSPC ratio in the two leaflets, as derived from the SLDs, was the same as in the 14 °C case, proving that the flip-flop mechanism is inhibited when one of the two leaflets is still in the gel phase (T_m = 50−51 °C for DSPC-d_{83}).

The thermal cycle continued up to 60 °C, where both lipid species were in the fluid phase. In going from 30 to 60 °C, kinetics measurements were performed in D_2O to monitor possible changes in the reflectivity curves. The measured curves are plotted in Figure 4. All of the NR data sets measured between 30 and 50 °C were the same as the red line in the graph. For temperatures of 53 and 56 °C (blue and black lines, respectively), the NR signal changed abruptly, indicating the presence of a structural transition. It is also possible to see that the 53 °C reflectivity data are approximately in between with respect to the other two curves. This could be explained by taking into account that in the middle of the acquisition time (2.5 min) a full structural transition took place (i.e., after more or less than 1.25 min). Therefore, the reflectivity is the average of the two stable states at the border of this transition. In fact all of the measurements taken after the 53 °C run showed a stable

Table 2. Parameters Extracted from the Fits of the Cold Sample at Different Temperatures during the Thermal Cycle

	14 °C	30 °C	60 °C	14 °C back[a]
t_{h1} (Å)	10 ± 1	9 ± 1	9 ± 1	9 ± 1
f_{h1}	0.30 ± 0.05	0.30 ± 0.05	0.52 ± 0.08	0.40 ± 0.05
σ_{h1} (Å)	2 ± 1	4 ± 1	4 ± 1	3 ± 1
t_{t1} (Å)	24.7 ± 0.5	25 ± 1	18.5 ± 0.2	18.7 ± 0.5
f_{t1}	0.12 ± 0.04	0.12 ± 0.05	0.05 ± 0.05	0.12 ± 0.05
σ_{t1} (Å)	2 ± 1	3 ± 1	4 ± 1	4 ± 1
t_{t2} (Å)	13.0 ± 0.9	12 ± 1	18.5 ± 0.2	18.7 ± 0.5
f_{t2}	0.12 ± 0.05	0.12 ± 0.05	0.00 ± 0.05	0.12 ± 0.05
σ_{t2} (Å)	2 ± 1	3 ± 1	4 ± 1	3 ± 1
t_{h2} (Å)	10 ± 2	9 ± 1	8 ± 1	9 ± 1
f_{h2}	0.3 ± 0.1	0.3 ± 0.1	0.5 ± 0.1	0.4 ± 0.1
σ_{h2} (Å)	3 ± 1	4 ± 1	3 ± 1	4 ± 1

[a]Measurements performed at the end of the cycle.

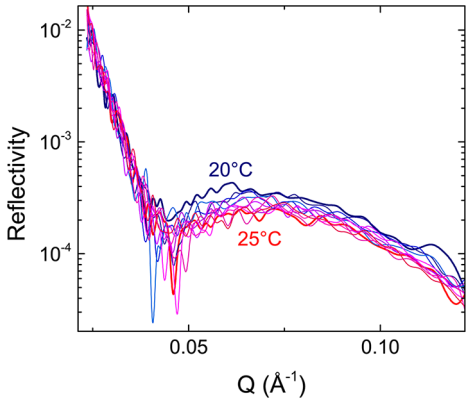

Figure 3. Reflectivity curves in D_2O for the cold sample measured during the heating cycle in the range of 20−25 °C. Below and above these two temperatures, the reflectivity curves were equal within errors to those taken at 14 and 30 °C, respectively. The acquisition time of each curve was 2.5 min.

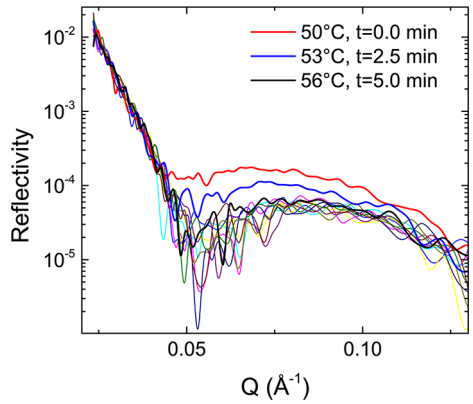

Figure 4. Reflectivity curves in D_2O for the cold sample measured during the heating cycle in the range of 50−60 °C. The red and black lines show the reflectivity before and after the transition, where the structure was stable. The blue line does not represent an actual stable structure or a single step across the transition, but it should be correctly interpreted as a time-weighted average of the initial and final states. The other curves show the measurements taken at T > 56 °C where the stable final state was already reached.

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reflectivity, superposable with the one taken at higher statistics at 60 °C. The features of this transition are completely different from those of the previous transition concerning not only the time scale but also the magnitude of the effect. It is clearly faster, and the reflected intensity is affected in a stronger way, decreasing by almost 1 order of magnitude in the region close to the first minimum. These features cannot be explained by assuming only the melting of the DSPC lipid molecules. A full characterization was necessary, and it was performed at 60 °C with both angular setups and in three different solvents. The reflectivity curves are reported in Figure 5. The final structure

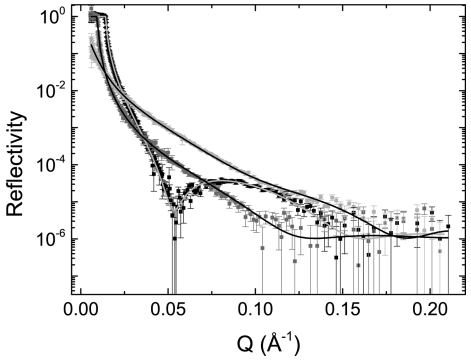

Figure 5. Reflectivity curves for the DSPC-d_{83}/DMPC asymmetric depositions measured at 60 °C in three different media: D_2O (black), 4MW (gray), and H_2O (light gray). The solid lines are the fits obtained with the common set of parameters listed in the third column of Table 2. The related SLD profiles are shown in Figure 6.

of the bilayer was completely different from the asymmetric state described before; in this case, it was completely symmetric. This is a confirmation that the melting of both lipid moieties activated the lipid translocation within the bilayer. The SLD of the final symmetric structure is depicted in Figure 6.

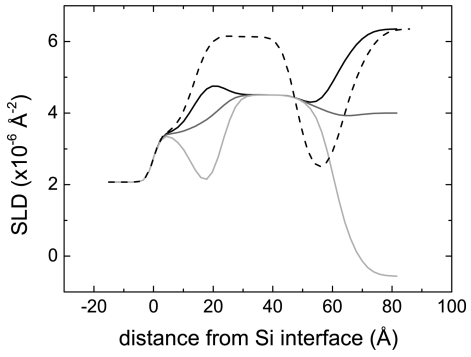

Figure 6. SLD profiles for the cold sample measured at 60 °C in D_2O (black), 4MW (gray), and H_2O (light gray). The asymmetric SLD found in D_2O at 14 °C is shown as a comparison (dashed line).

The difference in SLD of the headgroup region among different solvents is typical for NR measurements and is caused by the presence of water molecules in this region. This feature must not be misinterpreted as a marker of residual asymmetry. The most clear indication of the symmetric structure can be found in the hydrophobic region of the bilayer (30 Å < z < 50 Å in Figure 6), where water is almost absent and there are no differences between the inner and outer leaflets. The structural parameters characterizing the symmetric bilayer are reported together in the last column of Table 2. A similar NR experiment was carried out for DSPC bilayers prepared in the gel phase with an asymmetric distribution of cholesterol.[43] This structural asymmetry was kept in the gel phase, but it completely disappeared during annealing to a temperature higher than the melting point of the phospholipid molecules.

Preparation at *RT*. In the previous section, the temperature evolution of the so-called cold sample was described. In this section, the influence of the sample preparation, regarding the state of the lipid molecules during the deposition, is described. The sample was prepared with room and trough temperatures at 25 °C. Under this condition, the DMPC was no longer in a gel-like state but in a fluid-like state once the monolayer was formed. Besides this difference, all of the steps in the LB−LS procedure were the same as for the cold sample. The first NR measurement was performed at t = 30 °C shortly after the sample was prepared. The curves, taken as for the cold sample in three different solvents, could not be modeled using the parameters found in the previous case at the same temperature; in fact, a higher degree of mixing between the two lipid moieties was necessary. A comparison between the SLD profiles derived in the two cases is shown in Figure 7 for the

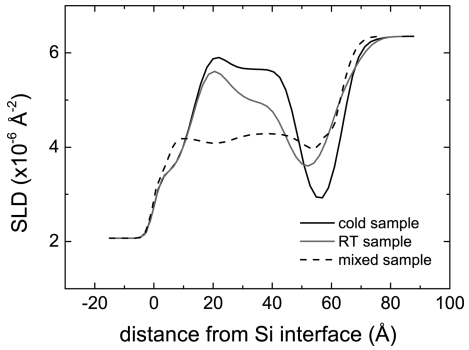

Figure 7. SLD profiles for the cold (black) and *RT* (gray) samples measured at 30 °C in D_2O. The SLD profile for an as-prepared mixed sample is shown with a dashed line.

D_2O measurements. It is clear that the structure of the *RT* bilayer is less asymmetric even if the DSPC molecules are still in the gel phase. This is a clear indication of a mixing induced by the mechanical shock of the Schaefer stage and not a real flip-flop process. From the SLD values, the amount of mixing for the as-prepared sample was estimated to be 30 ± 5%. This behavior could confirm that the 10% mixing observed for the cold sample has the same origin.

CONCLUSIONS

As already mentioned, there are different hypothesis on the time scale, occurrence, and features of the flip-flop mechanism in natural membranes and in model bilayers. The nature of the topic requires the study of the evolution from a nonequilibrated structure to a stable one. Few experimental techniques are suitable for such a study, and neutron reflectometry is one of these. Sum-frequency vibrational spectroscopy studies on solid-supported lipid bilayers[26−28] are the closest, in terms of the sample preparation and environment, to NR. Nevertheless, large discrepancies between the results arising from these two techniques are observed; as mentioned in the Introduction, SFVS shows that the lipid translocation takes place when the lipid bilayer is in the gel-phase with a typical time scale on the

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order of several minutes. The time scale is shortened when approaching the gel-to-liquid phase-transition temperature. Moreover, all studies performed by this technique were carried out with the lipids in the fluid phase. In the current work, it was shown that the sample prepared with all of the lipid molecules in the gel phase did not show any flip-flop process on a time scale of several hours when at least one of the leaflet was in the gel phase. The system was stable, in terms of lipid mixing, during the first 10 h of measurements as shown by the structural characterizations performed at 14 and at 30 °C. Then, once the melting transition temperatures of both lipid species were crossed, the flip-flop was activated and the lipids mixed completely on the 1′ to 2′ time scale. Therefore, it can be concluded that to be active flip-flop requires that both the leaflets or that at least the majority of the lipids in both leaflets are in the fluid phase.

The comparison between the two preparation methods showed that the thermodynamic properties of the Langmuir film are peculiar to obtaining a valid initial asymmetric bilayer. It was shown that the Langmuir−Schaefer stage could lead to contamination between the molecules composing the two leaflets if the packing of one of the monolayers was not tight enough. This was the case of the sample prepared with DMPC in the liquid state and DSPC in the gel state. The mixing was strongly reduced, preparing both leaflets in the gel state where only partial and negligible mixing occurred. This is a clear indication that to prepare a fully asymmetric solid-supported bilayer many conditions have to be satisfied and the preparation protocol must be improved to reduce to the minimum external perturbation such as the mechanical shock induced by the Langmuir/Schaefer stage.

■ ASSOCIATED CONTENT

Supporting Information

Detailed description of the *RT* sample and its comparison to the cold one. This material is available free of charge via the Internet at http://pubs.acs.org/.

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Notes

The authors declare no competing financial interest.

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