Effect of Low Amounts of Cholesterol on the Swelling Behavior of Floating Bilayers

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The effect of the addition of 1, 2, 4, and 6 mol % cholesterol to 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) floating bilayers has been investigated by neutron reflectivity. All samples exhibited fully stable and reversible gel and fluid phases. Around the main lipid phase transition temperature, DPPC double bilayers exhibit large increases in the water layer separating the bilayers and the upper bilayer roughness. The inclusion of low amounts of cholesterol reduced the swelling of the water layer between the bilayers and the upper bilayer roughness and progressively widened the temperature range over which swelling occurs. Results from asymmetric bilayers are also reported. A higher amount of cholesterol in the lower bilayer induces a smaller swelling of the water layer between the bilayers than in the symmetric case. Finally, the effect of the inclusion of a leaflet of 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE) was investigated. The presence of a leaflet with a higher gel-transition temperature (T_m) modifies the phase behavior of the lower T_m leaflet.

Introduction

Recent years have witnessed increasing attention to the study of lipid-cholesterol interactions, as the lateral organization of lipids seems to result from preferential packing of sphingolipids and cholesterol into rafts onto which proteins interact specifically with the bilayer.1 Model membrane systems represent a useful tool to understand those interactions. Cholesterol is universally present in the plasma membrane of eukaryote organisms. It is orientated in membranes such that its long axis lies parallel to the lipid chains. This has the effect of increasing order in the upper part, while decreasing the packing constraints at the terminal methyl groups. The phase diagram of phosphatidylcholines—cholesterol systems has been determined.2,3 The three common phases observed in many phosphatidylcholines systems are the gel and ripple phases, where the chains are conformationally ordered, and the fluid phase, where the chains are disordered. Low amounts of cholesterol (0–6 mol %) have little influence on the phase equilibria, where only a minute depression of the gel–fluid transition is observed and a very narrow coexistence is detected. Domain formation of cholesterol rich and poor regions has been observed for ratios ≥8 mol %.4

With the aim of investigating the possibility to prepare mixed planar floating bilayers as well as the effect of low amounts of cholesterol on the swelling behavior of phosphatidylcholine model systems, cholesterol concentrations of 1, 2, 4, and 6 mol % were incorporated into 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC) double bilayers. Figure 1 shows a cartoon of the double bilayer system. Specular neutron reflectivity was used to elucidate the structures between 25 and 48 °C, which covered the gel and fluid lipid phases as well as the main transition region.

It is experimentally interesting to be able to prepare samples with variable and well controlled composition and in this respect Langmuir–Blodgett and Langmuir–Schaefer techniques are very useful. They have been used in the past for the preparation of double bilayer systems.5 These consist of two lipid bilayers deposited on solid substrates and are stable in bulk water. The upper bilayer is separated from the lower one by a water layer of thickness, D_w, between 20 and 30 Å, whereas the lower bilayer is separated from the substrate by a water layer of 5–10 Å. The upper bilayer is freer to fluctuate than single adsorbed ones,6,7 and the presence of the water layer

Figure 1. Cartoon of a floating bilayer system containing cholesterol.
separating the two bilayers should enable the incorporation of trans-membrane proteins into the system, which is difficult with supported single bilayers because of their strong interaction with the substrate. Since the second bilayer is only weakly bound, the preparation is delicate but results are reproducible, with the samples exhibiting fully stable and reversible gel and fluid phases.

Furthermore, the system allows the application of a range of interfacial and surface techniques. The structural behavior as a function of temperature of double bilayers of phosphatidylcholines with saturated acyl chains of different length (varying between 16 and 20 carbons) has been characterized by neutron reflectivity measurements. This allowed the determination of the average and root-mean-square position of the floating bilayer, which was interpreted in terms of the competition between the interbilayer potential and membrane fluctuations and used to estimate the bending rigidity of the bilayer. Large swelling of the water layer was observed just below the main transition temperature, $T_m$, for all of these chain lengths, which corresponded to a minimum of the bending modulus in that region. Specular and off-specular synchrotron radiation measurements have confirmed the structural parameters of the systems obtained with neutron reflectivity and allowed the calculation of the membrane tension, bending modulus, and free energy.

The addition of 10 mol % cholesterol to the double bilayer system has resulted in the suppression of the swelling of the water layer at the gel-fluid phase transition. We will see below that the reduction of the swelling and modifications of the bilayer structural parameters happen progressively with the addition of cholesterol.

Asymmetric bilayers were also investigated. Results are presented on the effect on the phase behavior of DPPC double bilayers with different ratios of cholesterol in the lower (6 mol %) and upper (1 mol %) bilayers. This enabled the effect of cholesterol upon the interspacing of the bilayers to be studied.

Finally, results from asymmetric bilayers containing a leaflet of phosphatidyl ethanolamine will be presented. In many membranes, phosphatidylcholines and phosphatidylethanolamines are asymmetrically distributed across the leaflets of the bilayer. In the human erythrocyte membrane and rat liver plasma membrane, phosphatidylcholines are predominantly located in the exoplasmic facing leaflet and phosphatidylethanolamines in the cytoplasm-facing leaflet. The asymmetric distribution of phospholipids is a fundamental feature of normal cell operation and has been found to be necessary in exocytosis (fusión of membranes and secretory vesicles) and intra-cellular fusion processes, and also in lipid–protein interactions and signal transduction pathways. The asymmetric nature of membranes is generated by the activity of an adenosine triphosphate (ATP)-dependent amino-phospholipid translocase that specifically transports specific types of lipids between bilayer leaflets.

To model the asymmetric distribution of phosphatidylcholines and phosphatidylethanolaminolmes of certain membranes, a sample consisting of a lower bilayer of DPPC and 10 mol % cholesterol and an upper bilayer with an inner leaflet of DPPE and outer leaflet of DPPC and 10 mol % cholesterol was studied. Cholesterol was not included in the DPPE leaflet as it has been found to increase the instability of planar DPPE double bilayers.

Materials and Methods

Lipids and Substrates. DPPC (purity >99%) was purchased from Avanti Polar Lipids (Alabaster, AL). DPPE and cholesterol (purities >99%) were purchased from Sigma Chemicals. All chemicals were used without further purification. The silicon substrates (8 cm × 5 cm × 2 cm) were polished on one side to an average root-mean-square roughness of $\pm 1\ \AA$ by the ESRF optics laboratory (Grenoble, France). Prior to deposition the silicon substrates were cleaned in chloroform, ethanol, and ultrapure water in an ultrasonic bath for 15 min per solvent. All solvents used were ultrapure grade. Deuterated water of Millipore grade (18 MΩ cm) and D₂O (99% purity) was supplied by the Institut Laue-Langevin. A highly hydrophilic surface was formed on the silicon substrates by exposure to UV/ozone for 30 min. The monolayers were deposited immediately after.

Bilayer Deposition. Double bilayer samples containing DPPC and 0, 1, 2, 4, and 6 mol % cholesterol were prepared. Furthermore, a DPPC double bilayer containing 6 mol % cholesterol in the lower bilayer and 3 mol % cholesterol in the upper bilayer (asymmetric bilayer 1) was prepared as well as a double bilayer with a lower bilayer formed by DPPC and 10 mol % cholesterol and an upper bilayer with an inner leaflet of DPPE and outer leaflet of DPPC and 10 mol % cholesterol (asymmetric bilayer 2). Ratios of DPPC and cholesterol were dissolved in chloroform and co-spread on a Nima LB trough (Nima technology, Coventry, U.K.) allowing 20 min for the solvent to evaporate. Depositions were done at 17°C at a deposition speed of 5 mm/min with the monolayer held at a constant surface pressure of 40 mN/m. The monolayers were deposited using three vertical Langmuir–Blodgett depositions and one horizontal Langmuir–Schaefer deposition. An in-house-built microtable-controlled manual dipper with precision adjusted verticality was used for the last deposition to achieve the necessary very high degree of horizontality and low speed of about 0.3 mm/s. No trend was observed in the monolayer transfer versus cholesterol content for 1–6 mol % cholesterol and DPPC for any of the depositions. To fabricate the asymmetric sample 1, the 6 mol % monolayer was removed after the second deposition and replaced with a DPPC monolayer containing 1 mol % cholesterol for the final two depositions. Similarly, for the DPPC/DPPE sample, the monolayer of DPPC and 10 mol % cholesterol was removed after the second deposition and replaced with a monolayer of DPPC for the third deposition. A monolayer of DPPC and 10 mol % cholesterol was then re-spread for the final deposition. The exchange of monolayers and the deposition of different types of monolayers did not adversely affect the monolayer transfer.

Neutron Reflectivity Measurements. Neutron reflectivity is widely used for the investigation of buried interfaces, allowing the determination of the structure of matter perpendicular to a surface or an interface. For biological systems its main advantages are that light elements such as H, C, O, and N are strong scatterers and different isotopes of the same element have different scattering lengths so that isotopic substitution may be used to highlight different parts of the interface. Measurements can be performed in situ, and only very small amounts of material are required. The technique is very sensitive to the thickness of the layer, the composition, and the roughness of the interfaces. Specular reflectivity, defined as the ratio between the reflected and the incoming intensities of a neutron beam, is measured as a function of the wave vector transfer, $Q = 4\pi|\lambda|\sin \theta$ (where $\lambda$ is the wavelength and $\theta$ is the angle of the incoming beam to the surface) and is proportional to the Fourier transform of the first derivative of the scattering length density.

Swelling Behavior of Floating Bilayers

Table 1. Scattering Length Densities of Materials Used

<table>
<thead>
<tr>
<th>material</th>
<th>SLD (10^{-6} \text{ Å}^{-2})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Si</td>
<td>2.07</td>
</tr>
<tr>
<td>SiO2</td>
<td>3.41</td>
</tr>
<tr>
<td>H2O</td>
<td>-0.56</td>
</tr>
<tr>
<td>D2O</td>
<td>6.25</td>
</tr>
<tr>
<td>palmitoyl chain C16H32O2 : gel</td>
<td>-0.41</td>
</tr>
<tr>
<td>fluid</td>
<td>-0.32</td>
</tr>
<tr>
<td>PC head-group C16H32O4PN</td>
<td>1.74</td>
</tr>
<tr>
<td>PE head-group C16H32O4PN</td>
<td>2.66</td>
</tr>
<tr>
<td>cholesterol</td>
<td>0.22</td>
</tr>
</tbody>
</table>

* All values are from ref 22 except cholesterol from ref 30. The fluid phase chain value is calculated using volume of 1000 Å^3, whereas the gel used 800 Å^3.

SLD, perpendicular to the interface. The SLD of the material at the interface depends on the composition density by SLD = \( \sum b_j n_j \), where \( b_j \) is the scattering length of nucleus \( j \) and \( n_j \) is the number of nuclei per unit volume. Neutron specular reflectivity measurements were performed on the high flux D17 reflectometer at the Institut Laue-Langevin (Grenoble, France) in time-of-flight mode using a spread of wavelengths between 2 and 20 Å with two incoming angles of 0.7° and 4°. Background from the solvent in the sample usually limited the useful \( q \) range to 0.25 Å\(^{-1}\). The samples were measured at gel, transition, and fluid phases upon heating and cooling. All samples were allowed to equilibrate for about 15 min at each temperature before measuring the reflectivity profile.

The analysis of reflectivity data of lipid systems has attracted considerable attention. One of the most common ways is the construction of a model of the sample that is then parsed into a series of parallel layers of homogeneous material. Each layer is characterized by a SLD and a thickness, which are used to calculate a model reflectivity profile by means of the optical matrix method. Interfacial roughness between any two consecutive layers may also be included in the model by the Abeles method. The parameters of the model are varied within physically realistic constraints until the calculated reflectivity profile matches the measured profile. The quality of the fit is assessed by using \( \chi^2 \) in the least-squares method. In previous lipid bilayer studies, multiple contrast neutron measurements have allowed the determination within angstrom precision of the profile of adsorbed and floating bilayers. Following those previous studies, here each bilayer was divided into two headgroup regions and a chain region. The water layer separating the bilayers and the water layer separating the lower bilayer from the substrate constituted two layers. The thin silicon oxide layer of the substrate was treated as a single layer. The scattering length density of the chain region was adjusted to be located predominantly in the chain region of the bilayers. The scattering length density of the chain region was adjusted to take into account the concentration and volume of the cholesterol. The chain region of the upper bilayer of the sample containing the DPPE leaflet was separated into a further two layers to take into account the differences in the scattering length densities.

Results

0–6 mol % Cholesterol Samples. The high monolayer transfer (followed by ratio of decrease in monolayer area to surface area of substrate) and the bilayer parameters obtained from the reflectivity data show that it is possible to fabricate high quality DPPC double bilayers containing very low amounts of cholesterol.

All samples were measured at gel and fluid phase and transition region temperatures, and the reversibility was assessed. Figure 2 shows reflectivity profiles collected at all cholesterol content at temperatures typical of the transition region and the fluid phase. The structural parameters used to model the data are listed in Table 2. The inclusion of 1–6 mol % cholesterol slightly lowered the DPPC gel–fluid transition temperature \( T_m \) of the upper bilayers, although no trend was discernible as a function of cholesterol content, see Figure 4. The minute depression of \( T_m \) has previously been observed in similar systems.

From the thickness of the lipid chains decreasing from about 35 Å in the gel phase to about 30 Å in the fluid phase, we could estimate the \( T_m \) of the lower and upper bilayers separately (data not shown). Although the upper bilayers had transition temperatures similar to literature values obtained from lamellar systems for all cholesterol ratios, the lower bilayers consistently had higher \( T_m \). The lower bilayer containing 0–2 mol % cholesterol had a \( T_m \) of 4–6 °C higher than the upper bilayer, whereas those containing 4–6 mol % had a \( T_m \) of 2–3 °C higher. By a ratio of 10 mol % cholesterol the lower bilayer had the same \( T_m \) of the upper bilayer.

The gel phase structures of the lower water layer and lower bilayer did not change with the incorporation of cholesterol, whereas the main water layer and upper bilayer structures did. The thickness of the water layer

References

the chains are not higher ordered or tilted in the fluid phase. 

Upon becoming fluid, the upper bilayer chain thickness decreased by $4-7\text{ Å}$. No trend was observed in the thickness as a function of cholesterol concentration. The lower bilayers thickness decreased by $4\text{ Å}$ regardless of cholesterol concentration. Despite the presence of cholesterol, the fluid phase chain thickness of the two bilayers was similar to that of DPPC bilayers in multilamellar vesicles ([29 Å][26]) and adsorbed bilayers of [28 Å][27]. This is expected as the chains are not higher ordered or tilted in the fluid phase[28] and cholesterol is not expected to influence the thickness.

The thickness of the main water layer of the samples containing 4 and 6 mol % cholesterol did not change for the gel and fluid phases, whereas in the samples containing 0–2 mol % cholesterol, it increased by 4–6 Å upon the bilayers entering the fluid phase.

The thickness of the water layer in the fluid phase as a function of cholesterol concentration follows the trend present in the gel phase samples (Figure 2). The reason for the presence of a minimum at 2 mol % cholesterol is unclear. One possible explanation is an increase of the thermal fluctuations at those low cholesterol contents that could lead to the bilayers coming closer. Indeed, very low amounts of cholesterol ($\leq 3$ mol %) have been observed to have an effect on transition events, such as the phenomenon of anomalous swelling observed near $T_m$ and interpreted as due to an increased softening of the bilayers by the cholesterol near this event.[25,29]

Behavior around the Main Lipid Phase Transition. Large increases in the thickness of the main water layer and the upper bilayer roughness during the transition region have previously been reported for double bilayers of DPPC.[6] Without cholesterol, the water layer was observed to increase by $\sim 15\text{ Å}$, whereas the upper bilayer roughness increased by $\sim 10\text{ Å}$. The changes in these parameters as a function of cholesterol content are listed in Table 3 with the change in the water layer thickness as a function of temperature given in Figure 4. The maximum increase in Dw and u-Rou as a function of cholesterol content are given in Figure 5. The maximum increases in the parameters of the sample with no cholesterol agree with previous measurements.[6] Addition of cholesterol progressively decreases the extent of swelling and by 6 mol % only an increase of 4 Å is discernible. By 10 mol %, it was not possible to discern any increase.[8] When the increases are plotted as a function of cholesterol content, and linear regression lines are calculated, the maximum increase in the water layer decreases nearly linearly with cholesterol concentration with a $r^2$ of 0.966, and the roughness decreases almost linearly with a $r^2$ of 0.985. There is a clear overall broadening of the width of the transition as a function of cholesterol content, seen in Figure 4, but the data is too scattered to make a quantitative link. Data shown in Figure 4 is from samples being warmed from gel phase temperatures to fluid phase ones. The behavior upon cooling, although qualitatively agrees with that upon heating, is more difficult to analyze. The box model used is not valid anymore and different analysis tools are being tried. Fluctuations seem to be enhanced when samples are cooled.

Asymmetric Sample 1: 6 mol %/1 mol % DPPC Double Bilayers. To understand the effect on the phase behavior of different cholesterol concentrations in the

$^a$ 6 mol % 1 mol % Asym denotes double bilayer with 6 mol % cholesterol in lower bilayer and 1 mol % in upper bilayer. PE/PC Asym denotes asymmetric bilayer of DPPC/10 mol % cholesterol and DPPE. The prefix u refers to the upper bilayer and l the lower. dw is the water layer thickness separating the lower bilayer from the substrate, Dw is the chain region thickness, Rou is bilayer roughness, and Dw is the water layer thickness separating the two bilayers. All values are in Å. The error values are the error determined by modeling.
upper and lower bilayer, a DPPC sample containing 6 mol% of cholesterol in the lower bilayer and 1 mol% of cholesterol in the upper bilayer was investigated. The fabrication results were similar to those of the respective cholesterol containing samples, showing that it is possible to fabricate successfully double bilayers with different concentrations of cholesterol in each bilayer. Figure 6 shows reflectivity measurements and fitted profiles for this sample in the gel and fluid phases and in the transition region. The gel and fluid phase parameters used to model the reflectivity profiles are shown in Table 2. The two bilayers exhibit similar gel and fluid phase structures to their respective single cholesterol concentration double bilayers. The bilayer roughness is slightly higher and maybe due

Figure 4. Dw as a function of temperature for samples at different cholesterol content: (●) 0 mol%; (○) 1 mol%; (♦) 2 mol%; (◇) 4 mol%; (■) 6 mol%; and (△) asymmetric sample 1. The T_m of DPPC is marked as a dotted line.

Table 3. Gel Phase Thickness of Water Layer, Maximum Thickness, and Maximum Increase

<table>
<thead>
<tr>
<th>mol%</th>
<th>main water layer (Å)</th>
<th>upper bilayer roughness (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>gel phase value (°C)</td>
<td>maximum value</td>
</tr>
<tr>
<td></td>
<td></td>
<td>max water layer (Å)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>gel phase value (°C)</td>
</tr>
<tr>
<td>0</td>
<td>27 ± 1</td>
<td>43 ± 1</td>
</tr>
<tr>
<td>1</td>
<td>22 ± 1</td>
<td>37 ± 1</td>
</tr>
<tr>
<td>2</td>
<td>23 ± 1</td>
<td>33 ± 1</td>
</tr>
<tr>
<td>4</td>
<td>29 ± 1</td>
<td>37 ± 1</td>
</tr>
<tr>
<td>6</td>
<td>33 ± 1</td>
<td>37 ± 1</td>
</tr>
<tr>
<td>6% 1% Asym</td>
<td>28 ± 1</td>
<td>35 ± 1</td>
</tr>
</tbody>
</table>

Figure 5. Maximum increase in the (●) water layer, Dw, and (○) upper bilayer roughness, u-Rou, as a function of cholesterol content in the transition region.

Figure 6. Reflectivity profiles from asymmetric sample 1 in the gel phase at 26 °C (●), in the transition region at 37 °C (◇) and fluid phase at 42 °C (○).
to differences in fabrication. The main water layer had a thickness similar to the higher cholesterol content samples (28Å).

The transition phase parameters are listed in Table 3 and the water layer thickness as a function of temperature is shown in Figure 4. The maximum increase in the water layer thickness was half that observed in the 1 mol % double bilayer, but higher than the 6 mol % double bilayer. The maximum increase in the upper bilayer roughness was a third that of the 1 mol % double bilayer of 9 ± 2 Å, but higher than the 6 mol % double bilayer. The thickness of the water layer as a function of temperature follows closely the trend of the 6 mol % double bilayer in extent and the temperature range over which it is observed.

**Asymmetric Sample 2: DPPC 10 mol % Cholesterol/DPPE.** The asymmetric sample consisted of a lower bilayer of DPPC and 10 mol % cholesterol and an upper bilayer with an inner leaflet of DPPE and outer leaflet of DPPC and 10 mol % cholesterol. The sample was successfully fabricated, showing for first time that it is possible to make asymmetric double bilayers with different lipid components in the leaflets. Figure 7 shows reflectivity measurements and fitted profiles for this sample in the gel and DPPC fluid phases and in the transition region.

The gel phase parameters used to model the reflectivity profiles are listed in Table 2. The structure of the lower bilayer was similar to that of the 10 mol % double bilayer, with a chain thickness of 35 ± 1 Å and roughness of 12 ± 1 Å. The upper bilayer chain region had a similar thickness to the 10 mol % double bilayer, which had a chain thickness of 37 ± 1 Å and roughness of 16 ± 1 Å. The similarities of the chain thicknesses are expected as unlike phosphatidylcholines phosphatidylethanolamine molecules are not tilted in the gel phase. The high roughness of the upper bilayer though is intriguing. The upper bilayers of DPPE have been found to have a low roughness of 5 Å. The results here indicate that the bilayer roughness is determined either by the DPPC–10 mol % cholesterol leaflet or by the asymmetric nature of the bilayer. Domain formation is known to occur at this ratio of cholesterol, so it is more likely that the higher roughness is due to the influence of the DPPC–10 mol % leaflet.

The upper bilayer of a double bilayer of DPPC–10 mol % has been observed to have a gel–fluid transition temperature of 40–43.5 °C, whereas double bilayers of DPPE had a Tm between 62 and 64 °C. In this asymmetric sample, the lower bilayer becomes fluid between 43.4 and 47.1 °C. The chain thickness decreases by 4 Å and the overall bilayer roughness by 4 Å. The decrease in roughness is expected, as, in the fluid phase, cholesterol and DPPC are completely miscible at this ratio and are thus expected to have a lower roughness. This contrasts with the gel phase. The upper bilayer behaved differently to the lower bilayer. Its structure remained gel even up to 15 °C above the Tm of the 10 mol % double bilayer (see Figure 8). The presence of the DPPE leaflet in the bilayer clearly influences the phase behavior of the DPPC–10 mol % leaflet. It was not possible to ascertain whether exchange of cholesterol between leaflets occurred in the upper bilayer.

**Conclusions and Perspectives**

In this paper, results have been reported on the phase behavior of DPPC double bilayers containing low amounts of cholesterol, up to 6 mol %, as well as two examples of asymmetric bilayers, one formed by two bilayers with different cholesterol content, 6 mol % in the lower layer and 1 mol % in the upper layer (asymmetric sample 1), and the other having a leaflet of the external bilayer formed by phospholipid with a phosphatidylethanolamine head-group, whereas the other layers consist of DPPC and 10 mol % cholesterol (asymmetric sample 2). It was found that these very low amounts of cholesterol affect the phase behavior of DPPC double bilayers, indicating that cholesterol is successfully incorporated into samples. The effect over the phase transition region is summarized in Figure 4.

The thickness of the water layer separating the bilayers in the fluid phase goes through a minimum at the value of 2 mol %. Swelling of the water layer between the bilayers has been observed in the past in pure phosphatidylcholine samples and was attributed to a lowering of the bilayer bending modulus at the phase transition in agreement with literature data. In these results, a widening of the temperature region over which swelling occurs, as well as a decrease in the extent of swelling as the cholesterol amount increases were observed. The effect of adding cholesterol can thus be interpreted as an enhancement of the thermal fluctuations in the model membrane system. If cholesterol at these low concentrations makes the membrane more fluidlike, the critical behavior at the transition may be somewhat diminished, the membrane fluctuates less, and the swelling is smaller with respect to the pure phospholipid system.

In asymmetric bilayers with an inner bilayer containing 6% and an outer bilayer of 1% of cholesterol, there was a lower degree of swelling than when the two bilayers have the same composition. In the gel and fluid phases, which as demonstrated here have similarities in the structures to their single concentration counterparts, there...
is no evidence that the forces are modified. The difference in cholesterol concentration only affects the transition phase behavior because of the large structural changes that occur in that phase. The transition phase behavior upon cooling is also modified and the results are currently being analyzed.

Asymmetric sample 2 contains the component DPPE for which less literature data is available. Phosphatidylethanolamines generally have higher $T_m$ than their phosphatidylcholine counterparts due to the stronger H-bonding possible between the headgroups. In this sample upon heating, while the lower bilayer became fluid, the upper bilayer stayed in the gel phase up to 15 °C above the DPPC chain melting temperature. As the DPPC and DPPE have similar chain lengths, the origin of this behavior on the $T_m$ is likely due to the DPPE headgroups. It is likely that the chains of the DPPE leaflet are interdigitated with the chains of the DPPC–cholesterol leaflet, thus influencing the behavior of that leaflet. This sample is part of ongoing studies into asymmetric mimics of bilayers.

The results described in this paper represent a step toward the improvement of double bilayers as mimics for membrane system. It has been already argued that this model system can be a useful tool for studies of transmembrane proteins and translocation across membranes. What makes the system interesting is not only the presence of a planar bilayer free to fluctuate and to be studied in the fraction of nanometer scale but also the possibility of varying its composition. We showed here that both the inclusion of cholesterol and the presence of asymmetric leaflets lead to stable systems and are potentially useful for membrane fluctuation studies.

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