Chapter 2

Influence of hydration on the phase behaviour of PC-cholesterol membranes

2.1 Introduction

Cholesterol is the most important sterol molecule from a biological perspective because of its strikingly singular presence in almost all higher order eukaryotic cell membranes. Phosphatidylcholine (PC) lipids are major components of cell membranes. Therefore, there have been many studies on PC-cholesterol model membranes to understand the influence of cholesterol on various membrane properties. In particular, the phase behaviour of lipid-cholesterol membranes has been given central importance in these studies. Various experimental techniques like DSC, NMR, x-ray and neutron scattering, microscopy etc. have been used to study the phase behaviour of lipid-cholesterol mixtures [1, 2, 3, 4, 5, 6, 7]. Phase diagrams constructed using various experimental techniques are given in chapter-1. The main results of these studies can be summarized as follows

1. A cholesterol rich \( \beta \) phase was found to coexist with both \( L_\alpha \) and \( L_{\beta'} \) phases over a wide cholesterol concentration range at temperatures above and below the main transition, respectively. Degree of ordering of the hydrocarbon chains and the in-plane diffusion rates in this phase were found to be intermediate between those in the fluid \( (L_\alpha) \) and gel \( (L_{\beta'}) \) phases.
2. At higher cholesterol concentrations (> 20 mol%) the $\beta$ phase exists throughout the temperature range. The $\beta$ and $L_\alpha$ phases are often referred to as the liquid ordered ($l_o$) phase and liquid disordered ($l_d$) phase respectively in the literature [4, 8, 9].

3. The two phase region above the main transition observed in NMR studies has not been seen in diffraction experiments [10, 11, 12, 13]. However, these studies have found two phase coexistence below the main transition at cholesterol concentration < 10 mol%. One of these two phases, which is presumably richer in cholesterol can swell more [10]. This phase persists even at higher cholesterol concentrations, whereas the cholesterol-poor phase disappears above 10 mol%.

4. The $l_o$ phase is believed to be rich in cholesterol, whereas the $l_d$ phase is poor in cholesterol. Hydrocarbon chain segmental order parameters in the $l_o$ phase is found to be almost twice compared to that in the pure lipid at temperatures above the chain melting transition [3].

X-ray diffraction studies on oriented bilayers of DPPC-cholesterol and DMPC-cholesterol mixtures at close to full hydration (98% relative humidity) was carried out by Karmakar et al. A novel modulated phase denoted as $P_\beta$ was observed at intermediate cholesterol concentrations [13].

Most of the above studies were carried out at full or close to full hydration, the reason being that the full hydration condition truly mimics the environment of the biological membranes. However the hydration levels strongly influences both the main and pre-transition temperatures of the pure lipid bilayers [14]. Also the chain tilt of the lipid decreases with decreasing degree of hydration. At very low hydration the tilt of the PC molecules vanishes [15, 12]. So it is an interesting problem to look at the effect of hydration on the phase behaviour of lipid-cholesterol membranes.

In this chapter we present our experimental results of x-ray diffraction studies on the structure and phase behaviour of dipalmitoyl phosphatidylecholine (DPPC) and dimiristyl phosphatidylecholine (DMPC) membranes at various cholesterol concentrations ($X_c$) as well.
as at different relative humidity (RH) conditions.

2.2 Experimental results

We have carried out x-ray diffraction studies on DPPC-cholesterol and DMPC-cholesterol mixtures in the form of both oriented and unoriented bilayers at various cholesterol concentrations. In this section we describe briefly the experimental methodology and results of our studies.

Both oriented and unoriented multilayers of DPPC-cholesterol and DMPC-cholesterol were prepared at required molar ratios as described in the previous chapter. Small angle x-ray scattering was used to probe these mixtures. The oriented samples were probed at various relative humidity conditions in a specially designed chamber. Various RHs were obtained by keeping a reservoir of saturated salt solutions in the chamber. Different salts give rise to different values of RH depending on their saturated vapor pressure. For example a saturated NaCl solution gives ~ 75% RH whereas CaCl₂ gives ~ 30% RH. A general empirical relation describing the relation of RH with the salt is given by $RH = ae^{-b/T}$. Where $a, b$ are characteristic constants for the particular salt.

2.2.1 Phase behaviour of DMPC-cholesterol mixtures at low humidity

Oriented bilayer stacks of DMPC-cholesterol mixtures were probed at various cholesterol concentrations and hydration levels. The partial phase diagrams were obtained by small-angle x-ray scattering studies. At low hydration levels a large number of Bragg peaks are observed even in the disordered fluid ($L_a$) phase. This is due to the fact that at low water content the bilayers come closer and hence the correlation length increases due to decreased thermal undulations of the bilayers. At full hydration or close to full hydration (98% RH) only ~ 4 Bragg peaks are obtained from the $L_a$ phase. Whereas in more rigid gel phase number of Bragg peaks is typically ~ 10. The phase behaviour of DMPC-cholesterol mixture at 65% RH and 30% RH are described below.
2.2.1.1 RH=65%

Partial phase diagram obtained from the diffraction study of DMPC-Cholesterol mixtures at 65% RH is shown in fig. 2.1. At 65% RH an increase of ~10°C was observed in the main transition temperature \( T_m \). For low cholesterol concentrations \( T_m \) was observed to be 35°C and the value was found to decrease with cholesterol content up to 12.5 mol% where the transition was completely abolished. Interestingly the pre-transition was completely abolished at all cholesterol concentration as the \( P_{\beta'} \) phase was not observed. As seen in the phase diagram a relatively broad region of two phase coexistence of \( L_{\beta'} \) and \( P_{\beta} \) was observed between 2.5 to 7.5 mol% of cholesterol. At cholesterol concentration ~ 10 mol% a narrow region of modulated phase was observed. The modulated phase was identified by the satellite peaks in the small angle region (see fig. 2.2).

2.2.1.2 RH=30%

Lowering the RH to 30% influences the phase behaviour of DMPC-cholesterol mixture significantly. Both main and pre-transitions were abolished at a very low cholesterol concentration. Even for the pure lipid the pre-transition was not observed which is in agreement with earlier studies by Smith et al. [14]. The small angle diffraction patterns showing the
Figure 2.2: Small angle diffraction pattern of the $P_\beta$ phase of DMPC–cholesterol mixtures at 65% RH. Cholesterol concentration = 10 mol%, $T = 10$ °C.

Figure 2.3: Diffraction pattern of DMPC bilayers at 30% RH (a) fluid phase ($L_a$) at $T = 47$ °C, (b) gel phase ($L_\beta$) at $T = 43$ °C, and (c) gel phase ($L_{\beta'}$) at $T = 20$ °C.
Figure 2.4: Diffraction pattern of DMPC-cholesterol mixture at 2.5 mol% of cholesterol at RH=30%. (a) fluid phase ($L_a$) at $T=55\, ^\circ C$, (b) gel phase ($L_{\beta}$) at $T=20\, ^\circ C$, and (c) gel phase ($L_{\beta'}$) at $T=5\, ^\circ C$.

phase transition of pure DMPC bilayers at 30% RH are shown in fig. 2.3. At 5 mol% of cholesterol we observe the fluid phase at all temperatures. Only at 2.5 mol% of cholesterol a fluid-gel transition was observed. The $T_m$ at that concentration was $\sim 50\, ^\circ C$. At 2.5 mol% of cholesterol the phase sequence $L_a \rightarrow L_{\beta} \rightarrow L_{\beta'}$ was observed. The $L_{\beta}$ and $L_{\beta'}$ phases were identified from the characteristic chain reflections in the wide angle region (see fig. 2.4).

Based on our diffraction study we have constructed a partial phase diagram (shown in fig. 2.5) for DMPC-cholesterol mixture at 30 % RH.

2.2.2 Phase behaviour of DPPC-cholesterol in excess water condition

Unoriented samples of DPPC-cholesterol mixtures at different cholesterol concentrations were probed in excess water. A partial phase diagram was constructed by analyzing the diffraction peaks and is shown in fig: 2.6.

The main transition temperature was found to decrease slightly with cholesterol content up to about 20 mol%, beyond which it drops sharply. At temperatures above $\sim 40\, ^\circ C$ the
Figure 2.5: Phase diagram of DMPC-cholesterol mixtures at 30% RH.

Figure 2.6: Phase diagram of DPPC-cholesterol mixtures in excess water.
Figure 2.7: I–q plots for DPPC-cholesterol mixtures at 20 mol% of cholesterol. (a) T=50°C, (b) T=30°C. Intensity scales are normalized.
Table 2.1: Lamellar spacings $d$ (Å) of DPPC-cholesterol mixtures as a function of temperature in excess water (100% RH). The error in $d$ is ± 0.3 Å.

<table>
<thead>
<tr>
<th>$T$ (°C)</th>
<th>$X_c$ (mol%)</th>
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<tr>
<td>2.5</td>
<td>63.2</td>
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<tr>
<td>5</td>
<td>64.5</td>
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<tr>
<td>7.5</td>
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<td>10</td>
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<td>63.7</td>
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The fluid phase ($L_g$) was observed at all cholesterol concentrations. For $X_c$ between 0 to 15 mol%, below $T_m$ the ripple phase ($P_{gb}$) was observed. We were not able to see the gel ($L_{gb}$) phase even at 2.5 mol% cholesterol. Also gel-fluid coexistence region was not observed.

The reason for this may be the coarse steps taken along the composition axis (~ 2.5 mol%). Interestingly the modulated phase ($P_{gb}$) was not seen in this phase diagram, rather that region of the phase diagram was occupied by a fluid phase which is denoted by $L_g'$. Such a notation was introduced to differentiate this region of phase diagram from that of the normal fluid phase $L_g$ region. Though $L_g'$ was characterised as a fluid phase but the lamellar periodicity is ~ 5Å larger than that of the $L_g$ phase. The $I - q$ plot at 20 mol% of cholesterol is shown in fig. 2.7 and the $I - q$ plot at 20 mol% of cholesterol as a function of temperature is shown in fig. 2.8. The jump in lamellar periodicity can be clearly seen from the plot of lamellar periodicity Vs temperature as shown in fig. 2.9. The abrupt increase in the lamellar periodicity by about 0.5 nm across the $L_g$ - $L_g'$ transition, suggests that the latter might be a distinct phase [16]. The lamellar periodicity data at various cholesterol concentrations and temperatures are given in table. 2.1.
Figure 2.8: $I - q$ plots as a function of temperature at 20 mo% of cholesterol.

Figure 2.9: Lamellar periodicity ($d$ in Å) as a function of temperature at various cholesterol concentration.
Figure 2.10: $T_m(\degree C)$ of DMPC at different RH s. Error bars in temperature axis signifies the coarse steps taken and error bar in RH axis arises from the accuracy of the RH meter.

Figure 2.11: Temperature-RH phase diagram of DMPC-water taken from reference [14]. Note the increase in $T_m$ as RH decreases and the absence of ripple phase at low RH.
2.3 Discussion

Phase behaviour of DMPC-cholesterol mixture at 98% RH was studied by Karmakar et al. [17] where the ripple phase was observed below $T_m$ for cholesterol concentration up to 20 mol%. Beyond that the main and pre-transitions are abolished. At intermediate cholesterol concentrations the $P_{\beta}$ phase was observed at low temperatures. Our studies on DMPC-cholesterol mixtures show that $T_m$ increases as RH decreases. A plot of $T_m$ as a function of RH is shown in fig. 2.10. The increase in the main transition temperature suggests that the gel phase ($L_{\beta}/L_{\beta'}$) is stabilized at lower humidities. This may be due to the fact that the effective lateral area per lipid molecule decreases as the hydration level decreases. This helps in the lipid packing facilitating the gel phase stabilization. We did not see the $P_{\beta}$ phase at both 65% and 30% RH. Which suggests that the pre-transition completely disappears at lower hydration. Our results are consistent with earlier studies on DMPC-water system [14].

The DMPC-RH phase diagram obtained by Smith et al. is given in fig. 2.11.

At 65% RH the phase behaviour of DMPC-cholesterol is very similar to DPPC-cholesterol mixture at 75% RH [17]. The modulated phase was observed at 65% RH. However at 30% RH we did not see the modulated phase. Earlier studies show that the tilt of the hydrocarbon chains of the PC molecule decreases as the hydration level decreases and at very low hydration the tilt completely vanishes [12]. Hence at low hydrations a PC molecule will behave similar to a lipid molecule having no tilt like the phosphatidylethanolamines (PEs). The phase behaviour of PC-cholesterol membrane should therefore be very similar to that of PE-cholesterol membranes. The phase behaviour PE-cholesterol membrane was reported by Karmakar et al. [17] where it was observed that cholesterol does not induce the $P_{\beta}$ phase in DLPE bilayers. We also obtain a very similar result from our studies on DMPC-cholesterol mixture at 30% RH where we observe a relatively broad region of the $L_{\beta}$ phase. This again ascertains the fact chain tilt plays an important role in inducing the modulated phase in lipid bilayers.

Consistent with earlier scattering studies on PC-cholesterol membranes we also do not
observe any $l_{o}$-$l_{d}$ co-existence above $T_{m}$ in DMPC-cholesterol bilayers. The observation of $l_{o}$-$l_{d}$ co-existence above $T_{m}$ from spectroscopic measurement may be attributed to the timescale of observation employed in such probes. We think that there are transient concentration fluctuations of cholesterol in the lipid bilayer above $T_{m}$. The lipid molecule in the vicinity of cholesterol will have higher chain ordering. This can be picked up by the spectroscopic probes. Whereas scattering techniques like x-ray which probe the system at a much longer timescale will average out all such transient behaviour [16]. The phase behaviour below $T_{m}$ depends on the cholesterol concentration and degree of hydration. Cholesterol concentration required to completely abolish the main transition decreases with decrease in degree of hydration (see fig. 2.12).

Another interesting aspect of our result is that phase behaviour of DPPC-cholesterol at excess water condition has certain differences from the phase behaviour at a slightly lower RH (98%) reported earlier [13]. In excess water we do not see the modulated phase $P_{β}$. That region of phase diagram is occupied by a fluid phase which we have denoted as $L'_{α}$. The reason for differentiating this phase from the normal fluid phase $L_{α}$ is that we see a jump of ~ 5Å in lamellar periodicity across $L_{α}$ $→$ $L'_{α}$ transition. The increase in lamellar periodicity may be attributed to the decrease in membrane rigidity. This can be understood from the
If we assume that the cholesterol concentration fluctuation can couple with the local bilayer curvature, as indicated by the formation of \( P_\beta \) phase, then we can write the expression for the free energy density \( f \) of the bilayer in terms of concentration fluctuation \( \delta X \) of cholesterol as:

\[
f = \frac{1}{2} \kappa C^2 + \alpha C \delta X + \beta (\delta X)^2
\]

where \( \kappa \) is the bending rigidity of a single bilayer, \( C \) is the mean curvature of the bilayer. \( \alpha \) and \( \beta \) are two parameters. Here \( \alpha \) can be either +ve or -ve depending on the particular systems, but \( \beta \) can only take +ve values since the system wants to maintain a homogeneous concentration.

Minimizing \( f \) w.r.t \( \delta X \) we get \( \delta X = -\frac{\alpha}{2\beta} \). Hence

\[
f = \frac{1}{2} (\kappa - \frac{\alpha^2}{2\beta}) C^2 = \frac{1}{2} \kappa' C^2
\]

where \( \kappa' = \kappa - \frac{\alpha^2}{2\beta} \) is the effective bending rigidity. Since the quantity \( \frac{\alpha^2}{2\beta} \) always assumes a +ve value for reasons mentioned above, \( \kappa' < \kappa \). Hence the bending rigidity of the bilayer decreases in presence of such concentration fluctuations, provided these fluctuations can be coupled to the local membrane curvature. Such a decrease in the bending rigidity can lead to increase in the steric repulsion between bilayers as the the undulation/steric repulsion interaction \( (f_U) \) per unit area is related to \( \kappa \) as-

\[
f_U \propto \frac{(K_B T)^2}{K_c a^2}
\]

where \( K_c = \kappa \times d \) represents the bending rigidity of the lamellar stack and \( a \) is the interbilayer separation, \( K_B \) is Boltzman constant, \( T \) is the temperature.

Therefore the decrease in \( \kappa \) can effectively increase the steric repulsion between the bilayers and hence can increase the inter bilayer separation. This can account for the observed jump in the lamellar periodicity across \( L_\alpha \to L'_\alpha \) transition.
2.4 Conclusion

We have studied the phase behaviour of DMPC-cholesterol mixtures at two different RHs (65 and 30%). We compared the results with earlier results obtained at 98% RH. Significant increase in $T_m$ was observed at low hydration. The gel phase is stabilized at low RH and we do not see the ripple phase at these low RHs. However the $P_{\beta}$ phase exists at low RH as long as the tilt angle of the chains in the gel phase is non zero. This result highlights the importance of the chain tilt in the formation of this phase. Our results on DPPC-cholesterol in excess water seems to suggest that the modulated phase ceases to exist in excess water. Instead we observe a fluid phase ($L_{\alpha}$) with higher lamellar periodicity, which can arise from a lowering of the membrane rigidity due to the coupling between cholesterol concentration and the tilt of the chain.
Bibliography


