Formation of the liquid-ordered phase in fully hydrated mixtures of cholesterol and lysopalmitoylphosphatidylcholine

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The role of cholesterol (Chol) in promoting lamellar phase formation in mixtures with 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (Lyso-PPC) in excess water was investigated using multinuclear solid-state NMR and X-ray scattering. It was found that mixtures containing Chol and Lyso-PPC form a liquid-ordered (L₀) lamellar phase over a range of temperatures and concentrations, as previously observed in mixtures of Chol with various diacylphospholipids. The maximum quadrupolar splitting of the ²H-NMR powder patterns for samples containing per-deuterated Lyso-PPC were 40–50 kHz which is strongly indicative of an L₀ phase. This evidence was supported by wide angle X-ray scattering data which showed a characteristic diffuse peak centred at 4.2 Å. The L₀ phase coexists with an isotropic Lyso-PPC phase at Chol concentrations up to 70 mol% Chol, and with Chol crystals at Chol concentrations above this value. Below 70 mol% Chol, an increase in the concentration of Chol in the system caused a corresponding increase in the proportion of the L₀ phase present compared with the amount of isotropic Lyso-PPC. The chemical-shift anisotropy (CSA) of the static ³¹P-NMR spectra of the L₀ phase showed the symmetry of a lamellar phase, but the linewidth, Δσ, was much narrower than CSA powder patterns obtained for diacylphospholipids in similar conditions, being ~20 ppm as opposed to ~40 ppm, respectively.

Introduction

Cholesterol (Chol) is the ubiquitous sterol in mammalian cell membranes. Its effects on the lipid bilayer are complex, and range from reducing the permeability of the bilayer, to moderating phase behaviour, and altering the bilayer micro-mechanical stability, bending moduli and area expansivity. Chol is also implicated in the formation of the biologically relevant liquid-ordered (L₀) phase. Along with all of these properties, Chol can also drive the formation of lamellar bilayer structures when mixed with non-lamellar amphiphiles such as fatty acids. It is this property in which we are most interested. In the case of both fatty acids and the 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (Lyso-PPC) studied here, the lamellar phase formed by the addition of Chol is the L₀ phase.

The L₀ phase is a lamellar liquid-crystalline phase with properties different to those of both the liquid-disordered (Lₐ) and the gel (L₉) phases. The L₀ phase has long-axis rotational correlation times, and lateral diffusion rates, comparable to the Lₐ phase, but the acyl chains of the amphiphiles present in the system adopt an ordered, largely all-trans conformation similar to that found in the L₉ phase, rather than the highly disordered conformations found in the Lₐ phase.

The L₀ phase has been linked to the phenomenon of ‘lipid rafts’ in biological membranes. In particular, it has been proposed that Chol interacts preferentially with certain long-chain saturated phospholipids such as sphingomyelins to form regions of the L₀ phase in a membrane which is predominantly in the Lₐ phase. This hypothesis arose as a result of the presence of high proportions of Chol and saturated long-chain phospholipids as insoluble fractions in biological extracts isolated with detergent from whole membranes. Some of these conclusions are now in doubt as a result of recent evidence suggesting that membrane extracts made using this technique contain artefacts. However, since this time, other studies have used fluorescence techniques to look directly at both intact and reconstituted biological membranes. In these systems it has been observed that the addition of cyclodextrin, which acts to remove Chol from the membrane, to a membrane exhibiting ‘raft-like’ behaviour often leads to the disruption of the rafts and the appearance of a more homogeneous phase.

The important role of Chol in these biologically occurring regions of ordered fluid lamellar phase has been supported and expanded upon by studies using model membranes. It has been found that Chol packs against the chains of saturated phospholipids and causes an increase in the order of the chains, as measured by ²H-NMR order parameters, S₀CD, where S₀CD reports on the orientational ordering of the C–H bonds in the methylene groups down the acyl chain, on the NMR timescale.

The phase behaviour of various mixtures of lysophospholipids, alkanes, alkanols and diacylphosphatidylcholine (PC) and diacylphosphatidylethanolamine (PE) has been studied previously. The ability of Chol to induce L₀ phase formation in fatty acid systems has also been reported. A previously recorded ³¹P-NMR spectrum of the single composi-
tion of a 1 : 1 mixture of Lyso-PPC and Chol showed a characteristic lamellar line-shape, with a $\Delta \sigma$ of 20 ppm, superimposed on an isotropic peak.29 The study of the effect of Chol on such amphiphiles may provide further insight into the precise nature of Chol’s phase-moderating behaviour, and thus into its effect on complex biological membranes.

Lysophospholipids are type I lipids, which readily form micelles at low concentrations when mixed with water.30 At low temperatures (below 5 °C for Lyso-PPC) an interdigitated $\text{L}_{\text{II}}$ gel phase is formed.31 The binary phase diagram of Lyso-PPC exhibits first a micellar cubic phase, then a type I hexagonal (H$_\text{I}$) phase, then a fluid lamellar phase upon reducing the water content.32 The two non-lamellar phases can be observed in excess aqueous solution by addition of polyethylene glycol.33

Lysophospholipids occur naturally in many cell species, in both protein-bound and free forms.34 They are formed in vivo by the action of the enzyme phospholipase A$_2$.35 They have been implicated in many cellular processes, particularly in relation to platelet-activating factor, immune responses and inflammation.36–39 Previous studies on the effects of lysophospholipids on model membranes have shown that the lysophospholipid enhances the permeability of the membrane, and can act as a detergent.28,40 Lyso-PPC induces concentration-dependent shape changes to giant unilamellar vesicles of dipalmitoylphosphatidylcholine (DPPC)–Chol or sphingomyelin (SM)–Chol in the $\text{L}_{\text{o}}$ phase.41 The data also indicated that the rate of transbilayer flip-flop of the Lyso-PPC across the $\text{L}_{\text{o}}$ membrane was very slow.

Experimental

Materials

1-Palmitoyl-2-hydroxy-sn-glycerol-3-phosphocholine (Lyso-PPC) and 1-palmitoyl-d$_{31}$-2-hydroxy-sn-glycerol-3-phosphocholine (Lyso-PPC-d$_{31}$) were bought from Avanti Lipids (Alabaster, AL, USA). Cholesterol (Chol) was bought from Aldrich (Gillingham, Dorset, UK) and D$_2$O (99.9 atom%) from Goss Scientific (Great Baddow, Essex, UK). All water used in sample preparation was deionised, HPLC-grade. All materials were used without further purification.

NMR acquisition

Spectra were acquired on a 600 MHz, narrow bore, Bruker (Karlsruhe, Germany) DRX spectrometer operating at 14.09 T with a $^1$H resonance of 600.1 MHz, a $^2$H resonance of 92.1 MHz, a $^{13}$C resonance of 150.9 MHz and a $^{31}$P resonance of 242.9 MHz. Relaxation times of 2.0 s were used for all static spectra, and 1.0 s for MAS spectra. The number of scans was adjusted to give a good signal-to-noise ratio for each sample. Phospholipid lamellar phases exhibit a characteristic shape of $^{31}$P spectra, due to partial averaging of the CSA by molecular motion.42 The ratio of the isotropic phase to the lamellar phase was calculated from the $^{31}$P-NMR spectra by spectral subtraction, using XWIN-NMR software (version 3.5) supplied by Bruker. Error bars represent ±5% of the isotropic value and were estimated from the variation in two different NMR spectra obtained for the same sample. Calculations of shielding tensors were performed in Microsoft Excel.2 H-NMR spectra were dePaked with Amix 3.6 software (Bruker) through 5 numerical iterations, and order parameters ($S_{CD}$) were calculated using established methodology.43

X-Ray scattering

Wide angle (WAXS) diffraction patterns were recorded on a film-based Guinier X-ray camera (Huber Diffractionstechnik, Rimsting, Germany). X-Rays were provided by a Philips generator running at 30 kV and 40 mA, with a fixed anode Cu X-ray tube. A quartz crystal monochromator provides a line-focused beam of K$_\text{a1}$ radiation ($\lambda = 1.5405$ Å). The films were digitised and then analysed using the software program AXcess, written by Andrew Heron using the IDL 6.0 (Research Systems) data-processing package.44

Small angle (SAXS) diffraction patterns were recorded on a Gemstar intensified CCD X-ray detector (Photonic Science Ltd, Battle, UK). X-Rays were provided by a Microsource X-ray generator (Bede Ltd, Durham, UK) running at 40 kV and 2 mA, producing X-rays with $\lambda = 1.54$ Å. Diffraction data were acquired and analysed using the AXcess software program.

All X-ray data were acquired at room temperature.

Results and discussion

$^{31}$P-NMR spectroscopy

The $^{31}$P-NMR spectra of the Lyso-PPC–Chol mixtures consist of two superimposed patterns for concentrations between 20 and 70 mol% Chol (Fig. 1A).

The isotropic peak is due to micellar Lyso-PPC which tumbles rapidly on the NMR timescale. Lyso-PPC has a low critical micellar concentration (CMC) in the micromolar range, and readily forms micelles in water.30,45,46 The broad peak has a CSA profile characteristic of phospholipids in a fluid lamellar phase. The ratio of the amounts of the two phases alters with changing Chol concentration (Fig. 1B). As the concentration of Chol increases in the Lyso-PPC–Chol mixtures, the proportion of the isotropic phase decreases from 100% at 0 mol% Chol to 0% by ~70 mol% Chol.

The width of the broad lamellar peak, from the shoulder at higher ppm to the peak at lower ppm, is described by the parameter $\Delta \sigma$. This is a measure of the difference between the perpendicular and parallel components of the chemical-shift tensor and provides information about the average orientation of the phosphate group. The $\Delta \sigma$ of the CSA of the lamellar phase fraction is unusual in that it is much smaller, at ~20 ppm, than is seen for diacylphospholipids. For example, a $\Delta \sigma$ of 40–50 ppm has been reported for DPPC–Chol,24 POPC–Chol, and POPC–SM–Chol,47 and for liposomes composed of lipids from erythrocyte ghosts.42 A small $\Delta \sigma$ has been observed previously in Lyso-PPC–palmitic acid mixtures,26 in egg PC–Lyso-PPC systems,28 and from our own experiments on Lyso-PPC–DPPC systems (data not shown). In the former system, which was found to form an $\text{L}_{\text{p}}$ phase above 45 °C, the unusually small $\Delta \sigma$ was attributed to a combination of a larger motional freedom and a different conformation of the Lyso-PPC headgroup compared with the diacyl lipid, DPPC.
There are several possible explanations for the small magnitude of the $D_s$ in Lyso-PPC–Chol mixtures. One is that the Lyso-PPC exchanges rapidly between the bilayer and the micellar phase, and that this causes partial averaging of the CSA. At high Chol concentrations, where the only phases present are the lamellar phase and Chol crystals and there can be no exchange with a micellar phase, the line-width is still $\sim 20$ ppm, so this explanation can be discounted.

Another possible explanation is that the averaging is due to increased motion about the phosphate headgroup due to the absence of an acyl chain on the $sn$-2 carbon of the glycerol region.

Alternatively there could be a narrowing due to a reorientaiton of the $^{31}$P chemical-shift tensor in the magnetic field, as has been reported for dialkylphosphate compounds.48,49

$^2$H-NMR spectroscopy

$^2$H-NMR experiments were carried out on several samples which contained Lyso-PPC with a per-deuterated acyl chain. All these samples showed a broad quadrupolar powder pattern with a maximum splitting of 50 kHz (Fig. 2A). The shape of the powder pattern is consistent with a lamellar lipid phase and the magnitudes of the quadrupolar splittings are characteristic of a $L_o$ phase.10,24 The width of the powder pattern indicates that the chains are essentially in an all-trans state close to the headgroup region. The well-resolved doublets associated with most of the $C-^2$H groups on the chain suggest that the individual $C-^2$H bonds retain a degree of motion (long-axis rotation) that is rapid on the NMR timescale.

Spectra were dePaked and the resultant splittings were used to calculate order parameters for each $C-^2$H group down the chain (Fig. 2B). A striking difference is seen in the order profile of the Lyso-PPC–Chol methylenes in comparison with those of DPPC and palmitic acid. The order parameters obtained for the first 6 or 7 methylene groups are comparable with those of the $L_o$ phase in DPPC–Chol, and palmitic acid–Chol systems. However, in the centre of the bilayer region, the magnitude of the $S_{CD}$ for equivalent carbons in the Lyso-PPC–Chol system is generally smaller than seen for DPPC or palmitic acid. This indicates that there is a greater amount of disorder or motion in the centre of the Lyso-PPC–Chol bilayer compared with the other systems.

We propose a simple model which could qualitatively explain both the unusual order parameter profile of the Lyso-PPC–Chol bilayers, and the narrow chemical-shift anisotropy of the $^{31}$P-NMR spectrum (Fig. 3). In this model, the glycerol region of the Lyso-PPC adopts a conformation in which its carbon backbone is almost parallel to the bilayer normal, and the Chol sits just below the phosphate group of the lipid headgroup, altering the orientation of the phosphate...
chemical-shift tensor, and restricting its conformational freedom. This model could explain the unusually narrow $^{31}$P-NMR chemical-shift anisotropy of the Lyso-PPC–Chol bilayer. Because the Chol molecules are situated towards the headgroup in this model, there is more conformational freedom for the methylene groups towards the centre of the bilayer, and this is reflected in the overall reduction in the magnitude of the $^2$H order parameters (C8–C16, Fig. 2A) and the resulting profile.

The spectral width of the $^2$H-NMR powder pattern increases slightly with increasing Chol concentration. This effect is small, with a difference of just 2–3 kHz over a concentration range of around 25 mol%. There is also a temperature effect, with the width of the powder pattern decreasing with increasing temperature (Fig. 4). This is due to an increase in the disorder of the lipid chains. This effect seemed to decrease slightly as the concentration of Chol increased.

X-Ray scattering

The WAXS diffraction patterns from mixtures of Lyso-PPC and Chol, when the Chol concentration lies within the range 24–74 mol%, show a single broad, asymmetric peak, centred at a spacing of approximately 4.2 Å, indicating that all of the Chol is mixed with the Lyso-PPC [Fig. 5]. Peaks in this wide-angle region arise mainly from lateral intermolecular packing. The relatively small value of 4.2 Å for the peak centre implies that the Lyso-PPC and Chol are more closely packed in the lateral direction than is normally seen in fluid L$_a$ phospholipid phases, where the broad WAXS peak is usually centred at approximately 4.6 Å.$^{50}$ This feature, together with its asymmetric appearance suggests qualitatively that this is a liquid-ordered L$_o$ phase rather than a fluid L$_a$ phase.

The SAXS data for samples with a Chol concentration of less than 85 mol% show the first and second order peaks of a lamellar phase, with a layer spacing in the range 67–69 Å (Fig. 6). This value is somewhat larger than either the layer spacing of pure DPPC in the L$_b$ gel phase in excess water (63.9 Å), or 1 : 1 DPPC–Chol in the L$_o$ phase (59.5 Å), at a relative humidity of 98% at 20°C.$^{51}$ The larger spacing when compared with the gel phase of DPPC could be attributed to the absence of chain tilt for the Lyso-PPC–Chol bilayers, and/or to an increased effective hydrophilicity of the phosphocholine group in the latter case, leading to an increased water-layer thickness. Future studies carried out over a range of hydrations should resolve this point. The 8–10 Å increase in spacing compared with 1 : 1 DPPC–Chol in the same L$_o$ phase is more difficult to understand, although it is possible that the reported spacing of 59.5 Å$^{51}$ was not the equilibrium spacing (samples in 98% relative humidity are not expected to hydrate fully). It should be noted that the second order peak from the Lyso-PPC–Chol L$_o$ phase occurs at approximately the same spacing (33.5–34.5 Å) as the first order d-spacing for crystalline Chol (34 Å), and so it is not possible to distinguish them from the small-angle data alone. The presence of crystalline Chol in the sample is, however, indicated by the appearance of multiple sharp peaks in the wide-angle region, and such peaks are only seen for Chol concentrations above 70 mol%. For the 92 mol% Chol sample (Fig. 6f), in addition to the 0.029 Å$^{-1}$ (34 Å) peak from Chol crystals, a small amount of L$_o$ phase is also present, but the small peak expected at 68 Å is not, in fact, visible under the noise.

Conclusions

We have shown that Chol can drive the formation of a stable lamellar phase with fully hydrated Lyso-PPC over a range of temperatures and Chol concentrations. We observed that the higher the Chol concentration, the greater the proportion of
observed. However, we have established that the $^2$H order parameter profile of the 92 mol% Chol sample, in addition to the 0.029 Å$^{-1}$ peak from Chol crystals, a small amount of Lo phase is present, but the small peak expected at 68 Å is not visible under the noise. The drop-off in intensity at values of 1/d of below 0.005 Å$^{-1}$ is due to the presence of a beam-stop.

We speculate that these results may shed an interesting light on studies of the action of phospholipase A$_2$ on biological membranes. It is possible that, in addition to the many other properties of Chol in biological membranes, it also acts to prevent the loss of lyso-lipids from cellular membranes into the external solution by acting to drive the formation of a lyso-containing lamellar phase.

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