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Cubic phase is induced by cholesterol in the dispersion of 1-palmitoyl-2-oleoyl-phosphatidylethanolamine

Xiaoyuan Wang [1], Peter J. Quinn *

Division of Life Sciences, King's College London, 150 Stamford Street, London SE1 9NN, UK

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Abstract

The effect of cholesterol, a major constituent of eukaryotic cell membranes, on the structure and thermotropic phase behaviour of 1-palmitoyl-2-oleoyl-phosphatidylethanolamine (POPE) dispersed in excess water was examined by synchrotron X-ray diffraction methods. Temperature scans over the range 10–75 °C showed that the gel to liquid–crystalline phase transition decreased from 25 to 10 °C in the presence of 20 mol% cholesterol, and no gel phase could be detected in the wide-angle X-ray scattering (WAXS) intensity profile of mixtures containing 35 mol% cholesterol. The small-angle X-ray scattering (SAXS) intensity profiles showed that the lamellar to nonlamellar phase transition temperature was also decreased in mixtures containing up to 30 mol% cholesterol but the trend was reversed in mixtures containing a higher proportion of cholesterol. There was evidence that the transition of the lamellar liquid–crystal phase is to cubic phases in mixtures containing less than 30 mol% cholesterol. The space group of one of these cubic phases was assigned as Pn3m. This effect of cholesterol on non-bilayer-forming phospholipids is considered in the context of the role of cholesterol in membrane organization and function.

Keywords: Membrane; Cholesterol; Phosphatidylethanolamine; Liposome dispersion; X-ray diffraction; Cubic phase

1. Introduction

Cholesterol is a major constituent of eukaryotic membranes. It serves as a precursor for the synthesis of steroid hormones, bile acids and lipoproteins. It fulfils a crucial role in membrane organization, dynamics, function and sorting [1–5]. Cholesterol has been implicated in embryonic development in *Drosophila* [6], and programmed cell death [7]. Its role in signal transduction is being increasingly recognized [8]. Cellular levels of cholesterol and its compartmentalization into different organelles are stringently controlled, and defects in its trafficking pathway have been shown to give rise to metabolic storage diseases such as Niemann–Pick type C [9]. Cholesterol is often found distributed nonrandomly in domains in biological and model membranes [2,5,10–13] and has been shown to preferentially interact with a subset of membrane lipids [14]. Indeed, structurally and kinetically distinct cholesterol-rich and -poor domains have been recognised in cellular membranes [15]. Dynamic clustering of cholesterol, along with other membrane components such as sphingolipids, has given rise to the idea of specialized microdomains or ''rafts'' in the cell membrane that might play crucial roles in cellular functions such as signal transduction, adhesion, motility, and sorting and trafficking of membrane components [5,12]. In addition, cholesterol has been reported to be distributed heterogeneously among various intracellular membranes. For example, along the biosynthetic route, the lowest cholesterol concentration is found in the membranes of the endoplasmic reticulum, which, interestingly, is the site of cholesterol biosynthesis [16]. Cholesterol concentration increases progressively along the cis-, medial-, and trans-Golgi stacks [16], whereas the highest concentration is found in the plasma membrane [17].

Cholesterol is believed to cause profound changes in the physical properties of membranes. Many studies have been

Abbreviations: POPE, 1-palmitoyl-2-oleoyl-*sn*-phosphatidylethanolamine; SAXS, small-angle X-ray scattering (2θ=0.043–7.9°); WAXS, wide-angle X-ray scattering (2θ=8–60°); Chol, cholesterol; L_α, lamellar liquid–crystalline phase; L_β, lamellar gel phase; H_{II}, inverted hexagonal phase

* Corresponding author. Tel.: +44-207-848-4408; fax: +44-207-848-4500.

E-mail address: p.quinn@kcl.ac.uk (P.J. Quinn).

[1] Present address: Department of Biochemistry and Molecular Biology, University of Texas-Houston, Medical School, Houston, TX 77225, USA.

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reported on the effect of cholesterol on structure and phase behaviour of phospholipid model membranes. Thus, in mixtures of phosphatidylcholine and cholesterol, a liquid-ordered phase was detected [18–22]. In mixtures of phosphatidylethanolamines and cholesterol the gel to liquid–crystalline phase transition is broadened and shifted toward low temperatures [23–25]. A limited amount of cholsterol leads to a decrease of the lamellar to inverted hexagonal phase transition temperature of phosphatidylethanolamines, but proportions of cholesterol higher than 30 mol% reverse this effect [24,26,27].

In the present study, the effects of cholesterol on the detailed structure and phase behaviour of 1-palmitoyl-2-oleoyl-phosphatidylethanolamine (POPE) dispersed in aqueous medium has been examined using synchrotron X-ray diffraction methods. It was found that the gel to liquid–crystalline transition shifted toward lower temperatures with increasing proportions of cholesterol up to 20 mol%. Mixtures containing less than 30 mol% cholesterol showed a decrease of lamellar to nonlamellar phase transition temperature, but proportions higher than 30 mol% cholesterol reversed this effect. Transitions from lamellar liquid–crystal to cubic phases were also identified at high temperatures in mixtures containing less than 30 mol% cholesterol.

2. Materials and methods

POPE was purchased from Avanti Polar Lipids (Alabaster), and cholesterol from Sigma. Both were used without further purification. The lipids were dissolved in chloroform and mixed in appropriate proportions to achieve the desired molar fractions. The solvent was evaporated under a stream of oxygen-free dry nitrogen and stored for 24 h under vacuum to remove remaining traces of solvent. The lipid mixtures were hydrated at 80 °C for at least 1 h and dispersed by a rotamixer until homogeneous dispersions were obtained. The lipid dispersions were stored at 4 °C for 24 h before examination. The method of preparation and storage resulted in reproducible phase behaviour when samples prepared at different times were examined by X-ray diffraction.

Synchrotron X-ray diffraction experiments were performed using a monochromatic (0.15405 nm) focused X-ray beam at station 8.2 of the Daresbury Synchrotron Radiation Laboratory, UK. The camera configuration allowed detection of small-angle and wide-angle X-ray scattering (SAXS and WAXS, respectively) with a minimum of parallax error [28]. The beamline generates a flux of 4×10^{10} photons per second with a focal spot size of 0.3×3 mm^2 ($V \times H$) when the synchrotron radiation source is operating at a nominal 200 mA. The samples were mounted in a slot (1×5 mm) cut in a 1-mm-thick copper plate sandwiched between a pair of thin mica sheets. The sandwich was clamped to the silver block containing the temperature sensing and modulating elements of a cryomicroscope stage (Linkam Scientific Instruments Ltd., UK). X-ray scattering intensities at small-angles ($2\theta = 0.043–7.9°$) were recorded using a multiwire quadrant detector. X-ray scattering intensities at wide angles ($2\theta = 8–60°$) were recorded using an INEL curved linear-wire detector (Instrumentation Electronique, France). Data were acquired in 400 consecutive time frames of 5 s and separated by a dead time between frames of 50 ms. Experimental data were analysed using the OTOKO software (EMBL, Hamburg, Germany) programme [29]. Scattering intensities at low angles were corrected for fluctuations in beam intensity and detector response recorded from an ^{55}Fe source. Spatial calibrations were obtained using 21 orders of wet rat-tail collagen ($d = 67$ nm) [30]. The scattering intensity data recorded by the INEL detector were corrected for scattering from an empty cell and spatial calibrations were established from high-density polyethylene (0.4166, 0.3780, 0.3014 nm) [31]. The reciprocal spacing $S = 1/d = 2\sin(\theta)/\lambda$, where d, λ, and θ are the repeat distance, X-ray wave length and the diffraction angle, respectively.

3. Results

X-ray diffraction patterns of the pure hydrated POPE recorded during a heating scan are shown in Fig. 1. It can be seen that the phospholipid undergoes a gel to liquid–crystalline ($L_\beta \rightarrow L_\alpha$) phase transition at 25 °C and a liquid–crystalline to inverted hexagonal ($L_\alpha \rightarrow H_{II}$) phase

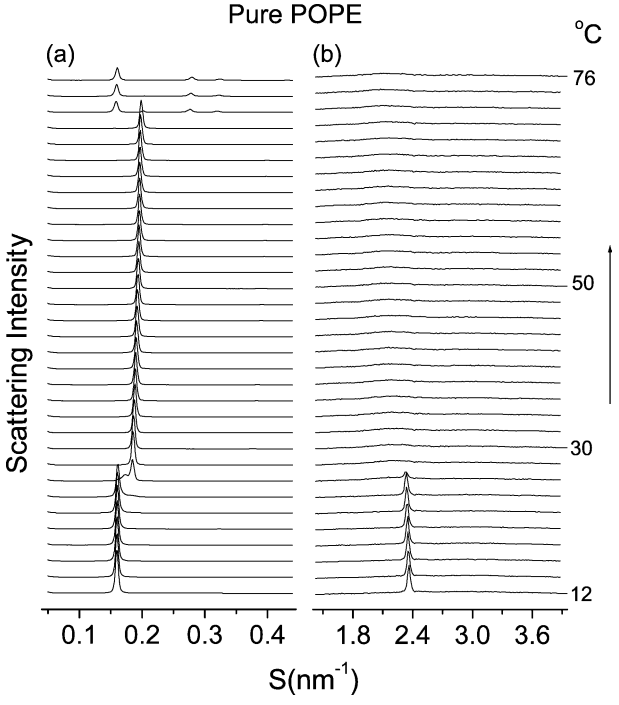

Fig. 1. Plots of SAXS (a) WAXS (b) intensity profiles versus reciprocal spacing as a function of temperature of an aqueous dispersion of POPE recorded during a heating scan at 2°/min from 12 to 76 °C. Each diffraction pattern represents scattering accumulated in 15 s.

transition at 71 °C. The former is characterised by a decrease of the lamellar repeat spacing from 6.3 nm (20 °C) to 5.3 nm (30 °C) in the SAXS region (Fig. 1a), which coincides with a change from a sharp symmetrical diffraction peak at 0.43 nm, typical of gel phases, to a broad peak centred at 0.46 nm in the WAXS region signifying disordered hydrocarbon chain packing (Fig. 1b). The H_{II} phase formed at temperatures greater than 71 °C is characterised by a series of reflections in the order of 1, $1/\sqrt{3}$, $1/\sqrt{4}$ in the SAXS region (Fig. 1a). The transition temperatures were determined as the midpoint of the change in *d*-spacing of the repeat lattice structure and, in the case of lamellar gel to liquid–crystal transitions, from the midpoint of change in intensity of the sharp WAXS peak at 0.43 nm. The temperature range over which coexistence of the phases can be detected is less than 2° for transitions between gel and fluid phases and for transitions between lamellar and nonlamellar phases it was in the order $8 \pm 2°$. The thermal protocol involved an initial heating scan followed immediately by a cooling and subsequent reheating scan. There was only a small hysteresis in phase transition temperatures recorded during heating and cooling scans and comparison of the two heating scans showed similar behaviour. Both lamellar gel to lamellar liquid–crystal and lamellar liquid–crystal to nonlamellar phase transition temperatures are in agreement with published data [32–34].

To determine the effect of cholesterol on the structure and thermotropic phase behaviour of POPE, mixed aqueous dispersions of the two lipids were examined during heating scans in the temperature range 10 to 74 °C using synchrotron X-ray diffraction methods. The X-ray diffraction pat-

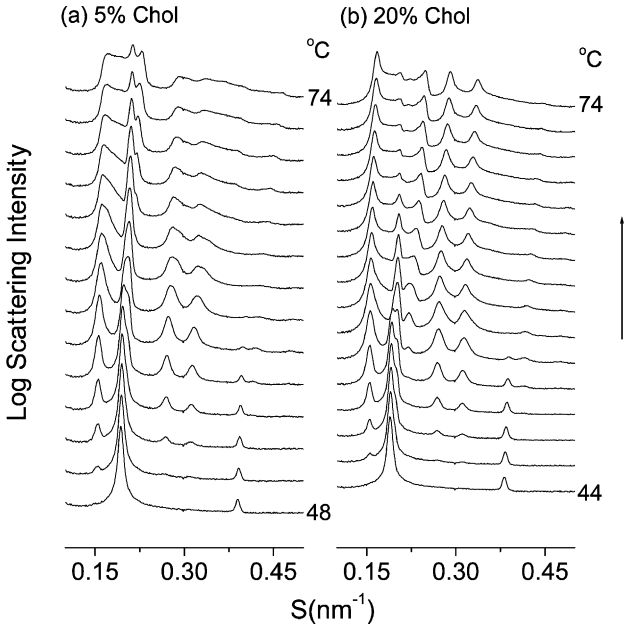

Fig. 3. Logarithmic plots of SAXS intensity profiles versus reciprocal spacing as a function of temperature of codispersions of POPE containing (a) 5 and (b) 20 mol% cholesterol in the region of the lamellar to nonlamellar phase transition.

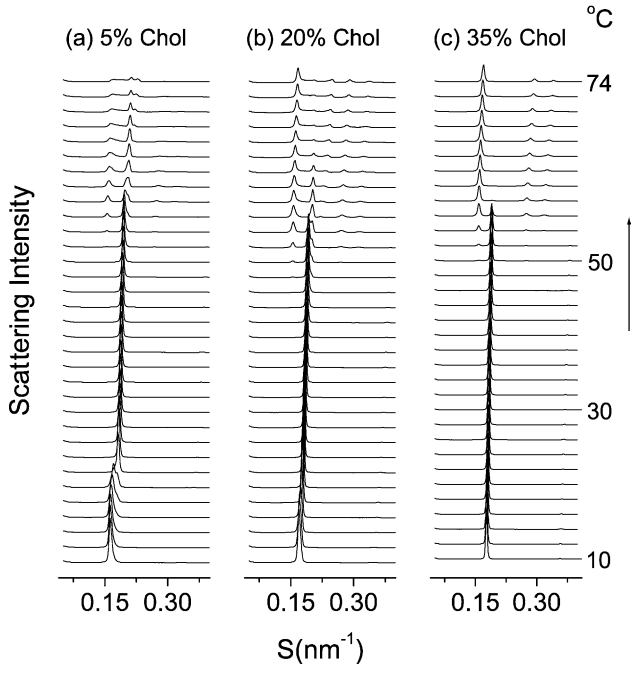

Fig. 2. Plots of SAXS intensity profiles versus reciprocal spacing as a function of temperature of codispersions of POPE with (a) 5, (b) 20 and (c) 35 mol% cholesterol recorded during heating scans at 1°/min between 10 and 74 °C. Each diffraction pattern represents scattering accumulated in 15 s.

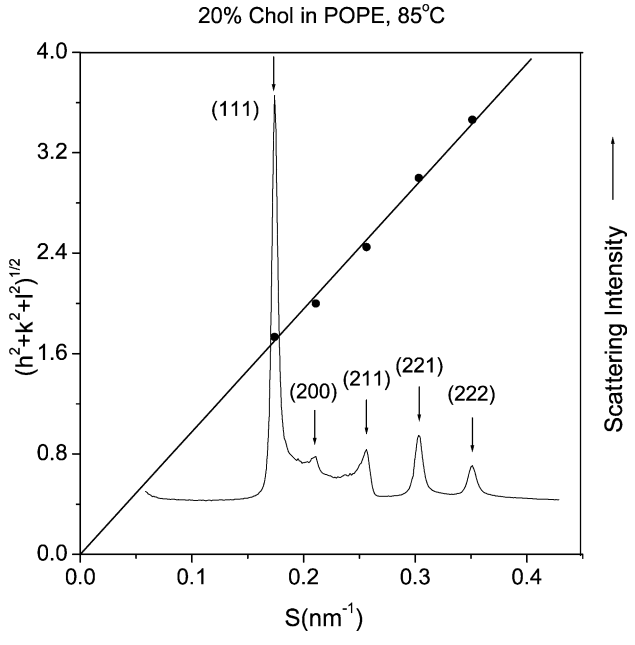

Fig. 4. A static SAXS pattern of a codispersion of POPE containing 20 mol% cholesterol recorded at 85 °C. Arrows locate expected positions of diffraction peaks of a Pn3m cubic lattice that are indexed (hkl). The $(h^2+k^2+l^2)^{1/2}$ for hkl indices vs. reciprocal spacings (S) fall on a straight line from which a lattice constant, $a = 9.88 \pm 0.01$ nm, is obtained from the reciprocal slope.

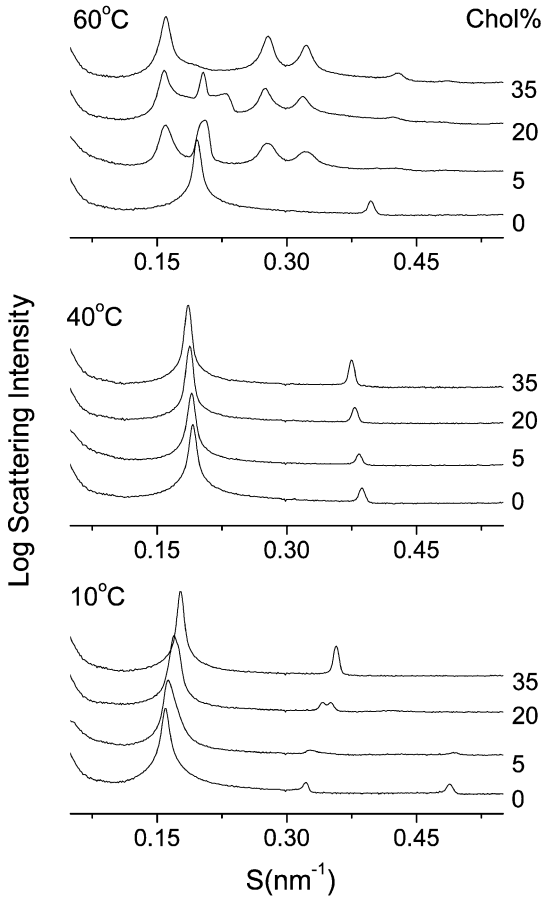

Fig. 5. Static SAXS intensity profiles recorded from codispersions of POPE with different proportions of cholesterol at three different temperatures. The profiles are plotted as the logarithm of intensity to emphasize the minor bands. The mol% cholesterol in the respective mixtures is indicated on each diffraction pattern.

terns of codispersions of POPE with 5, 20 and 35 mol% cholesterol heated from 10 to 74 °C are shown in Fig. 2a–c, respectively. It can be seen that the presence of cholesterol affects the temperature of both the L_β to L_α and the transition from L_α to the nonlamellar phase. The L_β to L_α transition, as judged by changes in WAXS intensity, decreased to about 21 and 10 °C in the presence of 5 and 20 mol% cholesterol in the respective mixtures; no gel phase could be detected in the WAXS profile in mixtures containing 35 mol% cholesterol. Transition of the L_α to a nonlamellar phase is observed at higher temperature and the structure of the nonlamellar phase depends on the amount of cholesterol in the mixture. The nonlamellar phase can be indexed to H_{II} phase for the mixture containing 35 mol% cholesterol by a series of higher-order reflections in the order of 1, $1/\sqrt{3}$, $1/\sqrt{4}$ (Fig. 2c). This is not the case for mixtures containing 5 and 20 mol% cholesterol (Fig. 2a and b). Moreover, in the mixture containing 20 mol% cholesterol, there is evidence that the initial nonlamellar phase transforms into another nonlamellar phase with increasing temperatures. This can be seen in the expanded plots of the logarithm of SAXS intensity covering the temperature range of the lamellar to nonlamellar phase transition presented in Fig. 3 for mixtures containing 5 and 20 mol% cholesterol. The higher temperature phase can be indexed to a cubic phase of space group Pn3m or Pn3 by five diffraction bands in a ratio of $1/\sqrt{3}:1/\sqrt{4}:1/\sqrt{6}:1/\sqrt{9}:1/\sqrt{12}$ (Fig. 4). The positions of diffraction peaks of a Pn3m cubic lattice that are indexed (hkl) show that reciprocal spacings vs. $(h^2+k^2+l^2)^{1/2}$ for hkl indices fall on a straight line passing through the origin.

Static X-ray diffraction intensity profiles were recorded from codispersions of POPE containing up to 35 mol% cholesterol equilibrated at three different temperatures and the results are presented in Fig. 5. At 10 °C, pure POPE and mixtures containing 5 mol% cholesterol exist in L_β phase although the presence of only a small proportion of cholesterol resulted in a significant broadening of the SAXS bands, indicating a disordering of the lamellar lattice spacing. In the mixture containing 20 mol% cholesterol, L_α phase coexists with L_β phase. With an increase in the proportion of cholesterol to 35 mol% there is only L_α phase. All of the mixtures exist in L_α phase at 40 °C. At 60 °C, however, the dispersion of pure POPE still showed L_α phase but in codispersions with 5 and 20 mol% cholesterol, a mixture of nonlamellar phases was observed. At the highest cholesterol content, four orders of an inverted H_{II} phase could be detected with no evidence of any additional nonlamellar phases.

A partial phase diagram of cholesterol codispersed with POPE has been constructed from the present X-ray data and results obtained from calorimetric studies of this dispersion reported by Epand and Bottega [35]. This is presented in

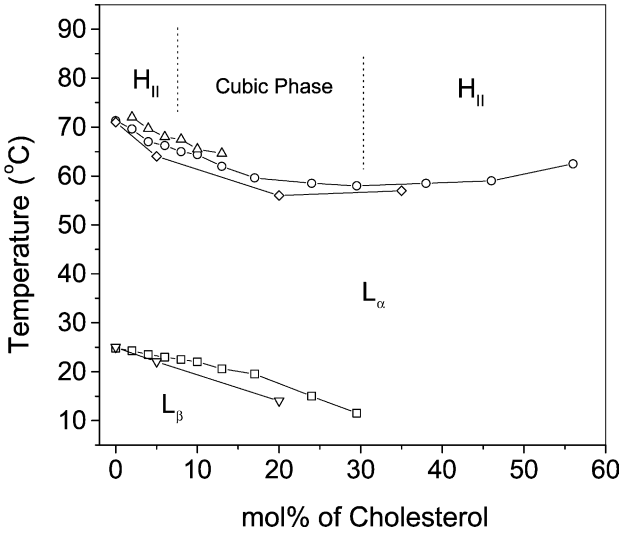

Fig. 6. Partial phase diagram of aqueous dispersions of POPE and cholesterol. Symbols ▽ and □ indicate L_β to L_α phase transitions; ◇, ○ and △ indicate lamellar to nonlamellar phase transition. The data symbolized by □, ○ and △ are from calorimetric data reported by Epand and Bottega [35].

Fig. 6. Both methods show a progressive decrease in temperature of L_β to L_α phase transition with increasing amounts of cholesterol up to about 30 mol% cholesterol, above which the gel phase can no longer be detected. There is also a progressive decrease in temperature of L_α to nonlamellar phase transition temperature, but where the transition is to an H_{II} phase in dispersions containing more than 30 mol% cholesterol, the trend is reversed such that increasing proportions of cholesterol result in an increase in transition temperature.

4. Discussion

Cholesterol is a prominent lipid constituent of cell membranes and in particular the plasma membrane. Due to the complex assortment of lipids that characterize membranes, it is difficult to ascertain the physical properties and functional roles of cholesterol and its precise mode of interaction with other lipids in biological membranes. Therefore, to establish the factors that govern the interaction of cholesterol with other membrane constituents, extensive use has been made of model systems consisting of dispersions of lipid mixtures with cholesterol in aqueous media. One of the specific physicochemical features of cholesterol is the planar steroid ring which imparts a relatively rigid structure to the molecule. This markedly influences the way cholesterol interactions with lipid molecules arranged in a bilayer configuration. The phases that form in mixed aqueous dispersions of cholesterol and phospholipid is a function of the relative proportion of cholesterol and the type of phospholipid as well as exhibiting thermotropic and lyotropic mesomorphism. The thermotropic phase behaviour of mixed aqueous dispersions of cholesterol with distearoyl and dielaidoyl molecular species of phophatidylcholine, phosphatidylethanolamine and phosphatidylserine have been examined using high sensitivity DSC [36]. These results indicate that the nature of the interaction between cholesterol and phospholipid and the miscibility of cholesterol in the bilayer, depend on both the structure of the phospholipid polar headgroup and whether the hydrocarbon chains were unsaturated or not. The role of phospholipid headgroup structure on the interaction has been studied in mixed monomolecular films of cholesterol with a set of acyl chain-matched phospholipids oriented at an air–water interface [37]. The interacting forces were assessed by measurement of the rate of desorption of cholesterol from cholesterol–phospholipid (1:1) monolayers to cyclodextrin in the subphase. The results showed that the interaction of cholesterol with the phospholipids decreased in the following order: sphingomyelin > phosphatidylcholine>phosphatidylserine>phosphatidylethanolamine. These findings are consistent with the limited miscibility of cholesterol in phosphatidylethanolamine bilayers, especially when the phospholipid is in the gel phase [25]. This has been explained, in part, by the suggestion that the strong interhead group electrostatic and hydrogen bonding interactions between neighbouring phosphatidylethanolamine molecules are stronger than the forces of interaction between cholesterol and the phospholipid. Cholesterol is more miscible with unsaturated phosphatidylethanolamine than it is in saturated molecular species as indicated by calorimetry [26,35,36], X-ray diffraction [24], NMR [38,39] and IR [27] measurements. This agrees with stronger intermolecular forces interacting between the more densely packed saturated phospholipid molecules compared with their unsaturated counterparts. At cholesterol contents less than 30 mol%, the incorporation of cholesterol progressively reduces the temperature of the lamellar to nonlamellar phase transition; however, above 30 mol% cholesterol, the lamellar to nonlamellar phase transition temperature increases with increasing cholesterol.

The synchrotron X-ray diffraction studies undertaken in the present work confirm that the presence of cholesterol perturbs the structure of the gel phase of POPE to such an extent, that a L_β to L_α transition can no longer be detected when the proportion of cholesterol in the mixture increases above 35 mol%. The lamellar phase in this region of the phase diagram exists in a liquid-ordered phase in which the acyl chains, although fluid, are still in a relatively ordered configuration [27]. Cholesterol also influences the propensity of POPE to form nonlamellar phases. Mixtures containing less than 30 mol% cholesterol showed a progressive decrease in temperature of transition from lamellar to nonlamellar phase whereas with mixtures containing higher proportions of cholesterol, there was an increase of the transition temperature. No evidence of any phase separation was observed in the present experiments in the temperature range 10 to 75 °C. One interpretation of these results is that the double bond of the oleate acylated to the *sn*-2 position of POPE decreases the van der Waals chain–chain interaction and creates sufficient space between POPE molecules where cholesterol could insert.

The detection of cubic phases in mixtures of POPE containing less than 30 mol% cholesterol has not been reported previously. The particular space grouping of the cubic phase depends on the amount of cholesterol and temperature. Cubic phases were usually observed as the first nonlamellar phase to appear with increasing temperature and they were not formed via H_{II} phase. The presence of relatively low proportions of cholesterol induced a cubic phase which resulted from destabilization of the lamellar, and with increasing cholesterol another cubic phase replaced that which was formed at lower cholesterol content. This was associated with a further reduction of the lamellar to nonlamellar phase transition temperature. This second cubic phase was indexed as a Pn3m structure. When the concentration of cholesterol was increased to more than 30 mol%, the inverted hexagonal phase dominated the phase structure and the lamellar to nonlamellar phase transition temperature increased with increasing cholesterol. It was not possible to conclude from the present experiments whether cubic

phases were formed as transient intermediates between the lamellar and H_{II} phase at higher cholesterol contents.

Epand and Bottega [35] examined the effect of cholesterol on the lamellar to nonlamellar phase transition temperature of POPE using high-sensitivity DSC and observed two endothermic peaks in mixtures containing less than 30 mol% cholesterol. They noted that the shape of these two peaks was more complex and differed from those expected from a typical lamellar to inverted hexagonal phase transition peak, which was usually sharper. The details of the transition are clear from the present X-ray diffraction data which shows that the first endothermic peak coincides with a transition of the lamellar phase to an as yet unidentified cubic phase, and the second peak is due to transition to a second cubic phase assigned as Pn3m space grouping. Studies of the structure of mixed aqueous dispersions of POPE and cholesterol by ^{2}H-NMR and IR have been reported by Pare and Lafleur [27] but the distinction of cubic phases from H_{II} is not straightforward when a mixture of phases coexists. El Jastimi and Lafleur [40] recorded the ^{31}P-NMR spectrum of a mixed aqueous dispersion of POPE containing 30 mol% cholesterol at 50 °C, and observed a narrow signal which increased in intensity with successive temperature cycles between 50 and 65 °C. The shape and position of these narrow signals were precisely the same as those recorded from a pure POPE sample at 65 °C, which had been subjected to temperature cycling between 65 and 75 °C. The narrow signal was attributed to the formation of an isotropic cubic phase consistent with X-ray diffraction studies of this system [41,42]. It is likely, therefore, that the narrow signal observed in the ^{31}P-NMR spectrum from POPE containing 30 mol% cholesterol is also due to the presence of a cubic phase. The effect of cholesterol on the phase behaviour of dielaidoylphosphatidylethanolmaine appears to be similar to that of POPE [24,35]. X-ray diffraction data from mixtures of cholesterol and dielaidoylphosphatidylethanolamine also showed that in the region of the L_{α}–H_{II} phase transition, there is coexistence of two phases in mixtures containing 10 and 20 mol% cholesterol [24]. Furthermore, an additional reflection with a *d*-spacing of about 5.6 nm was detected together with the reflections from the L_{α} and H_{II} phases in the temperature range of 62–65 °C. On the basis of the present evidence, this additional phase might be a cubic phase. It is noteworthy that such phases were observed only in mixtures of unsaturated phosphatidylethanolamine containing less than 30 mol% cholesterol. Likewise, in the cholesterol–dielaidoylphosphatidylethanolmine system, the lamellar to nonlamellar phase transition temperature also decreased with increasing cholesterol up to 30 mol%, then began to increase with addition of further cholesterol [35,36].

Because of its relatively small head group, cholesterol is often considered as a cone-shaped molecule and therefore able to promote nonlamellar phase formation in the phospholipid bilayer. Phospholipids with strong intermolecular interactions with one another, like saturated phosphatidylethanolamine, tend to exclude cholesterol from the bilayer [25,36]. In the bilayer formed by unsaturated phosphatidylethanolamine, the intermolecular interaction is weak and cholesterol could insert between phospholipid molecules and therefore promote nonlamellar phase formation. The formation of nonlamellar phase depends both on the curvature stress and bilayer thickness. Curvature stress in POPE–cholesterol mixtures is dependent on the proportion of cholesterol, which promotes the formation of different nonlamellar structures. Curvature stress is believed to be important in cellular processes such as the formation of small vesicles involved in subcellular membrane trafficing [43] and membrane sorting in the endosome compartment [44]. Modulation of the cholesterol content of subcellular membranes allied to the proportion of non-lamellar-forming phospholipid molecular species may represent a means of regulating sorting processes associated with secretion and membrane differentiation.

Acknowledgements

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