Phosphatidylcholine structure determines cholesterol solubility and lipid polymorphism

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Abstract

In the present work, we demonstrate that phosphatidylcholine with (16:1) 9 acyl chains undergoes polymorphic rearrangements in mixtures with 0.6–0.8 mol fraction cholesterol. Studies were performed using differential scanning calorimetry, X-ray diffraction, cryo-electron microscopy, 31 P NMR static powder patterns and 13 C MAS/NMR. Mixtures of phosphatidylcholine with (16:1) 9 acyl chains and 0.6 mol fraction cholesterol, after being heated to 100 °C, can form an ordered array with periodicity 14 nm which may be indicative of a cubic phase. Our results indicate that the formation of highly curved bilayer structures, such as those required for membrane fusion, can occur in mixtures of cholesterol with certain phosphatidylcholines that do not form non-lamellar structures in the absence of cholesterol.

We also determine the polymorphic behavior of mixtures of symmetric phosphatidylcholines with cholesterol. Species of phosphatidylcholine with (20:1) 11, (22:1) 13 or (24:1) 15 acyl chains in mixtures with 0.6–0.8 mol fraction cholesterol undergo a transition to the hexagonal phase at temperatures 70–80 °C. This is not the case for phosphatidylcholine with (18:1) 6 acyl chains which remains in the lamellar phase up to 100 °C when mixed with as much as 0.8 mol fraction cholesterol. Thus, the polymorphic behavior of mixtures of phosphatidylcholine and cholesterol is not uncommon and is dependent on the intrinsic curvature of the phospholipid.

Crystals of cholesterol can be detected in mixtures of all of these phosphatidylcholines at sufficiently high cholesterol mole fraction. What is unusual about the formation of these crystals in several cases is that cholesterol crystals are present in the

Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; Di(X:Y) Z PC, symmetric phosphatidylcholine with each acyl chain having X carbon atoms and Y double bonds at position Z in the acyl chain; CP, cross-polarization; DP, direct polarization; MAS, magic angle spinning; DSC, differential scanning calorimetry; cryo-TEM, cryo-transmission electron microscopy; ILA, interlamellar attachment site

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monohydrate form in preference to the anhydrous form. Furthermore, after heating to 100 °C and recooling, the cholesterol crystals are again observed to be in the monohydrate form, although pure cholesterol crystals require many hours to rehydrate after being heated to 100 °C. Both the nature of the acyl chain as well as the mole fraction cholesterol determine whether cholesterol crystals in mixtures with the phospholipids will be in the monohydrate or in the anhydrous form.

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There is currently considerable interest in understanding the arrangement of cholesterol in membranes. Cholesterol has been observed to enhance rates of membrane fusion in several diverse systems (Hope et al., 1977; Tarshis et al., 1993; Nussbaum et al., 1992; Surewicz et al., 1986). Intermediates in membrane fusion require a rearrangement of the morphology of the membrane.

The solubility of cholesterol in bilayers is dependent on the nature of the phospholipid. Cholesterol has high miscibility with sphingomyelin with which it has strong interactions (Guo et al., 2002; Epand, 2003; Matijus and Slotte, 1996; Brown, 1998; Ohvo-Rekila et al., 2002; Ramstedt and Slotte, 2002), although these interactions may not be specific (Holopainen et al., 2004). In contrast, it has also been found that cholesterol is preferentially excluded from interactions with phosphatidylcholines containing polyunsaturated acyl chains (Buzsikovitz et al., 2002a, 2002b; Epand et al., 2004). In addition to the acyl chain, the nature of the lipid headgroup also affects cholesterol solubility (Bach and Wachtel, 2003).

Several methods have been used to determine the miscibility limit of cholesterol with specific lipids. A general problem with measuring the phase behavior of lipid mixtures is that once the lipid is hydrated, its rearrangement can be very slow. The method of solvent removal has been found to have a small effect on cholesterol crystal formation (McMullen et al., 2000; Buboltz and Feigenson, 1999), but these effects are not significant when compared with differences in cholesterol solubility among lipid species (Epand et al., 2002). It is clear that the appearance of cholesterol crystals is not simply a consequence of their formation during solvent evaporation. The amount of crystals formed in mixtures with phosphatidylethanolamine is maximal at a 1:1 cholesterol:phosphatidylethanolamine molar ratio and does not increase as the cholesterol component increases (Epand et al., 2001). Cholesterol crystals formed in the presence of phospholipids can have altered temperatures of dehydration (Epand et al., 2001) or altered kinetics of hydration (Epand et al., 2003), indicating that these cholesterol crystals are intimately mixed with phospholipid. The solubility of cholesterol in dioleoyl phosphatidylcholine (Di(18:1)PC) has also been measured by optical microscopy and by light scattering (Parker et al., 2004). However, not surprisingly, the formation of cholesterol crystallites is initiated at much lower cholesterol mole fractions (Epand et al., 2003) than can be observed macroscopically (Parker et al., 2004).

In the present study, we investigate the properties of mixtures of cholesterol and phosphatidylcholines with systematic variations in acyl chain length and position of a single double bond. We demonstrate in this work that Di(16:1)PC can form structures whose morphology resembles the organization of lipid required for membrane fusion. We employ several physical methods to determine the presence of cholesterol crystals, the hydration and dehydration properties of these crystals, as well as the thermotropic behavior and morphology of the mixtures.

1. Experimental procedures

1.1. Materials

Phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL). Cholesterol was purchased from either Avanti Polar Lipids or from NuChek Prep (Elysian, MN).

1.2. Preparation of lipid films

Phospholipid and cholesterol were dissolved in chloroform/methanol (2/1, v/v) and mixed at appropriate ratios. The solvent was evaporated under a stream of nitrogen with constant rotation of a test tube so as
to deposit a uniform film of lipid over the bottom third of the tube. Last traces of solvent were removed by placing the tube under high vacuum for at least 2 h.

1.3. Hydration of lipid films for DSC and NMR experiments

The lipid film was hydrated with 20 mM PIPES, 1 mM EDTA, 150 mM NaCl with 0.002% NaN₃, pH 7.40 and suspended by intermittent vortexing and heating to 50–60 °C over a period of 30 min under argon.

1.4. Sample preparation for X-ray diffraction experiments

The dry phospholipids or phospholipid–cholesterol mixtures were introduced into X-ray capillaries and an excess of salt solution (150 mM NaCl in 10 mM Tris–HCl buffer, pH 7.4) was added, followed by incubation at ∼50 °C.

1.5. Sample preparation for cryo-transmission electron microscopy (cryo-TEM)

Lipid films of Di(16:1)₉ PC containing 0.6 mol fraction cholesterol, prepared as described above, were dispersed in 50 mM NaCl in 10 mM Tris–HCl buffer at a concentration of ∼10 mg/mL, incubated at 50 °C for 0.5 h and then heated in boiling water for 5 min. The lower salt concentration was dictated by the necessity of maximizing the contrast between the liposomes and the surrounding solvent. After storage at 4 °C for approximately 2 weeks, samples were prepared for cryo-TEM observation in a controlled environment vitrification system (CEVS) (Talmon, 1996). A 4 µL drop of sample was deposited on a lacy carbon film supported on a 200 mesh electron microscope grid (SPI Supplies, USA). The drop was then thinned by blotting with filter paper down to a layer thickness of 1000–3000 Å. The specimens were vitrified by plunging into liquid ethane at its melting point.

1.6. Cryo-TEM

Images were recorded using low dose cryo-conditions on a TVIPS slow scan 1k CCD camera utilizing an FEI Tecnai F20 microscope operating at 200 kV. Fast Fourier transforms of selected regions of the TEM images were performed using the public domain program ImageJ.

1.7. Differential scanning calorimetry (DSC)

Measurements were made using a Nano Differential Scanning Calorimeter (Calorimetry Sciences Corporation, American Fork, UT). The scan rate was 2 °C/min and there was a delay of 5 min between sequential scans in a series to allow for thermal equilibration. DSC curves were analyzed by using the fitting program, DA-2, provided by Microcal Inc. (Northampton, MA) and plotted with Origin, version 5.0.

1.8. ³¹P NMR

The ³¹P NMR spectra, from suspensions of about 25 mg of lipid in PIPES buffer, were obtained using a Bruker DRX-500 spectrometer operating at 202.45 MHz in a 5 mm broad band probe over a 49 kHz sweep width in 16 × 1024 data points. A 90° pulse width of 9.4 µs was used. The sample was contained in a 5 mm diameter Shigemi NMR tube (Shigemi Co., Tokyo, Japan). Composite pulse decoupling was used to remove any proton coupling. Generally, 800 free induction decays were processed using an exponential line broadening of 100 Hz prior to Fourier transformation. Probe temperature was maintained to ±0.2 °C by a Bruker BVT 3000 variable temperature unit. Temperatures were monitored with a calibrated thermocouple probe placed in the cavity of the NMR probe.

1.9. ¹³C MAS/NMR

Lipid suspensions in buffer were spun in an Eppendorf centrifuge at room temperature. The resulting hydrated pellet was transferred to a 18 mm × 4 mm ZrO₂ rotor, attempting to pack the maximal amount of lipid into the rotor while maintaining it wet. The rotor was placed in a Bruker Avance 300 spectrometer operating at 75.48 MHz for ¹³C and equipped with CP/MAS capabilities. The spectra were referenced to an external standard of glycine crystals, assigning a chemical shift of 176.14 ppm for the carbonyl carbon. Samples were spun at 8 kHz. The temperature inside the rotor was maintained at 25 °C by the variable temperature unit of the instrument and was calibrated by measuring the
chemical shift of ethylene glycol spinning at 5 kHz. The power levels used for cross-polarization corresponded to a 4 μs π/2 pulse. The Hartmann–Hahn match was established on the sample of glycine. Continuous-wave decoupling at an increased power level was used during acquisition. Some experiments were repeated to verify the stability and reproducibility of the cross-polarization. DP/MAS was obtained using single pulse excitation with high power proton decoupling using a 4 μs pulse for 13C and the proton frequency optimized for decoupling. A recycle time of 5 s was used. Generally each spectrum was obtained with 12,000 scans and processed with a 1 Hz line broadening. Resonances were assigned based on reports of phosphatidylcholine (Forbes et al., 1988) and cholesterol (Guo and Hamilton, 1996).

1.10. X-ray diffraction

Low angle X-ray diffraction measurements were performed as a function of temperature as described in Bach et al. (2002).

2. Results

2.1. Phase transitions determined by DSC

We measured the enthalpy and temperatures of the phase transitions of several PC species alone and admixed with various mol fractions of cholesterol by DSC. Values for the pure phospholipids used in these studies are shown in Table 1. The enthalpy and cooperativity of these transitions are decreased in the presence of cholesterol. When cholesterol passes its solubility limit in the membrane it can form crystallites, either attached to the membrane or free in solution. These also give rise to transition peaks in the DSC. There are two kinds of cholesterol crystals: anhydrous cholesterol and cholesterol monohydrate (Loomis et al., 1979). Anhydrous cholesterol crystals can be detected by the presence of the thermotropic transition between two polymorphic forms that occurs at about 38°C on heating and at about 20°C on cooling at the scan rate of 2°C/min (Epand et al., 2000). The endotherms caused by the transitions in heating scans are clearly separated from other transitions and are also characterized by the hysteresis observed between heating and cooling scans. In addition to anhydrous crystals, cholesterol can also crystallize as the monohydrate. This crystalline form generally undergoes dehydration at about 80°C on heating. With pure cholesterol monohydrate crystals, this transition is very slowly reversible, requiring many hours for rehydration even in the presence of excess water and therefore may not be observed after the first heating scan (Loomis et al., 1979). In the same temperature range that there is dehydration of crystals of cholesterol monohydrate, some mixtures of cholesterol and PC display polymorphic phase transitions of the lipid mixture. These polymorphic transitions are generally reversible and have a greater cooperativity compared with the dehydration of cholesterol that is broad and very slowly reversible over a period of hours (Epand et al., 2003). The transitions observed in this temperature range are further analyzed below using other methods to distinguish their origin.

Interestingly, with many of the forms of phosphatidylcholine used in the present work, no anhydrous cholesterol crystals can be detected even after heating mixtures containing 0.7 mol fraction of cholesterol to

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Trivial name of acyl chain</th>
<th>Transition temperature (°C)</th>
<th>Enthalpy (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D(24:1)PC</td>
<td>Nervonoyl</td>
<td>27.0</td>
<td>10.8</td>
</tr>
<tr>
<td>D(22:1)PC</td>
<td>Erucosenoyl</td>
<td>13.1</td>
<td>6.4</td>
</tr>
<tr>
<td>D(20:1)PC</td>
<td>Eicosenoyl</td>
<td>&lt;0</td>
<td>&lt;0</td>
</tr>
<tr>
<td>C18:1(9Z)PC</td>
<td>Oleoyl</td>
<td>−8</td>
<td>−4.5</td>
</tr>
<tr>
<td>C18:1(9Z)PC</td>
<td>Petroselinoyl</td>
<td>&lt;0</td>
<td>&lt;0</td>
</tr>
<tr>
<td>C16:0(9Z)PC</td>
<td>Palmitoyl</td>
<td>41.8</td>
<td>9.6</td>
</tr>
<tr>
<td>C16:1(9Z)PC</td>
<td>Palmitoleoleoyl</td>
<td>&lt;0</td>
<td>&lt;0</td>
</tr>
</tbody>
</table>

* Data from Cullis et al. (1978).
100 °C, which would be expected to cause the dehydration of cholesterol monohydrate crystals. We show the second heating and second cooling DSC scans for the homologous series of Di(24:1)₁₅ PC, Di(22:1)₁₃ PC and Di(20:1)₁₁ PC at a cholesterol mol fraction of 0.7 (Fig. 1). This is also the case with the next homolog in the series, Di(18:1)₉ PC, as has been previously reported (Epand et al., 2003). Several of these mixtures exhibit multiple transitions on the first heating scan (see below). However, after the second heating and cooling scans, subsequent scans are reversible.

We summarize the characteristics of the phase transitions in the region ∼75–90 °C for a homologous series of PCs differing from one another by two CH₂ groups in the length of each acyl chain and in the position of the double bond (Table 2). The transition in this temperature range could be caused by the dehydration of cholesterol monohydrate crystals or by a polymorphic phase transition of the phospholipid. If we calculate the enthalpy per mole cholesterol (rather than per mole PC as in Table 2), then the enthalpy decreases about two-fold in going from 0.6 to 0.8 mol fraction cholesterol, opposite to the direction one would expect if the transition arose solely from the dehydration of cholesterol monohydrate crystals. In contrast, when calculated per mole PC the enthalpy does not vary greatly with the mole fraction of cholesterol, although there is some tendency for the enthalpy to increase with increasing mole fraction of cholesterol. This suggests that this peak is caused by a polymorphic transition that

![Fig. 1. DSC heating scans of a sample of PC with 0.7 mol fraction of cholesterol. The curves are labeled with the acyl chain designation of each of the PCs shown. Curves shown are the second heating and second cooling scans of each of the samples. Lipid concentration 1.5 mg/mL in 20 mM PIPES, 1 mM EDTA, 150 mM NaCl with 0.002% NaN₃, pH 7.4. Scan rate 2 °C/min. Cp is given per mole cholesterol and curves have been displaced along the y-axis for presentation.](image-url)

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>Mole fraction of cholesterol</th>
<th>Transition temperature on heating</th>
<th>Transition temperature on cooling</th>
<th>ΔH (cal/mol PC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Di(24:1)₁₅ PC</td>
<td>0.6</td>
<td>89</td>
<td>85</td>
<td>1300</td>
</tr>
<tr>
<td></td>
<td>0.7</td>
<td>84.5</td>
<td>79</td>
<td>1500</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>78</td>
<td>75</td>
<td>1650</td>
</tr>
<tr>
<td>Di(22:1)₁₃ PC</td>
<td>0.6</td>
<td>85</td>
<td>80</td>
<td>825</td>
</tr>
<tr>
<td></td>
<td>0.7</td>
<td>80</td>
<td>74</td>
<td>1280</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>76</td>
<td>71</td>
<td>1000</td>
</tr>
<tr>
<td>Di(20:1)₁₁ PC</td>
<td>0.6</td>
<td>83</td>
<td>76</td>
<td>631</td>
</tr>
<tr>
<td></td>
<td>0.7</td>
<td>78</td>
<td>72</td>
<td>700</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>78</td>
<td>72</td>
<td>920</td>
</tr>
<tr>
<td>Di(18:1)₉ PC</td>
<td>0.6</td>
<td>84</td>
<td>75</td>
<td>560</td>
</tr>
<tr>
<td></td>
<td>0.7</td>
<td>78</td>
<td>65</td>
<td>560</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>78</td>
<td>65</td>
<td>560</td>
</tr>
</tbody>
</table>

* Data obtained from DSC measurements at a 2 °C/min scan rate.
* Data from Epand et al. (2003).
involves the phospholipid. There could also be a contribution from the dehydration of cholesterol monohydrate crystals to the observed transition, but we believe that it must be a minor component.

Considering that the chain melting transitions of Di(18:1)_{9}PC and Di(24:1)_{15}PC are −8°C (Cullis et al., 1978) and +24°C (Caffrey and Feigenson, 1981), respectively, the range of transition temperatures given in Table 2 is relatively small. Lengthening the acyl chain (i.e. introducing more hydrocarbon) generally lowers the temperature at which inverted phases form. However, moving the double bond from position 9 to 15 has the opposite effect (Epand et al., 1996). The overall effect is small, but there is some tendency for the transition temperature to increase with increasing acyl chain length as well as with increasing mole fraction of cholesterol. This is also consistent with the transition representing a polymorphic transition of the lipid mixture.

Di(18:1)_{6}PC and Di(16:0)PC were also studied, although they are not part of the homologous series of PCs. In the cases of Di(16:0)PC and Di(18:1)_{6}PC there is no evidence for non-lamellar structures in mixtures with cholesterol at any temperature (see below). For Di(16:0)PC, at mole fractions of cholesterol between 0.6 and 0.8, both anhydrous cholesterol crystals, as well as crystals of cholesterol monohydrate are formed. However, the transition observed at 72°C, which corresponds to the dehydration of cholesterol monohydrate, appears only in the first heating scan and not in any cooling scans or the subsequent heating scan. At 0.7 mol fraction cholesterol and Di(16:0)PC the polymorphic transition of anhydrous cholesterol crystals at 37°C is 130 cal/mol for each of the six scans that were run. This is in accord with the commonly observed behavior of cholesterol crystals, that the rehydration of anhydrous cholesterol is a very slow process requiring several hours to complete (Loomis et al., 1979). For Di(18:1)_{6}PC containing 0.6 mol fraction cholesterol transitions corresponding to the presence of both anhydrous and monohydrate crystals of cholesterol appear only in the first heating scan, subsequent scans do not exhibit any transitions. However, at 0.7 mol fraction cholesterol with Di(18:1)_{6}PC more complex behavior is observed (Fig. 2). After heating and cooling, subsequent heating scans show the disappearance of anhydrous cholesterol crystals (38°C transition) and an increase in the enthalpy of the transitions corresponding to the dehydration of cholesterol monohydrate crystals at about 80°C. The enthalpy of the polymorphic transition of anhydrous cholesterol at 36°C is 265 cal/mol cholesterol in the first heating scan but the anhydrous cholesterol is largely converted to cholesterol monohydrate on heating and cooling. The enthalpy of this transition on the second heating scan is only 40 cal/mol, which would correspond to only 4% of the total cholesterol. This is opposite to what usually occurs on repeated scanning of pure cholesterol crystals in which the monohydrate form is converted to anhydrous cholesterol (Epand et al., 2000). There is also a transition observed at 8.8°C on cooling whose enthalpy, 250 cal/mol, is the same as that for the 79°C transition observed in heating scans. Since the enthalpy of this transition is the same magnitude but opposite in sign to the one at 79°C and no polymorphic transition of anhydrous cholesterol crystals is observed in subsequent heating scans, we suggest...
this low temperature transition at 8.8 °C corresponds to the dehydration of cholesterol. The more rapid rehydration of cholesterol in the presence of Di(18:1)PC also accounts for the fact that the transition at 79 °C is irreversible over a short period of time on cooling and reheating. With Di(18:1)PC and a mole fraction of cholesterol of 0.8, transitions at both 36 °C for the polymorphic transition of anhydrous cholesterol and at 73 °C, corresponding to the dehydration of cholesterol monohydrate crystals, are observed on the first heating scan (data not shown). However, unlike the situation at 0.7 mol fraction cholesterol, only the polymorphic transition of anhydrous cholesterol is observed at 22.1 °C on cooling and on subsequent heating the only transition observed is at 35.3 °C with the same enthalpy as the cooling transition. Hence for this lipid mixture, dehydration of anhydrous cholesterol is not rapid and only the polymorphic transition of anhydrous cholesterol is observed after the first heating scan.

Because mixtures of Di(16:1)PC and cholesterol exhibit particularly interesting morphological behavior (see below) and because it is formally not part of the series of PCs with acyl chains (18:1), (20:1), (22:1), or (24:1) since the 16 carbon homolog in this series would be Di(16:1)PC, we describe the DSC behavior of this lipid separately and in more detail (Fig. 3). At 0.6 and 0.7 mol fraction cholesterol there is a transition at 38.7 °C, corresponding to the polymorphic transition of anhydrous cholesterol crystals only in the first heating scan. However at 0.8 mol fraction cholesterol this transition appears in every heating scan together with the corresponding 23 °C transition in each of the cooling scans. Probably at this mole fraction of cholesterol, cholesterol crystallites, appear free in solution and their rehydration is a slow process as for pure cholesterol crystals. There is also a large transition at 87 °C in heating and 79 °C in cooling for each of the mole fractions of cholesterol. The enthalpy of this transition per mole phospholipid is 1000 cal/mol. Both the temperature and enthalpy of this transition are similar to transitions in other lipid mixtures that we ascribe to a polymorphic transition of the lipid mixture (Table 2). The sample of Di(16:1)PC with 0.8 mol fraction cholesterol also has a broader transition at 80 °C in the first heating scan that is not present in subsequent transitions. We suggest that this transition corresponds to the dehydration transition of cholesterol monohydrate crystals since it is irreversible and results in an increase in the enthalpy of the polymorphic transition of anhydrous cholesterol from 106 cal/mol cholesterol on the first heating scan at 36 °C to 210 cal/mol on subsequent heating and cooling scans.

2.2. Phase behavior of the samples monitored by 31P NMR

We studied the thermotropic phase behavior of mixtures of Di(16:1)PC with varying mole fractions of cholesterol. Samples with 0, 0.3 or 0.4 mol fraction of cholesterol remained in lamellar form when heated to 95 °C and upon recooling to 25 °C (not shown). For samples containing between 0.5 and 0.7 mol fraction cholesterol, a non-lamellar phase began to form in the temperature range of 80–90 °C (Fig. 4). At 0.5 mol fraction cholesterol, an isotropic component contributes to the lamellar powder pattern that increases with increasing temperature up to 95 °C (cf. X-ray diffraction results). Some of the isotropic signal persists after recooling to 25 °C. At 0.7 mol fraction cholesterol only the hexagonal phase forms at higher temperatures, but it reconverts to a lamellar phase with some isotropic signal on recoiling. The Di(16:1)PC with 0.6 mol fraction of cholesterol forms both hexagonal and isotropic phases at higher temperature but converts to all isotropic (albeit with a broader resonance line) on recoiling.

2.3. Phase behavior of the samples monitored by X-ray diffraction

We have studied the X-ray diffraction from mixtures of cholesterol with Di(16:1)PC, Di(18:1)PC, Di(20:1)PC, Di(22:1)PC, and Di(24:1)PC. The mole fractions of cholesterol varied from 0.5 to 0.82 but the whole range was not checked for all phospholipids. At room temperature, the lipid mixtures exhibited a diffraction pattern characteristic of the lamellar phase with interlamellar spacing that varied between 60 and 75 Å. This value depended on the chain length and the mole fraction of cholesterol. The presence of cholesterol crystallites in the lamellar phase could be definitively identified for only two samples: Di(18:1)PC with mole fraction cholesterol 0.8, and Di(24:1)PC with mole fraction cholesterol 0.7. In the cases of Di(20:1)PC or Di(22:1)PC, the characteristic 34 Å
Fig. 3. DSC scans of Di(16:1)PC with 60, 70 and 80 mol% cholesterol as indicated in the figure. Lipid concentration 1.5 mg/mL in 20 mM PIPES, 1 mM EDTA, 150 mM NaCl with 0.002% NaN₃, pH 7.4. Scan rate 2 °C/min. For each panel the top three curves are heating scans and bottom three curves are cooling scans. The numbers indicate the order in which the scans were run. Curves have been displaced along the y-axis for presentation. Excess heat capacity is expressed per mole of cholesterol.
Fig. 4. 31P NMR spectra of Di(16:1)9 PC with different mol fractions of cholesterol as a function of temperature. The mole fraction of cholesterol is shown on top and the temperatures along the right-hand side of the spectra. For each sample, the spectrum was first accumulated at 25°C (lower spectrum) over a period of about 30 min and then the sample heated in approximately 15°C increments. This process was repeated over a range of temperatures. The order of spectral acquisition is from bottom to top. The top spectrum at 25°C was measured after heating to 95°C.

In all samples the lipid concentration was 50 mg in 400 μL.

diffraction peak of cholesterol crystals (either anhydrous or monohydrate), even if present, would not be resolvable from the second order of the interlamellar spacing.

Di(16:1)9 PC, Di(20:1)11 PC, Di(22:1)13 PC, and Di(24:1)15 PC all exhibited a lamellar to hexagonal phase transition when heated to 70–80°C in the presence of greater than equimolar amounts of cholesterol. Only mixtures of Di(18:1)6 PC with cholesterol did not undergo this transition upon heating. The appearance of the HII phase is identified by the three diffraction maxima with ratios of d-spacing 1:√3:2. The lattice constant of the hexagonal phase showed a weak dependence on cholesterol content, while also varying with chain length: e.g. 72 Å for Di(16:1)9 PC, mole fraction cholesterol 0.55 and 90 Å for Di(24:1)15 PC, mole fraction cholesterol 0.7. The observed change in symmetry confirms the assignment of the high temperature endotherms in the DSC heating traces of Figs. 1 and 3 as being due to polymorphic transitions of the phospholipids. On the other hand the high temperature peaks in Fig. 2 must be, as suggested above, due to dehydration of cholesterol monohydrate. Phase separation of cholesterol crystallites in the hexagonal phase could be detected by X-ray diffraction. For Di(20:1)11 PC, or Di(24:1)15 PC the characteristic cholesterol diffraction peak at 34 Å was observed in samples with 0.7 mol fraction cholesterol and above. For Di(20:1)11 PC the amount of cholesterol had to be increased to 0.82 in order for crystallization to be positively identified, while for Di(16:1)9 PC no cholesterol crystallite diffraction was observed in the hexagonal phase up to 0.7 mol fraction cholesterol. Although we looked for indication that cholesterol crystallite formation was either promoted or suppressed by the change in symmetry of the phospholipid phase, we did not find any unambiguous evidence for this. In addition to the lamellar and hexagonal phases, diffuse scattering from a non-ordered phase was observed at ~80°C for Di(16:1)9 PC with 0.5 mol fraction...
cholesterol and for Di(24:1)15 PC with 0.7 mol fraction cholesterol.

2.4. 13 C MAS/NMR

13 C MAS/NMR provides evidence for the presence of cholesterol monohydrate crystals after heating to 100 °C and recooling back to room temperature. We have measured the 13 C MAS/NMR of Di(16:1) 9 PC containing 0.6 mol fraction cholesterol both before and immediately after heating to 100 °C using both direct polarization (DP) as well as cross-polarization (CP) (Fig. 5). All measurements were done at 25 °C. The first spectrum was run immediately after heating to 100 °C for 5 min and recooling to 25 °C. The NMR spectrum in this case was acquired over a period of 2 h. Other spectra were acquired over a period of 8 h and have better signal/noise ratio. With DP one should observe all of the carbon atoms, providing the spin-lattice relaxation time (T1) is not too long. In general, the number of C atoms observed by DP is in good agreement with the expected values (Table 3). Note that the number of C atoms expected is adjusted for the cholesterol/PC ratio in the sample and that the observed number of carbons for the DP/MAS spectra were calculated in relation to the peak for the quaternary ammonium methyl groups. This group should be mobile and therefore not have a very long T1. The only groups that show lower intensity by DP/MAS are carbon atoms from some of the positions of cholesterol, particularly those of the B ring and C15 as well as the glycerol backbone of PC. These are likely more rigid positions that have long T1. A few general conclusions can be reached from the DP/MAS. The only crystalline form of cholesterol seen, either before or after heating is cholesterol monohydrate. Anhydrous cholesterol has a different spectrum, particularly for the C5, C6 and C18 carbons of cholesterol (Guo and Hamilton, 1996). The peaks are more split with anhydrous cholesterol, which may account for the fact that we do not detect the small fraction of anhydrous cholesterol seen by DSC before heating. In addition, however, there is a longer time delay before acquisition of the first NMR spectrum compared with DSC that may allow hydration to occur before or during the NMR measurement. Based on the C18 peak of cholesterol, we calculate that 37.5% of the cholesterol is in the form of cholesterol monohydrate crystals before heating and 30.0% after heating. DSC would easily ob-

**Fig. 5.** 13 C CP/MAS and DP/MAS NMR spectra of Di(16:1) 9 PC containing 0.6 mol fraction cholesterol. Spectra were all run at 25 °C of an unheated sample and of sample that was heated to 100 °C for 2 min and then recooled to 25 °C. Measurement made at 75.48 MHz with sample spinning at 5 kHz using a contact time of 1 ms for CP/MAS and a delay time of 5 s for DP/MAS.
Table 3

<table>
<thead>
<tr>
<th>Assignment</th>
<th>Chemical shift</th>
<th>Number of C atoms</th>
<th>DP MAS Before heat</th>
<th>After heat</th>
<th>CP MAS Before heat</th>
<th>Immediately after heat</th>
<th>Later after heat</th>
</tr>
</thead>
<tbody>
<tr>
<td>C(\text{O})</td>
<td>173.9</td>
<td>1</td>
<td>1.09</td>
<td>1.00</td>
<td>0.55</td>
<td>0</td>
<td>0</td>
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<tr>
<td>C(\text{O})</td>
<td>173.5</td>
<td>1</td>
<td>0.86</td>
<td>0.80</td>
<td>0.53</td>
<td>0</td>
<td>0</td>
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<tr>
<td>C5</td>
<td>142.1</td>
<td>1.5</td>
<td>0.36</td>
<td>0.20</td>
<td>0.57</td>
<td>0.38</td>
<td>0.57</td>
</tr>
<tr>
<td>C5</td>
<td>140.7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.49</td>
<td>0</td>
<td>0.55</td>
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<tr>
<td>C(\text{O}) acyl</td>
<td>130</td>
<td>2</td>
<td>1.79</td>
<td>1.64</td>
<td>2.24(b)</td>
<td>4(c)</td>
<td>4(c)</td>
</tr>
<tr>
<td>C(\text{O}) acyl</td>
<td>129.6</td>
<td>2</td>
<td>2.11</td>
<td>1.96</td>
<td>1.76(c)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C(\text{O})</td>
<td>120.7</td>
<td>1.5</td>
<td>0.56</td>
<td>0.64</td>
<td>1.02</td>
<td>1.46</td>
<td>1.16</td>
</tr>
<tr>
<td>Choline-(\beta)</td>
<td>66.3</td>
<td>1</td>
<td>0.88</td>
<td>0.85</td>
<td>0.49</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Glyceryl-C3</td>
<td>64.16</td>
<td>1</td>
<td>0.62</td>
<td>0.60</td>
<td>0.82</td>
<td>1.46</td>
<td>1.75</td>
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<tr>
<td>Glyceryl-C1</td>
<td>63.57</td>
<td>1</td>
<td>0.51</td>
<td>0.38</td>
<td>0.61</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Choline-(\alpha)</td>
<td>59.82</td>
<td>1</td>
<td>1.00</td>
<td>0.97</td>
<td>0.79</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Choline-(\gamma)-CH(3)</td>
<td>54.41</td>
<td>3</td>
<td>3.00(c)</td>
<td>3.00(c)</td>
<td>0.40</td>
<td>2.85</td>
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<tr>
<td>C9</td>
<td>50.61</td>
<td>1.5</td>
<td>0.77</td>
<td>0.86</td>
<td>1.80</td>
<td>5.31</td>
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<td>42.91</td>
<td>1.5</td>
<td>1.26</td>
<td>1.43</td>
<td>3.65</td>
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<tr>
<td>C4</td>
<td>41.84</td>
<td>1.5</td>
<td>0.73</td>
<td>0.79</td>
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<td>4.27</td>
<td>5.49</td>
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<td>C24</td>
<td>40.55</td>
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<td>0.64</td>
<td>0.72</td>
<td>2.00</td>
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<td>4.58</td>
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<td>39.94</td>
<td>1.5</td>
<td>1.03</td>
<td>1.11</td>
<td>2.87</td>
<td>4.89</td>
<td>5.73</td>
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<tr>
<td>C1</td>
<td>38.04</td>
<td>1.5</td>
<td>1.18</td>
<td>1.03</td>
<td>4.93</td>
<td>6.72</td>
<td>8.45</td>
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<tr>
<td>C5</td>
<td>24.23</td>
<td>1.5</td>
<td>0.55</td>
<td>0.39</td>
<td>1.77</td>
<td>1.83</td>
<td>2.30</td>
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<tr>
<td>C9</td>
<td>19.35</td>
<td>1.5</td>
<td>1.41</td>
<td>1.54</td>
<td>3.34</td>
<td>5.19</td>
<td>5.53</td>
</tr>
<tr>
<td>Acyl-terminal methyl</td>
<td>14.23</td>
<td>2</td>
<td>2.03</td>
<td>2.01</td>
<td>4.87</td>
<td>2.29</td>
<td>1.65</td>
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<td>CH(\text{O})-crystal</td>
<td>13.21</td>
<td>1.5(d)</td>
<td>0.52</td>
<td>0.27</td>
<td>1.28</td>
<td>1.16</td>
<td>1.66</td>
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<tr>
<td>CH(\text{O})</td>
<td>12.63</td>
<td>1.5(d)</td>
<td>0.98</td>
<td>1.03</td>
<td>1.70</td>
<td>3.05</td>
<td>2.96</td>
</tr>
<tr>
<td>CH(\text{O})-crystal</td>
<td>11.93</td>
<td>1.5(d)</td>
<td>0.27</td>
<td>0.17</td>
<td>1.61</td>
<td>1.42</td>
<td>1.83</td>
</tr>
</tbody>
</table>

a. C with a number after it refers to carbons of cholesterol; the others are carbons of the Di(16:1)PC.
b. Values calculated relative to the sum equaling 4.0.
c. Values calculated relative to this one.
d. Sum of intensities at 13.21, 12.63 and 11.93 = 1.5.

serve the dehydration of this large fraction of cholesterol monohydrate crystals, unless the transition was very broad. It is certain, however, that within the time to cool the sample from 100 °C and run the DP/MAS (about 30 min, for a spectrum with reasonable signal to noise ratio) essentially all of the cholesterol crystals are in the monohydrate form. This is also in agreement with the fact that we do not detect an exothermic transition at ~22 °C, corresponding to the polymorphic transition of anhydrous cholesterol crystals; i.e. the crystals must already be hydrated by the time they reach room temperature or they never are dehydrated during heating to 100 °C. This is markedly different from pure cholesterol crystals that remain in the anhydrous form for many hours after heating to 100 °C. It should be noted that these cholesterol crystals are not observed by X-ray diffraction, possibly because they are too small or disordered. The other solid state NMR method used is CP/MAS. CP is cross-polarization. In this method the intensity of the signals from carbon are enhanced by dipolar interactions with surrounding protons (Shekar et al., 2002). The enhancement is only effective if the carbon atom is not in rapid motion. Hence the choline CH\(3\) shows low intensity before heating because it is in rapid motion. It is also affected by the number of bonded protons with which it can generally undergo dipolar interactions more readily than with non-bonded protons. Hence the CH\(3\) gives a low magnitude peak because it has no bonded protons (although it does have protons in its vicinity that can contribute to cross-polarization and signal enhancement). Thus, the numbers of carbons observed are not necessarily the same as the number present in the sample or the number observed by DP/MAS. However, one can draw some qualitative conclusions about motional properties. We have compared the intensity of each of
the peaks with those of the C–C carbon atoms in the acyl chain. These should be relatively immobile because of the restricted rotation around the double bond and therefore give full intensity. The motion of the carbons in the ring system of cholesterol should also be restricted. They show greater intensity, corresponding to more restricted motion, than the C–C of the acyl chain. Furthermore, this difference increases as a result of heating, indicating that the motional properties of cholesterol become more restricted as a consequence of the heating. There are also changes in the motional properties of the phospholipid before and after heating. The resonances for the C=O, the α and β carbons of choline as well as the glycerol C1 are no longer detectable after heating. In addition the relative intensities of other carbon atoms of the phospholipid also change as a result of heating, some increasing and some decreasing. These results indicate that there is a marked rearrangement of the phospholipid as a result of heating, in accord with the morphological changes discussed above.

2.5. Cryo-transmission electron microscopy

Cryo-transmission electron microscopy of a sample of Di(16:1)PC with 0.6 mol fraction cholesterol, which had been kept in boiling water for 5 min and stored at 4 °C for 2 weeks, revealed that the lipids are organized in a variety of structural forms. These included single shell vesicles, multilamellar vesicles and arrays of relatively small lipid particles organized with varying degrees of order. The last of these constituted a minority population and they were generally found in the thinner layers of vitreous ice in which the material was embedded. One of the more well-ordered arrays is shown in Fig. 6. In this image, the small particles appear to be organized in a tetragonal lattice, which is most likely a projection of a three-dimensional structure. It is known, for instance, that a cubic phase can generate many different projections in the electron microscope, including one similar to Fig. 6, depending along which axis the unit cell is viewed. Fast Fourier transform of the selected region reveals two pairs of diffraction peaks oriented at approximately 90° to one another. The reciprocal lattice spacing of these peaks indicates that the center-to-center distance of the small lipid particles is approximately 14 nm. More exact identification of the structure of this minority phase could not be made as X-ray diffraction from the material used for microscopy was dominated by the multilamellar vesicles.

3. Discussion

3.1. Cholesterol and non-lamellar phases

The formation of cubic phase structures bears an important relationship to membrane fusion events.
This is because the connections between unit cells in a bicontinuous cubic phase resemble a fusion pore. A structure with hour-glass morphology, suggested to resemble a fusion stalk intermediate, has been found in partially dehydrated pure lipid systems (Yang and Huang, 2002). In addition, fusion pore-like interlamellar attachment sites (ILAs) have been observed with lipids such as Di(16:1)9PE (Siegel and Epand, 2000), monomethyldioleoylphosphatidylethanolamine (Siegel et al., 1994), or in a mixture of Di(18:1)sPE, and cholesterol (Frederik et al., 1991; Tlcsok et al., 1982; Johnson and Edwards, 2001; Koyanova et al., 1997). The formation of cubic phases has also been observed in mixtures of PEG-PE and dielaidoyl-PE (Koyanova et al., 1997). The evolution of this cubic phase through membrane fusion events has been studied by cryo-TEM (Johnson and Edwards, 2001). These studies involve either partial dehydration or the presence of PE, both of which strongly favor the formation of non-lamellar structures. In the present work we show that an ordered phase, probably organized by ILAs, can be formed with a phospholipid, Di(16:1)9PC, that has no tendency alone to form non-lamellar structures. In most biological membranes which is usually in the range of 0.3–0.4. However, it may not be high for cholesterol-rich domains believed to be present in biological membranes. Furthermore, the lipid composition of a biological membrane would not be expected to have a composition that would facilitate the process of membrane fusion.

We have previously reported that Di(18:1)sPC undergoes transitions to non-lamellar structures in mixtures with cholesterol (Epand et al., 2003). In this work we show that this is a common phenomenon with phosphatidylincholines having two acyl chains with a single double bond. However, it is not a universal property of such lipids since Di(18:1)sPC does not form non-lamellar phases. This lipid has less negative curvature strain due to the position of the double bond than Di(18:1)sPC (Epand et al., 1996) which may explain the fact that it does not form inverted structures when heated with cholesterol.

3.2. Characterization of cholesterol crystals

Previous studies have shown that mixtures of phospholipids and cholesterol in which cholesterol has surpassed its solubility limit in the membrane will result in the formation of cholesterol crystals. Depending on the nature of the phospholipid and the history of the sample, these crystals of cholesterol can be in the form of cholesterol monohydrate, anhydrous cholesterol or a mixture of both forms. However, after the sample has been heated once to 100°C, all of the cholesterol crystals are generally converted to the anhydrous form and cholesterol monohydrate crystals reform very slowly, over a period of hours. An exception to this is mixtures of cholesterol with Di(18:1)sPC in which cholesterol crystals are largely in the monohydrate form even after heating to 100°C (Epand et al., 2003). In the present work we show that this phenomenon is not unique to Di(18:1)sPC. We demonstrate that for mixtures of 0.7 mol fraction cholesterol with several of the phosphatidylincholines used in this work there is almost no appearance of anhydrous cholesterol crystals that are stable to heating to 100°C (Figs. 1–3). It is not likely that such a group of PCs that range in acyl chain length from 16 to 24 carbon atoms would all exhibit such unusually high solubility for cholesterol. In some cases anhydrous cholesterol crystals are detected by DSC in the first heating scan, but disappear with sequential scanning. We show in the case of Di(16:1)sPC with mole fraction cholesterol 0.6 that not all of the cholesterol is dissolved in the membrane, but rather a composition that would facilitate the process of membrane fusion.
The formation of cholesterol crystals, which are preferentially in the monohydrate, rather than the anhydrous form, is common in PCs with two mono unsaturated acyl chains. An intermediate case is PCs with polyunsaturated acyl chains. With these lipids, cholesterol monohydrate crystals are not observed after heating to 100 °C in mixtures with phospholipids having one (Epand et al., 2003) or two saturated (e.g. DL6:0:PC, this work) acyl chains. It does not require that the mixture form non-lamellar phases, since this property is also shared with DL6:1:0PC that remains lamellar to 100 °C. Even with lipids like DL6:1:0PC at 0.7 mol fraction cholesterol, where cholesterol monohydrate crystals are preferentially found (Fig. 5), conversion of cholesterol monohydrate crystals to the anhydrous form is observed at 0.8 mol fraction cholesterol (Fig. 5). This indicates that with the larger amount of cholesterol, the dehydration process is slow, indicative of cholesterol crystallites being free in solution, separated from the bilayer. We suggest that in mixtures with phospholipids, the hydration and dehydration processes of cholesterol crystals are determined by the interfacial properties of the membrane depending on both the chemical and physical nature of the lipids.

Acknowledgements

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References


