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Biophysical Chemistry 81 (1999) 33–44

Biophysical Chemistry

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Effects of alcohols on lipid bilayers with and without cholesterol: the dipalmitoylphosphatidylcholine system

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Received 13 January 1999; received in revised form 14 June 1999; accepted 14 June 1999

Abstract

Differential scanning calorimetry is a useful method to study the thermotropic phase transitions of a phospholipid bilayer. In the present study DSC is used to determine the effects of methanol and ethanol on DPPC and DPPC/2 mol% cholesterol bilayers. The biphasic effect of the main transition and the presence of an extra peak on the DSC cooling scans were observed above certain alcohol concentrations. In the presence of 2% cholesterol, the concentration at which the biphasic effect occurs is increased by both short-chain alcohols. 1,6-Diphenyl-1,3,5-hexatriene (DPH) is used as a fluorescent probe to directly determine the onset of interdigitation in these systems as reflected by a drop in the DPH fluorescence intensity. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Phospholipid; Cholesterol; Subgel; Interdigitation; DSC; Fluorescence

1. Introduction

Phospholipid bilayers have been widely used to elucidate the properties and functions of biological membranes. Biological membranes consist of a complex mixture of lipids, proteins, and carbohydrates. For example, a human red blood cell can contain up to 200 chemically distinct lipid molecules [1]. In order to understand how the form of the membrane may affect its function, many researchers have used single phospholipid or simple mixtures to study the phase transitions of the bilayer. This approach is justified by the observation that some model membrane systems have remarkably similar behavior to natural systems such as those found in myelin and erythrocyte membranes [2].

Rowe [3,4] first reported that the gel to liquid-crystalline phase transition temperature of phosphatidylcholine bilayers shifts in the presence of

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PII: S0301-4622(99)00081-2

ethanol. Using X-ray diffraction, McIntosh et al. [5] showed that several surface active molecules, including ethanol, caused 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) to interdigitate. The interdigitated phase was characterized by lipid molecules from opposing monolayers interpenetrating, thereby decreasing the bilayer thickness. In DPPC bilayers, the thickness is decreased to 30 Å vs. 42 Å in the planar gel phase [6]. It has been well established that [7] ethanol also induced a biphasic effect with phosphatidylcholine (PC) bilayers, where the main transition temperature first decreases with increasing alcohol concentration, but at higher alcohol concentrations, the trend reverses and the transition temperature increases with increasing alcohol concentration. This was correlated by Simon and McIntosh [6] with the induction of interdigitation.

Rowe [8] continued her studies with spectrophotometry to study both PCs and PEs in the presence of MeOH, EtOH, and propanol. She showed that PEs do not exhibit the biphasic effect, but that in PCs, all three alcohols induce the biphasic effect. Threshold concentrations of 1 M ethanol and 2.5 M methanol were reported for DPPC bilayers. At high alcohol concentrations, a large hysteresis of the main transition temperature was noted, while at low alcohol concentrations only a small hysteresis was seen [8,9].

The induction of the main transition hysteresis and the biphasic effect also correlates with the critical concentration of alcohol which abolishes the pretransition [10]. Two different pathways for the phase transitions were proposed depending on the alcohol concentration [11]. At lower alcohol concentrations, as the temperature is increased, the lipid bilayer undergoes a transition from the planar gel phase, L_{β}', to the ripple gel phase, P_{β}', and finally to the liquid-crystalline phase, L_{α}. At higher alcohol concentrations, the ripple gel phase is replaced by the interdigitated phase, $L_{\beta}I$.

It was noted that inducer molecules must be able to displace water from the interfacial region and not extend too deeply in the bilayer interior [12]. Many molecules such as acetylcholine, choline [13], anesthetics, alcohols, buffer molecules, anions, polymyxin B, and myelin basic protein ([14] and references therein) have since been shown to induce the interdigitated phase. It is important to note that the interdigitated phase could be induced in natural systems. Lewis et al. [15] used IR spectroscopy to show that ethanol could induce partial interdigitation in rat liver plasma membranes. A spin label study also suggested that polymyxin B induced small interdigitated domains in a bilayer formed from a lipid extract of Pseudomonas aeruginosa, a Gram-negative bacteria [16].

Many different techniques have been used to study the interdigitated phase. A light scattering method was used in 1992 to study the hydrocarbon tilt in DPPC membranes and detect chain interdigitation [17]. Nagel et al. concluded from this study that an excessive repulsion is created between the lipid molecules and the solute molecules. This repulsion in the interfacial region causes the lipid chains to tilt. At the point where the lipid molecules cannot tilt anymore, the interdigitated structure forms. In 1994, Barry and Gawrisch [18] used NMR to prove that ethanol did indeed bind in the lipid–water interface. The hydrophobic part of the ethanol interacts with the glycerol, while the hydrophilic part of the ethanol interacts with the hydroxyl group and a carbonyl or phosphate oxygen. It is possible that the ethanol molecule creates a small void that the hydrocarbon chains from a phospholipid in the opposing layer can enter. This interaction would be stabilized by van der Waals interactions between the acyl chains. It was shown by X-ray diffraction that two alcohol molecules would interact with the exposed methyl ends of the acyl chains. In this structure, the alcohols would also be surrounded by the lipid headgroups [19].

Fluorescence spectroscopy has been used extensively to look at the structure and dynamics of biological membranes. Probes such as pyrene and 1,6-diphenyl-1,3,5-hexatriene (DPH) have been described as good probes due to their sensitivity to the environment and the fact that they interfere very little with the lipid bilayer [20–22]. This study uses the method developed by Nambi et al. [22] with the DPH probe. This probe is normally located within the hydrophobic interior and oriented perpendicular to the acyl chains. Upon

interdigitation, the probe is shifted so that it lies parallel to the acyl chains. The probe is now exposed to the hydrophilic, aqueous environment and causes a sharp decrease in the fluorescence intensity. Due to the probe's sensitivity to its environment, the intensity changes as the bilayer undergoes phase transitions.

Cholesterol has been long recognized as an important component in membranes. The cholesterol content, however, varies from one type of membrane to another. Mitochondria have been shown to contain 6 mol% cholesterol while myelin membranes can contain up to 40 mol% [23]. Many studies have focused on the interactions and distribution of cholesterol in the membrane (for a review, see [24]). Cholesterol also affects the bilayers thermotropic transitions ([25] and references therein). The subtransition can persist up to 20 mol% cholesterol [26], and 50 mol% cholesterol has been shown to abolish the main transition in most PC bilayers [27]. Small amounts of cholesterol decrease the pretransition temperature and eventually lead to its disappearance at concentrations above 5 mol%. The ability of ethanol to induce interdigitation is prevented by 20 mol% cholesterol [22].

Most of the studies so far have focused on higher cholesterol concentrations. For a better understanding of the nature of this interesting effect and its potential physiological implications, this study provides a detailed analysis of the effect of 2 mol% cholesterol on the phase transitions of a DPPC bilayer. We have used DSC and fluorescence spectroscopy to examine both the interdigitated phase and subtransition of DPPC, and DPPC in 2 mol% cholesterol mixtures in the presence of methanol and ethanol. The reduced fluorescence emission is a good indication of the presence of the subgel (L_c) and provides a good method to study the subtransition. The DSC data from this study also provide some interesting insight on the DPPC/alcohol and DPPC/2%Chol/alcohol phase diagrams. The presence of an extra peak at the main transition in the cooling scan supports the presence of a mixed phase at high alcohol concentrations.

2. Materials and methods

2.1. Materials

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), purity 99 + %, was obtained from Avanti Polar Lipids (Alabaster, AL, USA). The fluorescence probe, 1,6-diphenyl-1,3,5-hexatriene (DPH), was purchased from Molecular Probes (Eugene, OR, USA), and the 5(6)-cholesten-3-ol (99 + %) was purchased from Sigma (St. Louis, MO, USA). All chemicals were used without further purification.

2.2. Differential scanning calorimetry measurements

DSC scans were made using a Calorimetry Sciences, Inc. Multi-cell DSC-HT Model 4100 at a scan rate of 10°C/h. The pure lipid samples were prepared by weighing out 2 mg of DPPC into a DSC ampoule and adding 100 μl of alcohol solution (alcohol in phosphate buffer, 25 mM, pH 7.0). The samples were hydrated at 45°C by vortexing. The samples used to study the subtransition were incubated at 5°C for at least 3 days prior to study. Scans were repeated at least three times to check for reproducibility, and that the samples were properly equilibrated.

Cholesterol samples (2 mol%) were made by first dissolving the appropriate amount of cholesterol in chloroform and then adding the chloroform solution to 2 mg of lipid. The chloroform was evaporated under nitrogen and then the sample was placed under high vacuum for at least 10 h to remove any residual chloroform. The lipid samples were then hydrated as described above.

2.3. Fluorescence assay

The fluorometer used was an ISS K2 MultiFrequency Cross-Correlation Phase and Modulation Fluorometer with a Xenon Arc Lamp as the light source. The excitation wavelength was set at 338 nm and the emission at 427 nm for all samples. The temperature bath, a Neslab RTE-111 with a microprocessor, was programmed to scan from 5 to 45°C at a rate of 10°C/h. A calibration between

the bath and cuvette temperature was used to correct all temperature data.

The DPH powder was dissolved in chloroform as well as the cholesterol. Appropriate amounts of the chloroform solutions were then added to the lipid powder to produce a 2 mol% cholesterol sample with a probe ratio of 500:1 DPPC/DPH. The chloroform solvent was removed by evaporation under nitrogen and the final traces were removed under high vacuum overnight. The samples were hydrated above the chain melting temperature with double distilled water for approximately 1 h, vortexing occasionally. The final fluorescence sample contained 0.64 mg/ml DPPC in varying amounts of alcohol. Samples were incubated for at least 3 days at 5°C. Nitrogen gas was administered through each sample for at least 1 min before and stirred continuously during data collection. All fluorescence data showing the subtransition were normalized before analysis in order to compare the percentage drop in intensity after the subtransition.

3. Results

3.1. Differential scanning calorimetry

Fig. 1 shows a typical DSC thermogram for the DPPC/alcohol system. In the presence of 0.74 M methanol, the three transitions centered at 20.1, 33.6, and 40.5°C correspond to the subtransition, pretransition, and main transition for DPPC. The enthalpy changes are 4.2, 1.4, and 7.8 kcal/mol, respectively, for this system. These thermal characteristics are similar to the pure DPPC system at this low methanol concentration. After the first heating scan, the subtransition disappears, indicating the necessity for long periods of low temperature incubation for the formation of the subgel.

In the lipid/alcohol system, the pretransition temperature decreases from 35 to 20.8°C as the methanol concentration is increased from 0 to 2.7 M for the heating scans. The pre-transition peak is not detectable beyond 2.7 M methanol. Similar results are observed for ethanol, with the disappearance of the pre-transition occurring at 1.4 M. The pre-transition typically disappears at lower alcohol concentrations during cooling scans due to the transition kinetics. Similar trends are observed in the samples containing 2% cholesterol. The peaks are slightly broader and the pre-transition is no longer observed upon heating beyond 1.5 M methanol and 1.4 M ethanol.

The main transition temperature also decreases with increasing alcohol concentration at lower alcohol concentrations. However, at higher alcohol concentrations the trend reverses and the main transition temperature increases (Fig. 2a,b). This behavior was classified as the biphasic effect by Rowe [7], and we refer to the concentration at

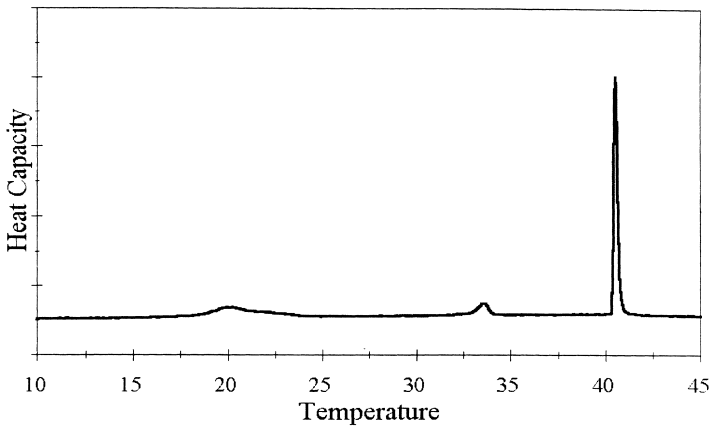

Fig. 1. DSC thermogram showing subtransition, pretransition, and main transition of DPPC/0.74 M MeOH sample (heating scan).

Table 1
Summary data for heating scans

System	Conc. at which pretransition disappears (M)	Conc. at which biphasic effect is observed (M)	Conc. at which fluorescence shows interdigitation (M)
DPPC/MeOH	2.7	2.7	2.7
DPPC/EtOH	1.4	1.0	1.0
DPPC/MeOH/2%Chol	1.5	3.2	2.5
DPPC/EtOH/2%Chol	1.4	1.4	0.9–1.0

which this reversal occurs as the threshold concentration. As previously reported [8], we saw that lower concentrations of ethanol than methanol were needed to induce this biphasic effect (results summarized in Table 1). Interestingly, the higher alcohol concentration cooling scans showed the presence of an extra peak by the main transition (Fig. 3). This extra peak appears after the biphasic effect occurs. Furthermore, this extra peak increases in size as the alcohol concentration increases.

Upon the addition of cholesterol, the main transition peak was slightly broader, and the disappearance of the pretransition occurred at lower methanol concentrations. With the DPPC/2%Chol/EtOH samples, however, the cholesterol did not significantly change the alcohol concentration at which the pretransition disappears. The threshold concentration for the induction of the main transition biphasic effect in the presence of 2 mol% cholesterol was increased from 2.7 M and 1 M for the DPPC/MeOH and DPPC/EtOH systems, respectively, to 3.2 M MeOH and 1.4 M EtOH for the DPPC/cholesterol system (Fig. 2c–d). The appearance of extra peaks on the cooling scan, however, did not occur in the DPPC/2 mol% chol samples until 3.9 M MeOH and 1.7 M EtOH. Shoulder peaks occasionally appeared on the heating scans with the DPPC/cholesterol samples. These peaks, however, were very small, and sometimes would only appear after the sample had been rerun. These heating scan shoulder peaks were more prominent with the ethanol/cholesterol samples than with the methanol/cholesterol samples.

Tables 1 and 2 summarize the data observed with each system for the heating and cooling scans, respectively. Overall, with the addition of cholesterol, the concentration at which the cooling scan shoulder peaks occur was increased. The alcohol concentration at which the pretransition disappears was decreased for methanol, and not significantly affected for the ethanol samples. The cholesterol also increased the alcohol concentration at which the biphasic effect is induced. While these results do provide many clues about the interactions of small amounts of cholesterol in a DPPC bilayer, they do not conclusively tell us how the addition of cholesterol is affecting the interdigitated phase. Therefore, fluorescence spectroscopy was used to determine whether or not interdigitation was occurring in the presence of 2% cholesterol, and if so, at what alcohol concentrations.

3.2. Fluorescence spectroscopy

Fig. 4 shows typical temperature scans for the DPPC/alcohol samples. The drastic increase in intensity at the beginning of the heating scan corresponds to the subtransition. The midpoint temperature of the intensity increase also corresponds to the subtransition temperatures observed by DSC. The subtransition is followed by a decrease in fluorescence intensity if a pretransition occurs. In the presence of higher concentrations of methanol, the pretransition disappears and at temperatures between the subtransition and the main transition, these samples showed intensity drops by as much as 50%. This drastic decrease showed that interdigitation was occurring. The 2.5 M MeOH sample shows a trend typically seen at concentrations near the threshold concentration. The pretransition has disappeared, but over the

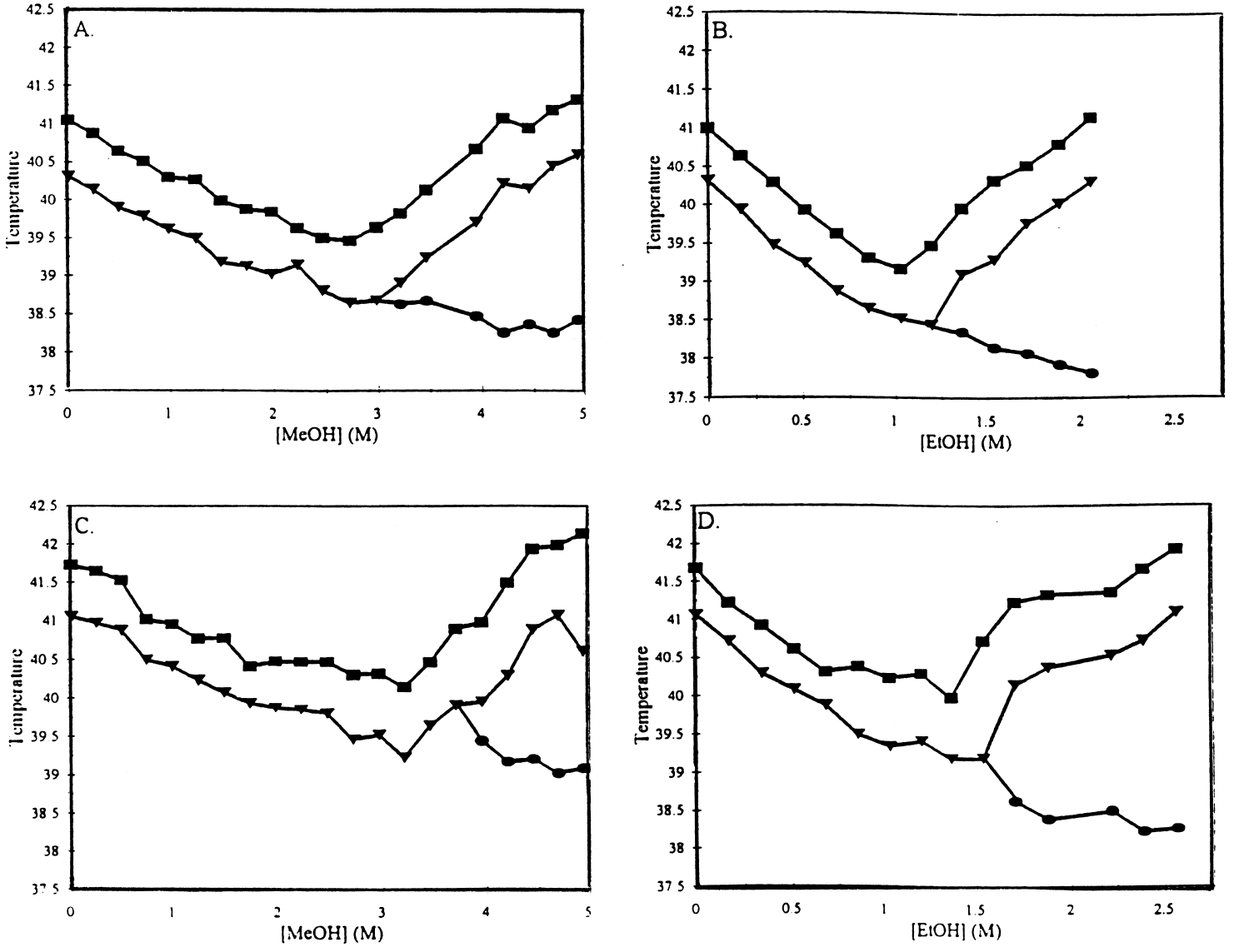

Fig. 2. Graphs of main transition temperature vs. alcohol concentration in the following systems: (A). DPPC/MeOH (B). DPPC/EtOH (C). DPPC/MeOH/2%Chol (D). DPPC/EtOH/2%Chol. (■, Heating scans; ▼, ●, cooling scans).

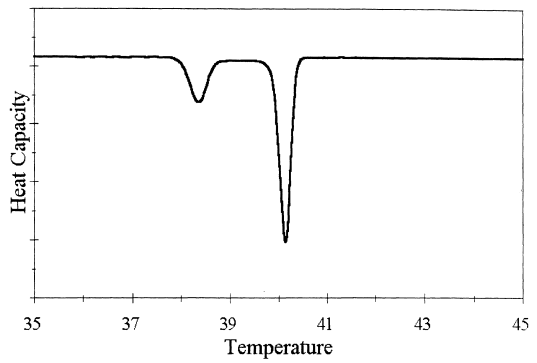

Fig. 3. A DSC thermogram of DPPC/4.5 M MeOH sample showing the presence of an extra peak at the cooling scan at a scan rate of 10°C/h. The hydrated sample had been heated from 20 to 50°C at 10°C/h immediately prior to cooling.

temperature range in which interdigitation usually occurs, there is an overall decrease in intensity without any singular drop. As the alcohol concentration is increased, the fluorescence scans show a clear trend toward interdigitation. Another observation was that once interdigitation occurs, the jump in intensity at the main transition increases by a greater amount than in the low alcohol concentration samples.

The DPPC/DPH/2%chol/alcohol samples also show the presence of the interdigitated phase (Figs. 5 and 6). The same general trends were observed for both sets of samples. At low alcohol concentrations we see the subtransition followed by a small decrease in intensity due to the pretransition; at intermediate alcohol concentrations the pretransition decrease either disappears or lessens; and, at higher alcohol concentrations, there is a drastic decrease in intensity showing the induction of the interdigitated phase. The drop in intensity seen for the ethanol/cholesterol samples is not as large as those seen in the methanol/cholesterol samples, but is clearly drastic enough to tell when interdigitation occurs. Thus, the large drop in intensity for the gel to interdigitated gel transition, accompanied by the large jump in intensity at the interdigitated gel to liquid-crystalline main transition became good indicators of interdigitation in these systems. The concentrations at which interdigitation first appeared on the fluorescence temperature scans are summarized in Table 1.

4. Discussion

4.1. The use of fluorescence spectroscopy to study the subtransition

The DSC data were not sufficiently reproducible for an accurate analysis of the subtransition. Some samples showed the presence of two peaks below the subtransition. Previous studies also reported multiple subtransition peaks [28,29]. There were many other problems encountered by groups who have previously studied the subtransition. When studied by DSC, the subtransition was reported to occur at 18°C, but X-ray diffraction and NMR showed the transition to be closer to 11°C [30]. Ruocco et al. [31] showed that the longer the DPPC sample is incubated, the higher the subtransition temperature. The fluorescence studies showed the presence of the subtransition by a large increase in intensity. By taking the midpoint of this increase, one could look at the effects a certain solute may have on the temperature of the subtransition. This technique also provides a method to determine if the subtransi-

Table 2
Summary data for cooling scans

System	Conc. at which pretransition disappears (M)	Conc. at which shoulder peaks first appear (M)	Conc. at which biphasic effect is observed (M)
DPPC/MeOH	2.2	3.2	3.0
DPPC/EtOH	1.2	1.4	1.2
DPPC/MeOH/2%Chol	1.5	3.9	3.2
DPPC/EtOH/2%Chol	1.4	1.7	1.4–1.5

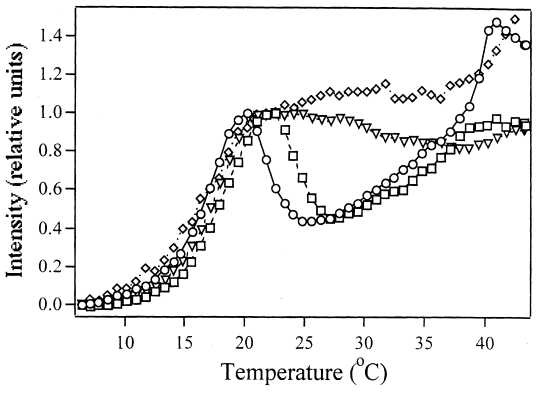

Fig. 4. Fluorescence temperature scans for DPPC/DPH/MeOH samples (◇, 1.7 M MeOH; ∇, 2.5 M MeOH; □, 3.0 M MeOH; ○, 3.5 M MeOH). The pretransition disappeared by 1.2 M MeOH and is evident on this graph with the 1.7 M MeOH sample.

tion even exists with a certain system. The low temperature and other complexities of the subtransition make it difficult to study by DSC, but fluorescence spectroscopy provides a new and useful method to characterize the subgel.

There are structural changes accompanying the subtransition which may help explain the response seen with the DPH intensity. The subgel phase is a highly ordered, condensed phase with fully extended hydrocarbon chains, with the long axes tilted to the bilayer normal and packed closer together in comparison with the L_{β}' phase [31–33]. This may force the DPH molecule to move closer to the interface so that its fluorescence intensity in the L_c phase is much lower than that in the L_{β}' phase. In the L_c phase, the phosphate head groups are relatively immobile [34] and the interfacial region of the bilayer is partially dehydrated [35]. Upon transition from the L_c phase to the L_{β}' phase, there is an increased mobility of the phosphate headgroup, a greater penetration of water into the interfacial region of the bilayer, and a corresponding migration of DPH molecules towards the non-polar region of the bilayer [34,36,37]. Therefore, the DPH fluorescence intensity increases with increasing temperature.

The fluorescence data showed that the subtransition temperature first increased by a small amount and then decreased with increasing alcohol concentrations for the DPPC/MeOH system. In the DPPC/EtOH system, the initial increase was not observable but a large decrease in the subtransition temperature was evident for samples with high EtOH concentrations (Fig. 6). This behavior was also reported by Slater and Huang [38] for the DPPC/ethanol system.

4.2. The presence of an extra peak on DSC cooling scans

An extra peak appeared on the DSC cooling scans for each system beyond the threshold alcohol concentration. The exact alcohol concentration at which the peaks appeared differed for each system, but it was always beyond the threshold concentration. We concluded that these peaks represented the presence of a mixed phase. It has been shown that the transition from the interdigitated phase to the liquid crystalline phase is not fully reversible [8]. Therefore, upon cooling it is difficult for the bilayer to enter the interdigitated phase. It is possible, however, that part of the bilayer undergoes a transition to the interdigitated phase, and then the lower temperature peak is due to the rest of the bilayer undergoing a similar transition. Another possibility is that the bilayer is following a pathway from the L_{α} to the P_{β}' to the $L_{\beta}I$ phase. Both pathways have been reported before with different systems.

Maruyama et al. [39] recently used high-pressure light transmission to observe a two-phase

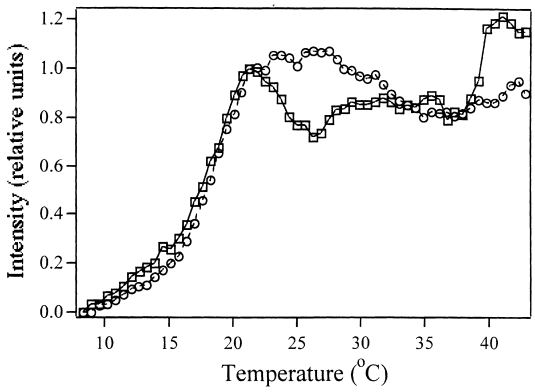

Fig. 5. DPPC/DPH/2% Chol/MeOH temperature scan (○, 1.5 M MeOH; □, 2.5 M MeOH).

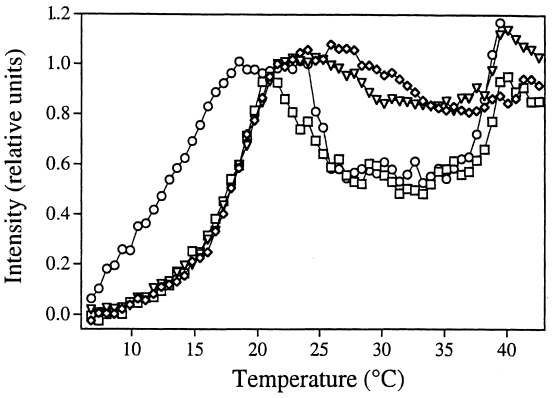

Fig. 6. DPPC/DPH/2% Chol/EtOH temperature scan (◇, 0.0 M EtOH; ∇, 0.7 M EtOH; □, 1.0 M EtOH; ○, 1.4 M EtOH).

coexistence with DPPC vesicles in the presence of the anesthetic tetracaine. They found coexisting phases of L_α with $L_\beta I$ and coexisting phases of L_β' and $L_\beta I$ phases. The $L_\beta I$ to P_β' to L_α pathway was observed with increasing temperature by an automated scanning density meter and differential scanning calorimeter [40]. Without further experimentation we cannot determine exactly which phase transition corresponds to the extra peak observed on the DSC cooling scan. In either case, our data do support the idea that the extra peak observed is due to a transition into the interdigitated phase since the enthalpy of that peak increases with increasing alcohol concentration.

4.3. The effect of cholesterol

When comparing the effects of methanol on the DSC data with and without cholesterol, the major difference is that the pretransition disappears at a much lower concentration and the main transition cooling scan shoulder peaks do not appear until a higher alcohol concentration in the presence of cholesterol. Upon the addition of cholesterol, though, the disappearance of the pretransition is no longer necessarily indicative of interdigitation due to the fact that typically, phospholipids only undergo a pretransition in its pure form. The shoulder peaks on the cooling scans appeared at 3.9 M MeOH with cholesterol and 3.2 M MeOH without cholesterol. This would suggest that in the presence of cholesterol, the mixed phase was not as prominent until the MeOH concentration reached 3.9 M.

Similar behavior is seen with the ethanol/cholesterol samples. The typical cooling scan shoulder peaks also appear with the ethanol/cholesterol samples at higher alcohol concentrations than they did in the absence of cholesterol. The threshold concentration is raised from 1 M to 1.4–1.5 M ethanol in the presence of cholesterol. These DSC data again suggest that the mixed phase is not present until higher ethanol concentrations are present.

The DPPC/alcohol/cholesterol samples differed significantly from the DPPC/alcohol samples due to the presence of shoulder peaks on the heating scans of the cholesterol samples. It is possible that the presence of these shoulder peaks is indicative of having two different regions in the bilayer. It is also shown that both cholesterol-poor and cholesterol-rich domains exist in bilayer systems [27,41–43]. It is likely that our DPPC/DPH/2%chol samples also have a cholesterol-rich domain, and a cholesterol-poor domain. These domains would interact differently with the alcohol thereby resulting in two transition temperatures. However, it is also possible that there is a mixed gel and liquid-crystalline phase due to the presence of the cholesterol domains. Vist et al. [44] identified three mixed phase regions in DPPC/cholesterol mixtures, one of which was an L_α/gel phase coexistence in the presence of 0–6 mol% cholesterol. This domain was found to lie right below the main transition temperature. This would explain the appearance of a shoulder peak on our DSC thermogram. Since our cholesterol concentration is so low, the extra peak only appears on occasional DSC runs. Interestingly, these shoulder peaks were more prominent with the ethanol samples than the methanol samples. The fluorescence data also showed that the ethanol samples were slightly more scattered than the methanol samples. This observation combined with the DSC data seems to suggest that 2 mol% cholesterol samples may interact differently with methanol and ethanol.

Generally, cholesterol is known to order cell membranes and ethanol is known to disorder cell membranes. Phospholipid desorption studies have shown that the presence of cholesterol did not significantly affect the phospholipid desorption, while ethanol did. From this study, it may be concluded that modifications to the lipid order by the cholesterol molecule do not have much impact on hydrogen bonding in the head region [45]. Cholesterol has also been shown to increase the orientational order of the L_α phase, but decrease the orientational order of the gel phase. The passive permeability of the membrane is similarly affected by cholesterol, but it is increased in the gel phase and decreased in the L_α phase [46,47]. If the permeability to ethanol in the gel phase is increased by the presence of cholesterol, then that would help explain why the fluorescence studies showed that a lower ethanol concentration was necessary to induce interdigitation in the presence of ethanol.

A few different groups have looked at the effect of ethanol in the presence of cholesterol, but conflicting results were obtained [47,48]. Chin and Goldstein studied the effect of ethanol at various depths in the membrane. They found that the effect of ethanol was greatest in the interior of cholesterol-poor membranes, but as the cholesterol concentration was increased, it dampened the effect of both ethanol and temperature. Interestingly, they also found that in the presence of low amounts of cholesterol, the 12-dioxy label showed diminished effects of ethanol. Barry and Gawrisch found similar results, except their NMR study showed that in low concentrations of cholesterol, the disordering effect of ethanol was enhanced at all positions along the acyl chain. They also showed that in DMPC bilayers with more than 30 mol% cholesterol, the effect of ethanol was curbed, but in the DMPC bilayers with less than 22 mol% cholesterol the effects of ethanol were enhanced [47].

In this study, DSC provides accurate measurements of phase boundaries and is particularly useful for the detection of mixed phases. Fluorescence spectroscopy has proved to be a very useful method to identify the phase structure. The fact that mixed phase regions occur beyond the threshold concentration adds to the success of the fluorescent probe. The combination of these two methods therefore, is very effective in analyzing the thermotropic behavior of lipid bilayers. The addition of higher concentrations of cholesterol can also be observed by fluorescence without the difficulties associated with broad transitions observed by calorimetry.

5. Nomenclature:1(0)

DPP: 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine
DMPC: 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine
PC: phosphatidylcholine
PE: phosphatidylethanolamine
DPH: 1,6-diphenyl-1,3,5-hexatriene
Chol: cholesterol
MeOH: methanol
EtOH: ethanol
DSC: differential scanning calorimetry
L_c: subgel phase
L_β': planar gel phase
P_β': ripple gel phase
L_α': liquid crystalline phase
$L_\beta I$: interdigitated phase.

Acknowledgements

We thank Tara Wyman and Christopher Cybulski for obtaining preliminary DSC data, and Dr Nathan Brandstater and Shirleen M. Ho for help with the figures. This work was supported in part by grants from the National Science Foundation (CHE-9531339), the ACS Petroleum Research Fund (31300-B4), the Ford Foundation and the Howard Hughes Medical Institute through the Undergraduate Biological Sciences Education Program.

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