Molecular Crystals and Liquid Crystals Incorporating Nonlinear Optics
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/gmcl17

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Version of record first published: 04 Oct 2006.

To cite this article: W. Tamura-Lis , L J. Lis , S. Qadri & P. J. Quinn (1990): Ethanol-Phosphatidylcholine Interactions: A Real Time X-Ray Diffraction Study, Molecular Crystals and Liquid Crystals Incorporating Nonlinear Optics, 178:1, 79-88

To link to this article: http://dx.doi.org/10.1080/00268949008042710

Mol. Cryst. Liq. Cryst., 1990, Vol. 178, pp. 79–88
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Printed in the United States of America

Ethanol-Phosphatidylcholine Interactions: A Real Time X-Ray Diffraction Study

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Dipalmitoylphosphatidylcholine has been previously shown to have a biphasic response to the presence of ethanol-water solutions as a function of ethanol concentration (E.S. Rowe, *Biochim. Biophys. Acta* **22** (1985) 3299–3305). Subsequently, Simon and McIntosh (*Biochim. Biophys. Acta* **773** (1984), 169–172) showed that DPPC bilayers formed an interdigitated multilamellar phase when the ethanol concentration was greater than 50 mg ethanol/ml water. We have used scanning calorimetry and real time x-ray diffraction to examine DPPC bilayers hydrated with 10 and 150 mg ethanol per ml water. When the lower concentration ethanol solution was present, DPPC bilayers formed subgel, gel, rippled, and liquid crystalline bilayer phases as the sample temperature was increased continuously. However, when the higher ethanol solution was used, DPPC bilayers formed subgel, gel (interdigitated), and liquid crystalline bilayer phases when driven by a temperature scan. In both cases, the subgel phase was not directly reversible upon cooling. Arguments are presented for the interpretation that both sub-gel phases consist of bilayers with interdigitated acyl chains. The transitions between the subgel and gel phases in both sample systems, and the pre-transition for DPPC in 150 mg ethanol/ml of water was shown to be second order while others proceeded via first order or two state mechanisms.

INTRODUCTION

The interaction of alcohols with lipids and membranes has been extensively studied, first as a model for anaesthetic action and then in the quest to determine the influence of alcohol (as a drug) on these systems. The physical chemistry of alcohol/phospholipid/water systems has been shown to be complex and unique.[1–6] The effect of ethanol on dipalmitoylphosphatidylcholine, for example, produced

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interdigitated gel phases at high ethanol concentrations.[2] The subsequently observed transition sequence[6] indicated that the presence of the interdigitated gel phase (1) decreased and/or eliminated the DPPC pretransition, depending on the ethanol concentration, and (2) the DPPC main transition was not reversible at high ethanol concentrations.

For this report, we used calorimetric and x-ray diffraction techniques to examine the structures and transitions for DPPC in high and low ethanol concentrations in water. The concentrations chosen were previously shown to be above and below the limit required to induce the gel bilayer interdigitated phase in DPPC. Samples, however, were equilibrated in such a manner as to induce a subgel bilayer phase.[7] Our aim was to determine the parameters related to the $L_C \rightarrow L_\beta$ (inter.) transition, as well as the commonly studied L_β (inter.) $\rightarrow L_\alpha$ phase transition. Comparisons are made to other thermodynamic and x-ray diffraction studies of solvent induced interdigitated gel bilayer phases.[8-10]

MATERIALS AND METHODS

All phospholipids used in this study were obtained from Avanti Polar Lipids (Birmingham, AL) and used without further purification. Lipid dispersions were prepared by suspending the lipid in ethanol-water mixtures. Ethanol was reagent grade and water was distilled. All samples had 80 vol/wt% solution. The samples were heated above their transition temperatures for one hour and then cooled to room temperature. The resulting samples appeared to be a homogeneous lipid-water mixture with no evidence of residual lipid powder. All dispersions were then equilibrated at ~0°C for at least 3 days. Samples for calorimetric examination were hermetically sealed in aluminum pans.

Transition temperatures and enthalpies were measured by a Perkin-Elmer DSC-2C with Thermal Analysis Data Station. Enthalpy per unit area was calibrated using indium (99.999% pure). Heating and cooling rates were 2.5°C/min for samples and calibration.

X-ray diffraction patterns were obtained using the 0.150 nm x-radiation at station 7.2/3 of the synchrotron radiation source at the SERC Daresbury Laboratory.[12] A cylindrically bent single crystal of Ge[13] and a long float mirror were used for monochromatization and horizontal focusing, providing about 2×10^9 photons·s^{-1} down a 0.2 mm collimator at 2.0 GeV and 100 to 200 mA of electron beam current. A Keele flat plate camera was used with a sample path of 1 mm. Scattered x-rays were recorded on a linear detector constructed at the Daresbury Laboratory. The dead time between data acquisition frames was 50μs with a temporal resolution of 2s for each frame. X-ray scattering has been plotted as a function of reciprocal space ($s = 2\sin\theta/\lambda$) using Teflon (0.48 nm) as a calibration standard.[14] All mesophase and subcell spacings were calculated using Bragg's Law.[15]

Temperature scans were produced by water baths connected internally to the sample mount of the x-ray camera. The temperature of the sample was monitored internally using a thermocouple placed adjacent to the sample in the x-ray sample holder.

RESULTS AND DISCUSSION

It has been previously determined using a variety of physical techniques that DPPC in water undergoes three lamellar phase transitions between equilibrium phases: L_C to $L_{\beta'}$ at 294°K, $L_{\beta'}$ to $P_{\beta'}$ at 308°K, and $P_{\beta'}$ to L_α at 314°K. The L_C phase can be initially induced by careful thermodynamic treatment.[7] We have previously shown[7] that during a temperature scan the transition sequence becomes $L_C \rightarrow L_\beta \rightarrow P_\beta \rightarrow L_\alpha$. It required minutes for the acyl chains to form the tilted $L_{\beta'}$ configuration. The total or partial substitution of water by a variety of solvents has been shown to alter both the phases present and transition sequences for DPPC. One of the more interesting observations has been the biphasic nature of the transition sequence for DPPC dispersed in varying ethanol-water concentrations, as described by light scattering.[1,3,5,6] When less than 50 mg ethanol per ml of water was used, DPPC underwent the pre- and main phase transitions originally reported for DPPC in water, however, when a greater ethanol concentration was used, the pretransition vanished as the ethanol concentration increased and the main transition became hysteretic.[6] There is no information about the effect of ethanol concentration on the phases and phase sequence in DPPC when the system is treated in a manner which normally induces a crystalline state (subgel) bilayer as the initial state.

Calorimetric scans have been recorded for DPPC dispersed in solutions containing 10 mg ethanol per ml of water and 150 mg ethanol per ml of water. Figure 1 shows the scans obtained in each system during the initial heating from the L_C phase and the subsequent reheating scan. It it unambiguous that upon initial heating, DPPC dispersed in 10 mg ethanol/ml water underwent the three transitions usually observed by DPPC in water. The temperature at which the pre-transition ($L_\beta \rightarrow P_\beta$) occurred was lowered by the addition of this small amount of ethanol, and the pretransition peak was clearly broadened. The transition temperatures for the DPPC sub- and main transitions were approximately the same in water and 10 mg ethanol per ml water. Upon reheating, the pre- and main phase transitions were shown to be reproducible with the subtransition eliminated. For DPPC dispersed in a solution containing 150 mg ethanol per ml of water, a single reversible transition was observed on all subsequent heating and cooling cycles. However, the kinetics of this transition were not fully explored by allowing long periods of time to elapse between scans. We have previously shown[11] that DPPC in 1M KSCN also produced a single phase transition which was characterized as being due to a transition from an interdigitated gel bilayer state to the L_α phase. A similar conclusion can be drawn from these observations for DPPC dispersed in 150 mg ethanol per ml of water. However, unlike DPPC in 1 M KSCN which produced an endotherm with a significant shoulder region, the endotherms produced for DPPC dispersed in a high ethanol concentration are extremely sharp with a greater enthalpy than observed for DPPC dispersed in water. These observations are consistent with the interpretation that the main phase transition for DPPC in 150 mg ethanol/ml water is a highly cooperative process.

Real time x-ray diffraction measurements were made while the above systems were undergoing temperature scans of approximately 8°C/min (Figures 2–5). The

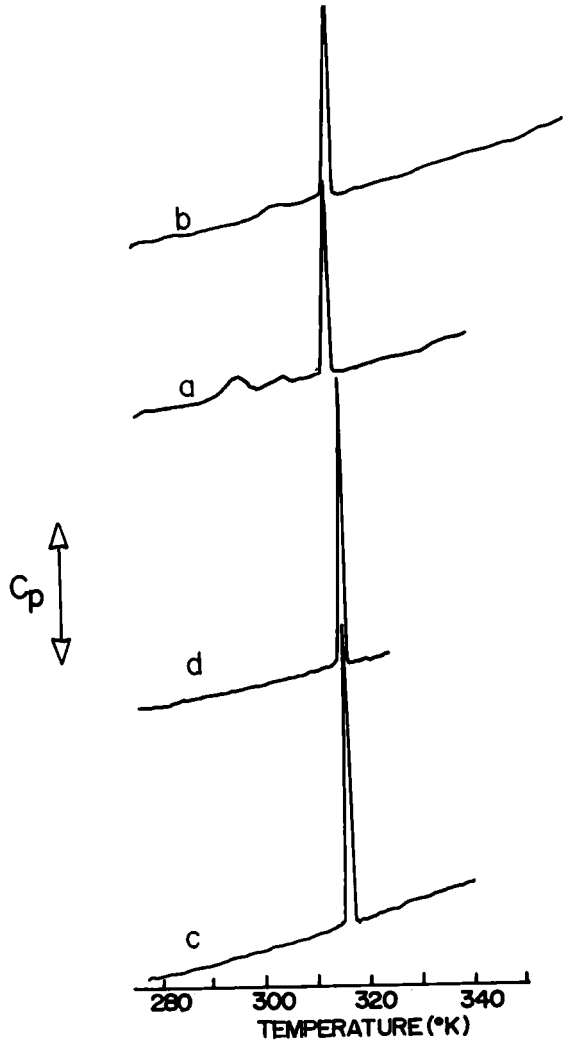

FIGURE 1. DSC scans for DPPC in 10mg EtOH/mℓ H_2O on a) initial heating, b) reheating and DPPC in 150mg EtOH/mℓ H_2O c) initial heating, and d) reheating. All samples were equilibrated at 0°C for at least one week. The scan rate was 2.5°C/min in all cases.

x-ray patterns produced by DPPC dispersed in 10 mg ethanol/ml H_2O are consistent with the phase sequence deduced from calorimetry, i.e., $L_C \rightarrow L_\beta \rightarrow P_\beta \rightarrow L_\alpha$ on initial heating. The measured mesophase and acyl chain repeat spacings for these phases are given in Table II. Although some differences in the DPPC bilayer structure exist with and without the presence of a low concentration of ethanol in the water phase, no significant differences in acyl chain packing as determined from the wide angle diffraction patterns were noted. The mesophase spacings for the fully hydrated subgel, gel and liquid crystalline phases of DPPC in water[7,11,18,19] have been previously shown to be greater than or equal to 60Å. Our observations of smaller d-spacings for the L_β and P_β phases when 10 mg ethanol/ml H_2O was

TABLE I

Thermodynamic Parameters for DPPC in H_2O, 10 mg EtOH/ml H_2O or 150 mg EtOH/ml H_2O

Solvent	T_1	ΔH_1	T_2	ΔH_2	T_3	ΔH_3
H_2O	294.0	2.53	308.3 308.6	0.98 0.67	314.3 314.2	7.26 4.28
10 mg Ethanol per ml H_2O	293.6	3.36	302.7 302.3	0.66 0.69	312.8 313.3	8.20 8.07
150 mg Ethanol per ml H_2O					315.4 315.4	10.49 10.51

Note: The first set of thermodynamic data is from the first heating of the sample after it is equilibrated at ~0°C for more than five days. The second set of data is obtained on the second heating obtained after the sample is cooled at 2.5°/min from above the main phase transition.

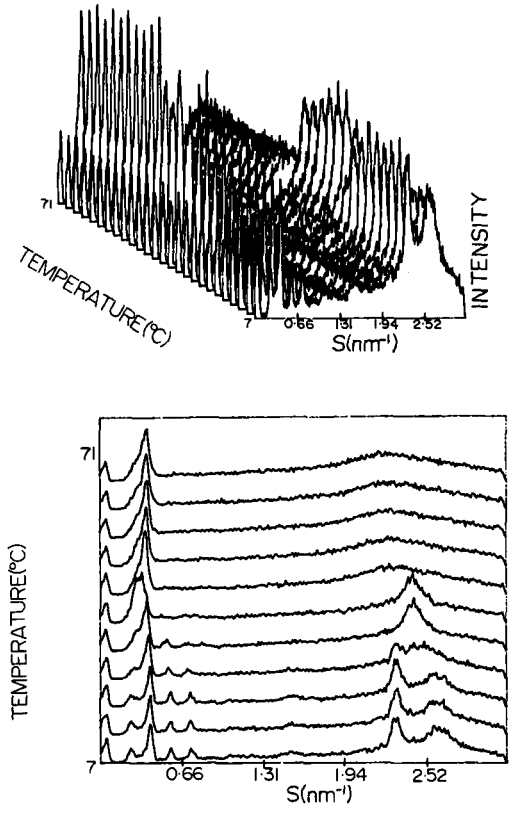

FIGURE 2. X-ray diffraction patterns collected for DPPC in 10mg EtOH per mℓ H_2O undergoing a temperature scan of ~7.5°C/min on initial heating after equilibration at ~0°C for three days. Every tenth frame of two second duration in a data set of 255 frames is shown. *Inset:* Every twenty-fifth frame of the data set is plotted as a function of inverse repeat spacing.

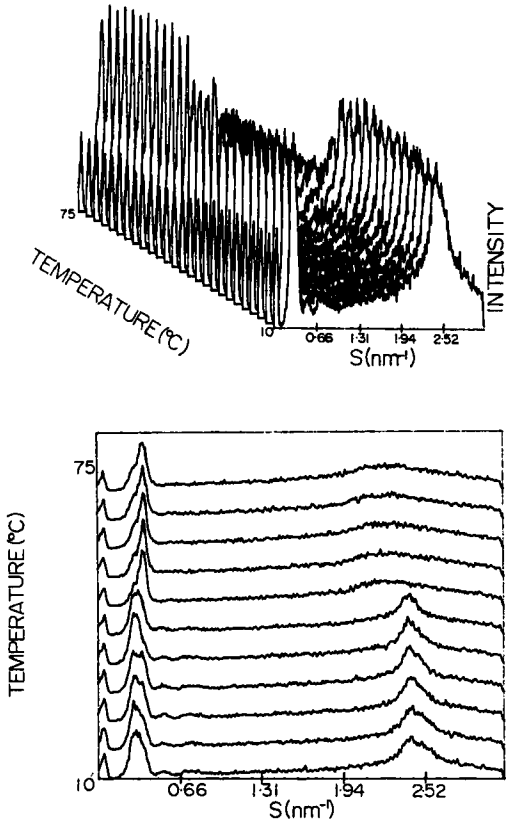

FIGURE 3. X-ray diffraction patterns collected for the sample described in Fig. 2 for a temperature scan of ~8.1°C/min on reheating. Every tenth frame of two second duration in a data set of 255 frames is shown. *Inset:* Every twenty-fifth frame of the data set is plotted as a function of inverse repeat spacing.

present may be due to the high scan rate used in our study. The mesophase spacing for the L_c phase which is also smaller than that observed for DPPC in water (60Å), however, was based on three diffraction orders which insures its reliability. It is interesting to speculate on the nature of the L_c phase for DPPC in low ethanol concentration aqueous solutions. Possible causes of the dramatic decrease in d-spacing for the DPPC L_c phase when low concentrations of ethanol are mixed with water are a limit in the concentration of solvent needed to fully hydrate the phase or the presence of a smaller bilayer thickness. It is unlikely that the solvent concentration was restricted since greater d-spacings are observed for the gel and liquid crystalline bilayer phases. It is more likely that the bilayer thickness has decreased with one possible source of this decrease being the formation of an interdigitated acyl chain bilayer phase. This is clearly an inference since there was insufficient data to obtain an electron density profile of the bilayer, and a gravimetric study of this system was not done. Upon reheating, the transition sequence $L_\beta \rightarrow P_\beta \rightarrow L_\alpha$ was observed (d-spacings are the same as in Table II). The transition between

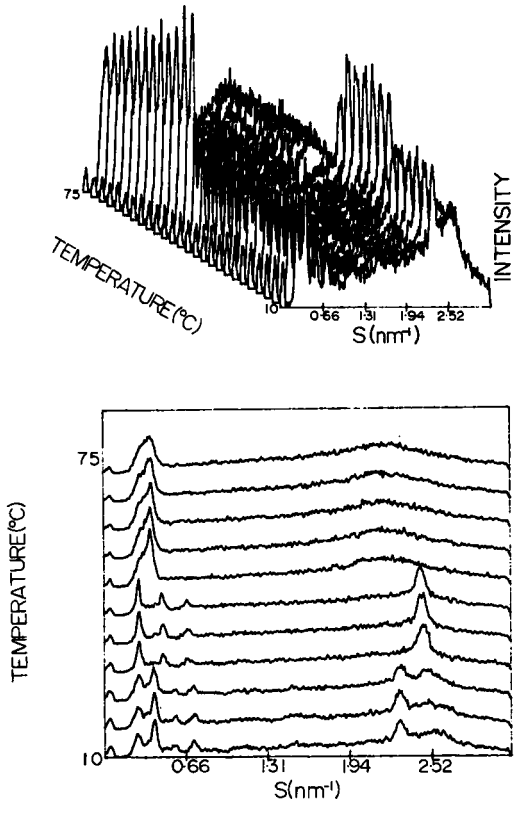

FIGURE 4. X-ray diffraction patterns collected for DPPC in 150mg EtOH/mℓ H_2O undergoing a temperature scan of ~8.0°C/min during an initial heating after the sample has been equilibrated at ~0°C for over three days. Every tenth frame of two second duration of a total data set of 255 frames is shown. *Inset:* Every twenty-fifth frame of the data set is plotted against inverse repeat spacing.

the subgel and gel phases proceeds via a second order thermodynamic process involving transitions between intermediate states in a manner similar to that previously reported for DPPC in water.[7] The transitions between the other phases proceed via first order or two state coexistence processes as was also previously reported for DPPC in water.[16] The primary diffraction markers are the small angle scattering region for the $L_\beta \rightarrow P_\beta$ transition and the wide angle scattering region for the other observed transitions.

The x-ray patterns produced by DPPC dispersed in 150 mg ethanol per ml of water suggest a different phase sequence than that obtained calorimetrically. A subgel phase was induced in this system which when heated was transformed first to an interdigitated bilayer phase and then to a liquid crystalline bilayer phase as the temperature was raised. The mesophase and acyl chain repeat spacings for this system are also listed in Table II. The d-spacings for the sub-gel and gel state bilayers were derived from at least three diffraction orders. The sub-gel state bilayer d-spacing is again lower than that reported for DPPC in water, while that for the

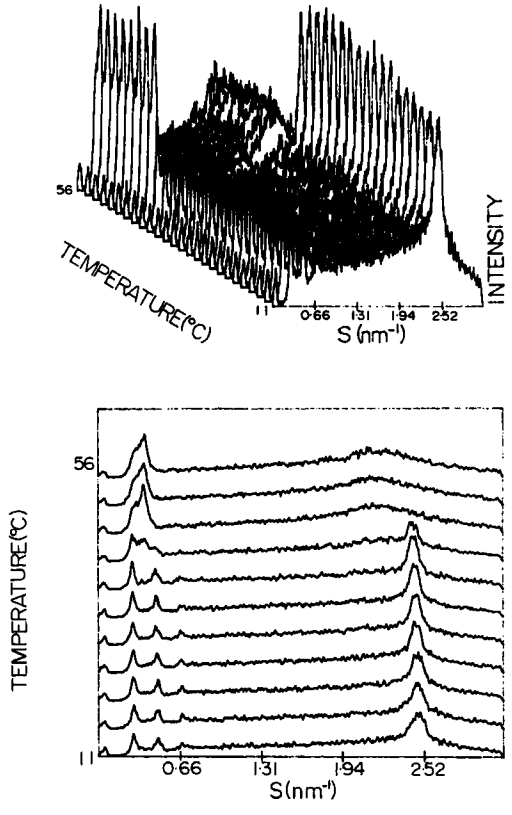

FIGURE 5. X-ray diffraction patterns collected for the sample described in Fig. 4 for a temperature scan of ~5.9°C/min on reheating. Every tenth frame of two second duration in a data set of 255 frames is shown. *Inset:* Every twenty-fifth frame of the data set is plotted as a function of inverse repeat spacing.

TABLE II

Structural Parameters of Phases Observed for Fully Hydrated DPPC in 10 or 150 mg EtOH per ml H_2O

Mg EtOH/ml H_2O	Phase	Repeat Spacing(s)	
		Mesophase (Å)	Acyl Chain (Å)
10	L_C (inter.)	50.0	3.84, 4.32
	L_β	57.2	4.12
	P_β	59.0	4.12
	L_α	63.6	4.47
150	L_C (inter.)	50.0	3.94, 4.34
	L_β (inter.)	50.8	4.04
	L_α	62.4	4.59

interdigitated gel state bilayer is lower than that reported by Simon and McIntosh[2] for a similar system but close to that reported by Cunningham and Lis[11] for an interdigitated DPPC gel state bilayer phase induced by the presence of 1M KSCN. The bilayer repeat spacing for the L_α phase is close to that reported previously for DPPC in water.[18] Since the change from the subgel to gel bilayer was not recorded by low resolution calorimetry, it can be assumed that it was either a low enthalpy process and/or a broad transition. This transition was recently observed calorimetrically by Slater and Huang[17] who examined this system under different conditions using a high resolution microcalorimeter at a slower scan rate. The temperature width for this transition as determined by the x-ray diffraction data confirmed that this was a transition similar to that observed, for example, for DPPC in water.[7] One explanation for a small enthalpic requirement for this transition is that the chains in the crystalline (subgel) bilayer are interdigitated. Our x-ray diffraction data are consistent with this interpretation since a mesophase d-spacing smaller than that of the fully hydrated DPPC in water L_C phase was observed. The transition between the subgel and interdigitated gel states proceeded via a continuous change in the acyl chain packing as first observed for DPPC in water[7] (i.e. a second order thermodynamic process). The transition between the gel and liquid crystalline phases proceeded via a two state coexistence or first order process. There was also evidence for a pre-transition in our time resolved data which involved a continuous change in the acyl chain subcell dimension from 4.04 to 4.09Å before the L_α phase chain packing peak appeared. This continuous transition was clearly a broad but low enthalpic process due to its second order transition character. A similar mechanism was observed for the pre-transition of DPPC in 10mM $CaCl_2$ where a broad pretransition calorimetry peak was also recorded.[20] The L_β (inter.) $\rightarrow$ L_α phase transition for DPPC in 150 mg ethanol/ml water was observed on reheating.

CONCLUSION

Structural and thermodynamic parameters for phase transitions induced by temperature scans were obtained for DPPC in 10 mg ethanol/ml water, and 150 mg ethanol/ml water and compared to similar quantities obtained for DPPC dispersed in water. No real changes in thermodynamic parameters were observed for the pre- and main phase transitions observed for DPPC in water or 10 mg ethanol/ml water. However, a broadening of the DPPC pretransition peak with a lowering of the transition temperature was observed when 10 mg ethanol/ml water replaced water as the lipid solvent. Structural data support the interpretation that the phase transition sequence for DPPC in 10 mg ethanol/ml water with increasing temperature was L_C (interdigitated) $\rightarrow$ L_β $\rightarrow$ P_β $\rightarrow$ L_α as compared to the previously reported[7] $L_C \rightarrow L_\beta \rightarrow P_\beta \rightarrow L_\alpha$ sequence reported for DPPC in water. This is the first inference that an interdigitated crystalline bilayer phase may be induced for DPPC in 10 mg ethanol/ml water, and that it can transform into a non-interdigitated gel state bilayer phase.

When DPPC was dispersed in 150 mg ethanol/ml water, thermodynamic parameters were obtained for only a single transition with a transition temperature approximately that of the main transition for DPPC in water but with a greater enthalpy. Structural parameters, however, can be interpreted to indicate the presence of the phase sequence: L_C (interdigitated) $\rightarrow$ L_β (interdigitated) $\rightarrow$ L_α for DPPC in 150 mg ethanol/ml water undergoing a temperature scan. This is the first inference of the formation of an interdigitated crystalline bilayer phase for DPPC in 150 mg ethanol/ml water, with a subsequent transition into an interdigitated gel-state bilayer phase. In addition, a "pre-transitional" second order continuous expansion of the acyl chain packing was observed for this system between the L_β (interdigitated) and L_α phases.

It can be concluded that the presence of even small amounts of alcohol is sufficient to cause a change in the acyl chain packing of the DPPC crystalline bilayer phase. Larger quantities of alcohol are needed to induce the presence of an interdigitated gel-state bilayer phase for DPPC, and to change the transition mechanism leading to the formation of the L_α phase from the two-state or first order process observed for DPPC in water to a second order mechanism involving the formation of intermediate states between the gel-state and the disordered acyl chains.

Acknowledgments

We would like to thank Ira Levin for initially suggesting that we apply these techniques to these systems and for his encouragement throughout.

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