Enigmatic Thermotropic Phase Behavior of Highly Asymmetric Mixed-Chain Phosphatidylcholines That Form Mixed-Interdigitated Gel Phases

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ABSTRACT Twelve saturated mixed-chain phosphatidylcholines have been identified for which the thermotropic phase behavior observed upon cooling from the L_α phase is dependent upon the thermal history of the sample in the gel phase. If fully hydrated samples of these lipids are cooled and soon thereafter examined by differential scanning calorimetry, one observes a single highly cooperative endotherm (the chain-melting phase transition) upon heating, and on subsequent cooling, a single exotherm that may occur at temperatures as much as 4–6°C below that of the single endotherm observed upon heating. In contrast, if the samples are incubated in the gel state at low temperatures for prolonged periods of time, one observes a single heating endotherm as before, but two sharp exotherms upon cooling. The latter transitions occur at temperatures close to that of the single endotherm observed upon heating and the single cooling exotherm observed prior to incubation in the gel state. The combined enthalpy of the two cooling exotherms is the same as that of the single heating endotherm or the single cooling exotherm initially observed. Infrared spectroscopic and X-ray diffraction studies indicate that the structural conversions characteristic of liquid-crystalline/gel phase transitions occur at both of those cooling exotherms. Of the 12 lipids that exhibit this unusual behavior, nine fulfill the previously defined structural requirements for the formation of the so-called mixed-interdigitated gel phase, and there is evidence in the literature that one of the three remaining lipids also forms such a structure. Infrared spectroscopic studies of the other two lipids indicate that their gel phases exhibit spectroscopic features that closely resemble those of lipids that meet the previously defined structural criteria for the formation of mixed-interdigitated gel phases and that differ markedly from those of both saturated symmetric-chain and saturated mixed-chain phosphatidylcholines that do not normally form mixed-interdigitated gel phases. Also, electron density reconstructions based on small-angle X-ray diffraction studies of the gel phases of those two lipids indicate that the thickness of their gel phase bilayers is consistent with their forming mixed-interdigitated gel phases. Thus the unusual thermotropic phase behavior described here may be a general characteristic of phosphatidylcholines that form mixed-interdigitated gel phases. This unusual behavior is not associated with any major change in any of several physical properties of these lipid bilayers but may arise from an alteration of the size and/or structure of microdomains present in the liquid-crystalline phase.

INTRODUCTION

Glycerolipids in which different fatty acyl chains are esterified to the *sn*1 and *sn*2 positions of the glycerol backbone (i.e., mixed-chain glycerolipids) are major constituents of most natural cell membranes. The most thoroughly studied of this class of mixed-chain glycerolipids are the *n*-saturated mixed-chain phospahtidylcholines (PCs), the behavior of which has been extensively studied as models of the more complex, naturally occurring, mixed-chain phospholipids (for reviews see Huang and Mason, 1986; Huang, 1990). To date, most of the studies of mixed-chain PCs have been primarily directed at a characterization of the way in which chain length asymmetry affects the thermotropic phase properties (particularly the T_m) of PC bilayers (see Huang, 1991, and others cited therein). Such studies have identified an interesting class PCs for which the effective length[1] of one of its acyl chains is almost twice that of the other (Hui et al., 1984; McIntosh et al., 1984; Xu and Huang, 1987; Xu et al., 1987; Mattai et al., 1987; Shah et al., 1990; Slater et al., 1992). Studies of fully hydrated bilayers of such lipids have shown that the thermodynamic aspects of their thermotropic phase behavior are discontinuous from trends extrapolated from studies of both symmetric-chain and other mixed-chain PCs (Huang and Mason, 1986; Huang, 1990, 1991; Marsh, 1992), observations that have been correlated with an unusual packing mode of these lipids when cooled to temperatures below T_m (Huang and Mason, 1986; Huang, 1990). This particular type of bilayer packing, the so-called mixed-interdigitated gel phase, has been studied by a wide range of physical techniques (Hui et al., 1984; McIntosh et al., 1984;

Received for publication 23 August 1993 and in final form 19 October 1993.

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Abbreviations used: DSC, differential scanning calorimetry; PC, phosphatidylcholine; Lyso PC, 1-*O*-acyl lysophosphatidylcholine; DPPC, dipalmitoyl phosphatidylcholine; FTIR, Fourier transform infrared; T_m, gel/liquid-crystalline phase transition temperature (°C); ΔC/CL, chain length inequivalence parameter defined by Huang (1990), where ΔC is the difference between the effective lengths of the *sn*1 and *sn*2 fatty acyl chains (defined as $|n1 - n2 + 1.5|$ where *n*1 and *n*2 are the number of carbon atoms in the *sn*1 and *sn*1 fatty acyl chains, respectively) and CL is the effective length of the longer of the two chains in C-C bonds.

0006-3495/94/01/207/10 $2.00

[1] Acyl chain length after correction for the conformational inequivalence between the *sn*1 and *sn*2 fatty acyl chains.

Huang and Mason, 1986; Mattai et al., 1987; Huang, 1990; Shah et al., 1990; Lewis and McElhaney, 1993; Zhu and Caffrey, 1993), and a fairly detailed structural picture has emerged (see Lewis and McElhaney, 1993, and references cited therein). In this form of lipid bilayer packing, the bilayer is organized as an assembly of dimeric repeat units. The two PC molecules forming the unit are arranged with the longer of their two acyl chains fully interdigitated across the entire bilayer and with the methyl termini of the shorter chains opposed across the center of the bilayer. Evidence has been recently been presented that the hydrocarbon chains of each repeat units are probably parallel to each other and that the repeat units are packed perpendicular to form a global motif of perpendicularly packed hydrocarbon chains (Lewis and McElhaney, 1993).

A picture of the structural requirements for the formation of the mixed-interdigitated gel phase has also emerged from the studies of various PCs that do or do not appear to form mixed-interdigitated gel phases (Huang, 1990). The current consensus is that the ideal structural requirement for forming such a phase is that the effective length of one acyl chain should be almost twice that of the other and that the propensity to form such phases decreases with structural deviations from this ideal (Huang, 1990). This has conveniently been expressed in terms of a chain equivalence parameter (ΔC/CL) as defined by Huang and co-workers (see Huang and Mason, 1986; Huang, 1990, and references cited therein). They proposed that the structural requirements for the formation of mixed-interdigitated gel phases are very stringent and that such phases can only be formed with lipids for which ΔC/CL falls within the range 0.44 to 0.57 (Huang, 1990). We report here that a number of highly asymmetric saturated mixed-chain PCs exhibit unusual thermotropic phase behavior. Specifically, the observed cooling behavior is determined by the thermal history of the sample in the gel phase prior to the conversion to the liquid-crystalline state. We also find that although most of the lipids exhibiting this enigmatic phase behavior meet the above structural requirements for forming a mixed-interdigitated gel phase, some of them do not. We present evidence that the particular lipids that exhibit this unusual behavior but do not meet the ΔC/CL criteria for forming a mixed-interdigitated gel phase do in fact form such structures. We also suggest that this unusual thermotropic phase behavior may be a general characteristic of PCs that form mixed-interdigitated gel phases.

MATERIALS AND METHODS

The mixed-chain PCs used in this study were synthesized by the acylation of appropriate lyso PCs using the required fatty acid anhydride and 4-pyrrolidino pyridine as a catalyst (Lewis and McElhaney, 1992) and subsequently purified as described previously (Lewis and McElhaney, 1985). DSC studies were performed with a Hart Scientific high-sensitivity scanning calorimeter and a Perkin Elmer DSC-2C low-sensitivity scanning calorimeter operating at scan rates of 10°C (Hart Scientific) and 18.75°C (Perkin Elmer) per hour. Sample preparation for DSC was as follows: 2–3 mg of dry lipid was hydrated by vigorously vortexing with excess water (50 μl for DSC-2C or 0.5 ml for Hart Scientific DSC) at temperatures well above the T_m of the given lipid. The samples were then sealed within appropriate containers (Hastelloy capsules for Hart Scientific, 75-μl stainless steel sample capsules for DSC-2C). With both instruments, data were acquired by means of dedicated microcomputer-controlled data acquisition systems. The data obtained was analyzed with software supplied by the respective instrument manufacturers and with other computer programs available in the laboratory. Samples were quantified using gas chromatography as previously described (Lewis and McElhaney, 1985). For the infrared spectroscopic experiments, 2–3 mg of the dried lipid sample was dispersed in 50 μl D_2O by vigorously vortexing at temperatures well above the T_m of the lipid. The dispersion was then squeezed between the BaF_2 windows of a heatable liquid cell (equipped with a teflon spacer) to form a 10-μm film. Once mounted in the sample holder of the instrument, the sample temperature could be controlled (between –20° and 90°C) by an external, computer-controlled circulating water bath. The infrared spectra were recorded with a Digilab FTS-40 infrared spectrometer using the acquisition parameters previously described by Mantsch et al. (1985). The spectra obtained were analyzed using software supplied by Digilab, Inc., and other computer programs obtained from the National Research Council of Canada. Small and wide-angle X-ray diffraction patterns were recorded with the Princeton SIV X-ray beam lines using the sample preparation and data acquisition methodologies described previously (Lewis et al., 1989). The X-ray data used for the reconstruction of electron density profiles were recorded and stored as two-dimensional image files and were azimuthally integrated to yield the scattered intensity as a function of scattering angle. The positions and intensities of the diffraction peaks were determined by fitting the observed peaks to Lorentzian peak shapes and, after application of a Lorentz correction, the corrected peak intensities were used in a Fourier reconstruction (see below).

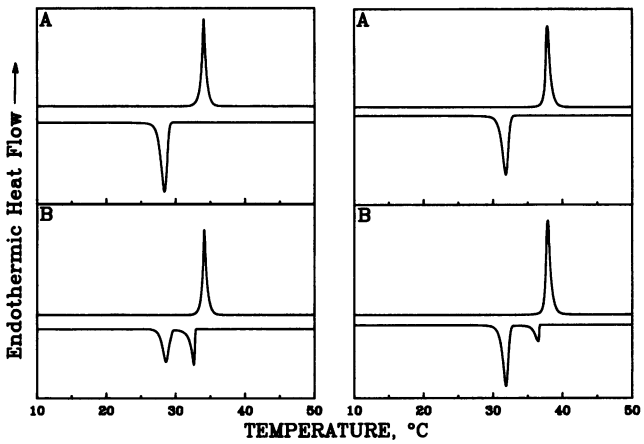

FIGURE 1 High sensitivity DSC thermograms of fully hydrated samples of 20:12 PC (*left*) and 10:22 PC (*right*). For each lipid the top panel (*A*) illustrates the heating (*upper curve*) and cooling (*lower curve*) behavior observed upon initial hydration of the dry lipids, and the bottom panel (*B*) illustrates the heating and cooling behavior observed after incubation of the samples at –20°C.

RESULTS

Fig. 1 shows the DSC heating and cooling thermograms exhibited by fully hydrated samples of 1-*O*-decanoyl, 2-*O*-docosanoyl PC (10:22 PC)[2] and 20:12 PC. These particular lipids have been studied previously (Xu et al., 1987; Lewis

[2] The shorthand notation used to describe these mixed-chain lipids consists of two numbers separated by a colon. The first number refers to the number of carbons in the *sn*1 fatty acyl chain and the second number refers to the number of carbons in the *sn*2 fatty acyl chain.

and McElhaney, 1993) and meet previously defined ΔC/CL structural criteria for the formation of mixed-interdigitated gel phases (Huang, 1990). As illustrated in Fig. 1, they exhibit single endothermic transitions upon heating irrespective of the thermal history of the sample. These transitions coincide with the conversion of their respective mixed-interdigitated gel phases to the liquid-crystalline forms of these lipids. However, when analyzed in the cooling mode, the observed behavior is clearly dependent upon the thermal history of the sample. For fully hydrated samples that have not been previously incubated at low temperatures, single cooling exotherms are observed with maxima some 4–6°C below those of the single endotherms observed in the heating mode. These single cooling exotherms are consistently observed with repeated scanning of the samples through their respective gel/liquid-crystalline phase transitions. Also, the difference between the heating and cooling peak maxima does not appear to be a simple kinetic artifact because conversion to the gel phases of these lipids does not occur when samples are cooled to temperatures between those of the heating and cooling peaks and held isothermally for extended periods. We find, however, that the cooling behavior of these lipids is irreversibly changed after prolonged low-temperature incubation (i.e., overnight at −20°C or several weeks at 0–4°C). As illustrated in Fig. 1, samples that have been incubated at low temperatures still exhibit single heating endotherms, the properties of which are virtually identical to those initially observed. However, in the cooling mode, two discrete cooling exotherms are observed. One of these peaks is observed at temperatures similar to that of the single cooling exotherm that is observed before low-temperature incubation, and the other occurs at temperatures slightly below that of the single endothermic peak observed upon heating. We also find that once the sample has been incubated at low temperatures, the two cooling exothermic peaks persist even if the sample is incubated in the liquid-crystalline phase (T_m + 10°C) for up to 2 days. Despite the difference in behavior, however, the total enthalpy measured (i.e., the sum of both cooling exothermic transitions) is essentially the same as that of the single heating endotherm or the single cooling exotherm initially observed (see Table 1).

The above observations are atypical of the behavior of saturated symmetric-chain PCs (see Lewis et al., 1987, and references cited therein) and indeed, of most of the other mixed-chain PCs that have been studied so far (see Serralach et al., 1984; Huang and Mason, 1986; Mattai et al., 1987; Lin et al., 1990; Shah et al., 1990; Blutmann et al., 1991, and references cited therein). This observation prompted further studies aimed at a determination of the structural requirements for such behavior. Our studies of the series of saturated, even-numbered mixed-chain PCs with acyl chains ranging from 10 to 22 carbon atoms (unpublished data) have identified 12 examples of lipids exhibiting such behavior. A list of these lipids and their thermodynamic properties is presented in Table 1. The lipids that exhibit this unusual behavior are of highly asymmetric chain lengths with ΔC/CL values ranging from 0.42 to 0.64. Huang and co-workers have recently postulated that the structural requirements for the formation of mixed-interdigitated gel phases are very stringent and that PCs will spontaneously form such structures only if their the chain length inequivalence parameters (ΔC/CL) range between 0.44 and 0.57 (Huang, 1990). An examination of the ΔC/CL values of the lipids listed in Table 1 shows that all of the PCs listed therein meet the ΔC/CL structural criteria for forming a mixed-interdigitated gel phase with the exception of 10:18 PC, 20:10 PC, and 22:10 PC. Some of the lipids listed in Table 1 have been studied elsewhere and exhibit properties consistent with their forming a mixed-interdigitated gel phase at temperatures well below T_m (Hui et al., 1984; McIntosh et al., 1984; Mattai et al., 1987; Xu and Huang, 1987; Xu et al., 1987; Huang, 1990; Lin et al., 1990; Shah et al., 1990; Lewis and McElhaney, 1993; Zhu and Caffrey, 1993). Also, one of the samples (10:18 PC, ΔC/CL = 0.419) that does not meet structural criteria proposed by Huang et al. appears to form a mixed-interdigitated gel phase (Mattai et al., 1987; Shah et al., 1990). However, the mixed-interdigitated gel phase of 10:18 PC may be thermodynamically unstable with respect to a quasi-crystalline phase, to which it converts upon prolonged incubation at low temperatures. Thus, 20:10 PC and 22:10 PC (ΔC/CL = 0.605 and 0.643, respectively) are the only lipids listed in Table 1 that do not meet the ΔC/CL criteria for the spontaneous formation of a mixed-interdigitated gel phase and for which there is no literature evidence for their forming such structures. The fact that these PCs do exhibit the same unusual thermotropic properties as species of lipids that are either expected to form or have demonstrably formed a mixed-interdigitated gel phase raises the possibility that they may also form a mixed-interdigitated

TABLE 1 Thermodynamic properties of highly asymmetric mixed-chain phosphatidylcholines

		Heating		1st cooling		2nd cooling	
Sample	ΔC/CL	T_m	ΔH	T_m	ΔH	T_m	ΔH
10:18 PC	0.419	12.1	6.4	10.3	6.4	11.2	0.6
						10.4	5.8
10:20 PC	0.486	27.6	9.9	23.1	9.9	26.3	3.5
						23.4	6.4
10:22 PC	0.538	37.9	13.0	31.8	13.0	36.5	2.3
						31.8	10.7
12:22 PC	0.436	38.2	11.2	33.6	11.2	37.0	2.8
						33.6	8.3
16:10 PC	0.500	5.9	6.7	1.9	6.7	4.4	3.4
						1.9	3.3
18:10 PC	0.559	19.3	10.1	14.9	10.1	18.2	0.5
						14.9	9.6
18:12 PC	0.441	17.4	8.9	15.3	8.9	16.1	1.1
						15.3	7.8
20:10 PC	0.605	26.3	10.6	21.2	10.6	25.1	3.1
						21.3	7.5
20:12 PC	0.500	34.1	12.2	28.4	12.2	32.6	4.9
						28.6	7.3
22:10 PC	0.643	29.4	12.3	25.5	12.3	27.7	6.0
						25.6	6.3
22:12 PC	0.548	43.5	13.4	36.9	13.4	37.3	6.2
						42.7	7.2
22:14 PC	0.452	41.9	11.2	37.0	11.2	37.1	5.9
						40.6	5.3

gel phase when cooled to temperatures below T_m. This possibility, which is relevant to the validity of previously proposed structural requirements for forming the so-called mixed-interdigitated gel phase, was examined further by infrared spectroscopy and X-ray diffraction.

Fig. 2 shows the CH_2 deformation and C=O stretching regions of the infrared spectra of the gel phases of 22:10 PC (Fig. 2 *B*) and 20:10 PC (Fig. 2 *C*) at temperatures near −20°C. The corresponding regions of the infrared spectra of 20:12 PC (Fig. 2 *A*), 22:16 PC (Fig. 2 *D*), and DPPC (Fig. 2 *E*) are also shown to enable comparison of these spectra with those PCs known to form the mixed-interdigitated gel phase, with mixed-chain PCs that are not expected to form mixed-interdigitated gel phases, and with saturated symmetric-chain PCs, respectively. It is evident that at temperatures well below T_m the spectroscopic features of the gel phases of 20:10 PC and 22:10 PC are very similar to those exhibited by 20:12 PC (a PC known to form a mixed-interdigitated gel phase) but distinct from those exhibited by DPPC and 22:16 PC (lipids that do not normally form such structures). Specifically, the CH_2 scissoring bands of 20:10 PC, 22:10 PC, and 20:12 PC are strongly split into components that are of comparable integrated intensity and centered near 1466 and 1472 cm^{-1}. The emergence of such a strong splitting of the CH_2 scissoring band is indicative of a compact perpendicular packing of the hydrocarbon chains as in an orthorhombic⊥ type of hydrocarbon subcell (Snyder, 1961, 1979). In contrast, the observed splitting is considerably weaker for 22:16 PC and DPPC. Also, a marked asymmetry in the contours of the C=O stretching band of 20:10 PC, 22:10 PC, and 20:12 PC is observed, and this appears to be attributable to a relative increase in the intensity of the high-frequency components of the absorption band (see Fig. 2). These features are noticeably different from those of DPPC and 22:16 PC, for which a more symmetric C=O stretching band is observed. The infrared spectroscopic features that are exhibited by 20:12 PC, 22:10 PC, and 22:10 PC have recently been characterized using unlabeled, specifically ^{13}C=O-labeled, and specifically chain-perdeuterated analogues (Lewis and McElhaney, 1993). Such work indicates that the spectroscopic features shown in Fig. 2 *A* are consistent with the structure proposed for the mixed-interdigitated gel phase and can be used to construct a fairly detailed structural picture of this phase (for details see Lewis and McElhaney, 1993). The fact that 20:10 PC and 22:10 PC exhibit spectroscopic features similar to that exhibited by PCs that form mixed-interdigitated phases and also exhibit anomalous thermotropic behavior suggest that these lipids may also form mixed-interdigitated gel phases when cooled to temperatures well below T_m.

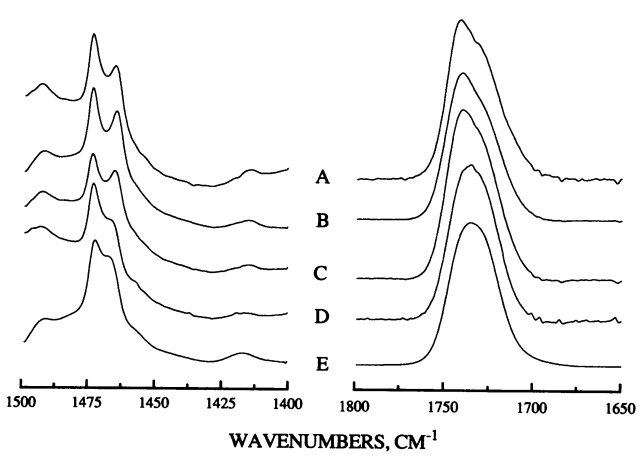

FIGURE 2 The CH_2 deformation (*left*) and C=O stretching (*right*) regions of the infrared spectra of gel phases of some asymmetric mixed-chain phosphatidylcholines. The absorbance spectra shown were acquired at −20°C for samples of: (*A*) 1-*O*-eicosanoyl, 2-*O*-dodecanoyl PC (20:12 PC); (*B*) 1-*O*-docosanoyl, 2-*O*-decanoyl PC (22:10 PC); (*C*) 1-*O*-eicosanoyl, 2-*O*-decanoyl PC (20:10 PC); (*D*) 1-*O*-docosanoyl, 2-*O*-hexadecanoyl PC (22:16 PC); (*E*) 1-*O*-hexadecanoyl, 2-*O*-hexadecanoyl PC (DPPC).

Further evidence that 20:10 PC and 22:10 PC form mixed-interdigitated gel phases when cooled to temperature well below T_m also emerges from an examination of the wide-angle region of the X-ray diffraction pattern. At temperatures just below T_m, these two lipids each exhibit a single, strong, wide-angle reflection near 4.1 Å. This reflection has been observed in all wide-angle X-ray diffraction studies of so-called mixed-interdigitated gel phases (Hui et al., 1984; McIntosh et al., 1984; Mattai et al., 1987; Shah et al., 1990; Zhu and Caffrey, 1993) and is attributed to hexagonal packing of the hydrocarbon chains (McIntosh et al., 1984) at temperatures near T_m. An interesting though peripheral aspect of our wide-angle X-ray diffraction measurements is the observation that at temperatures well below T_m, there is also the emergence of an additional reflection at 3.7–3.8 Å (data not shown). At temperatures near −30°C this reflection is observed near 3.7 Å and, as the temperature increases, its position progressively approaches 3.8 Å and eventually disappears at temperatures above 0°C. It has recently been predicted that all-*trans* polmethylene chains packed in an orthorhombic ⊥ subcell should exhibit reflections near 4.1 and 3.7 Å (Maulik et al., 1990) and that the position of the reflection near 3.7 Å should progressively approach that of the reflection near 4.1 Å as the orthorhombic ⊥ subcell is progressively distorted to form a hexagonally packed subcell. The possibility that in the mixed-interdigitated gel phases of these lipids, the hydrocarbon chains spontaneously assemble into compact orthorhombic ⊥ subcells when reorientational fluctuations are damped by either low temperatures or high pressures has been inferred from previous FTIR spectroscopic studies (Lewis and McElhaney, 1993; Wong and Huang, 1989). These X-ray diffraction measurements provide direct experimental evidence in support of such a conclusion.

To confirm that 20:12 and 22:12 PCs do form mixed-interdigitated gel phases, we also constructed one-dimensional electron density profiles from the small-angle X-ray diffraction measurements of these lipids. Four peak intensities were measured for these Fourier reconstructions of the electron density profiles, leading to 16 possible phase combinations that would yield eight unique electron density profiles. Of the various phase combinations, only two (−, −,

+, – and –, +, +, –) yielded electron density profiles consistent with that of a hydrated lipid bilayer. In the gel phase of a hydrated bilayer, one expects that thermally induced swelling of the layer of interlamellar water would result in temperature-dependent changes in the thickness of the regions of the electron density profile attributable to the water layer. This was only observed with the electron density profiles calculated using the phase combination –, –, +, –. This phase combination was determined by McIntosh et al. (1984) in their studies of 18:10 PC, in which case it was derived from the water-swelling method outlined by Worthington et al. (1973). Illustrated in Fig. 3 are the one-dimensional electron density profiles calculated for 20:10 PC and 22:10 PC at temperatures near –10°C. The observed pattern is similar to that observed in comparable studies of the mixed-interdigitated gel phases of other asymmetric-chain PCs (McIntosh et al., 1984; Mattai et al., 1987; Shah et al., 1990; Zhu and Caffrey, 1993). From the electron density profiles we estimate that the mean headgroup/headgroup separations (d_L) across the mixed-interdigitated bilayers of 20:10 PC and 22:10 PC are 32.8 Å and 34.9 Å, respectively. Given the acyl chain compositions of these particular PCs, it is evident that our estimates of d_L can only be accommodated within the context of a mixed-interdigitated bilayer or perhaps a so-called partially interdigitated bilayer in which the hydrocarbon chains are extraordinarily tilted. The latter possibility is effectively eliminated by the absence of the pattern of characteristic wide-angle reflections between 4.5 Å and 4.6 Å that are attributable to chain tilting (see Tardieu et al., 1973).

The spectroscopic and X-ray diffraction data presented above clearly indicate that 20:10 PC and 22:10 PC do form a mixed-interdigitated gel phase, despite the fact that they do not meet the ΔC/CL criteria for forming such a structure. Thus, all of the lipids that exhibit the unusual thermotropic phase behavior described above do form mixed-interdigitated gel phases when cooled to temperatures below their respective T_m's. This conclusion itself suggests that such unusual thermotropic phase behavior may be a property of PCs that can form mixed-interdigitated gel phases and prompted further FTIR spectroscopic and X-ray diffraction studies to probe the structural basis of the observed phenomenon. Fig. 4 shows a plot of the temperature dependence of the CH_2 symmetric stretching band of 20:12 PC as observed in the heating mode and in the cooling mode before and after the sample was incubated at low temperature. In the heating mode, the single endothermic transition observed by DSC coincides with a sharp increase (≅2.5 cm^{-1}; Fig. 3, *top*) in the frequency of the CH_2 symmetric stretching band and an increase in bandwidth (data not shown). Infrared spectroscopic changes of this type and magnitude have been observed at the chain-melting phase transitions of virtually all lipid bilayers (Mendelsohn and Mantsch, 1986; Mantsch and McElhaney, 1992) and are diagnostic of the increase in conformational disorder coincident with the melting of polymethylene chains (Snyder, 1967). Thus, as expected, these data indicate that chain melting is an integral part of the thermotropic processes that are occurring. In the cooling experiments, we find that prior to low-temperature incubation

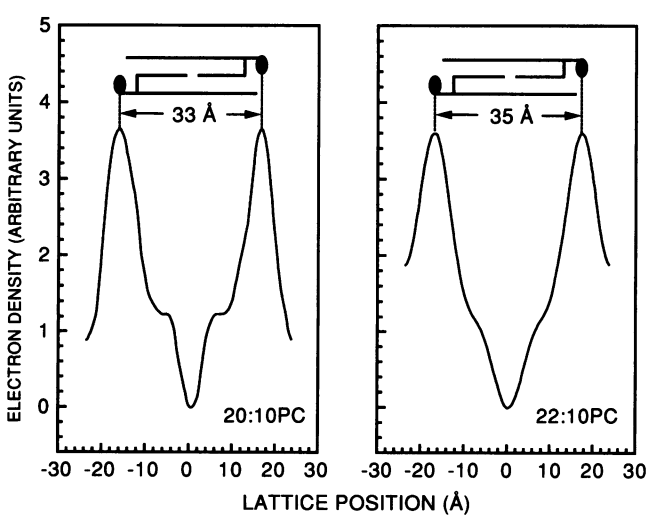

FIGURE 3 One-dimensional electron density profiles constructed from the small angle X-ray scattering patterns exhibited by the mixed-interdigitated gel phases of 20:10 PC and 22:10 PC in excess water.

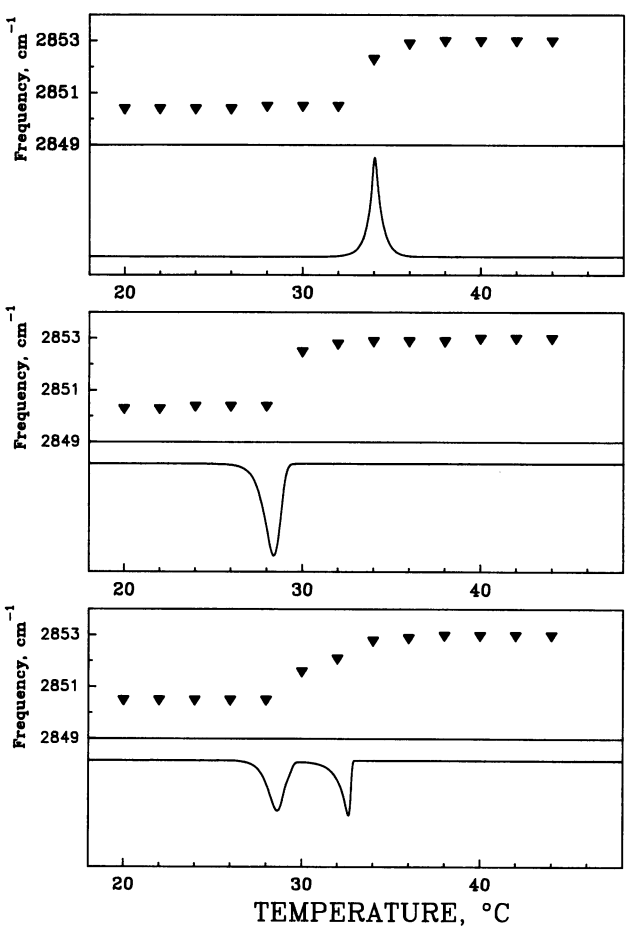

FIGURE 4 Temperature dependence of the CH_2 symmetric stretching bands of 20:12 PC. The corresponding DSC thermograms are also shown below the frequency plots. *Top:* heating mode experiment. *Middle:* cooling mode experiment prior to low-temperature incubation. *Bottom:* cooling mode experiment after low-temperature incubation.

of the samples, the FTIR spectroscopic changes observed upon heating are fully reversed at temperatures that coincide with the single cooling exotherm observed under such conditions (see Fig. 3, *middle*). However, after low-temperature incubation of these samples, the decrease in frequency diagnostic of the "freezing" of the polymethylene chains is spread out over a temperature range that covers the two exothermic transitions observed (see Fig. 3, *bottom*). Moreover, in our studies of the various lipids, we find that the fractional change in frequency observed at the completion temperature of the higher-temperature cooling exotherm correlates well with the fractional enthalpy change (relative to the combined enthalpy of the two cooling exotherms) at that temperature. This result clearly suggests that processes involving the "freezing" of the hydrocarbon chains are probably occurring during both of the cooling exothermic events that are observed.

Parallel small-angle X-ray diffraction experiments were performed with 10:20 PC. This particular lipid is well suited to this type of study because DSC studies indicate that there is baseline separation of the two cooling exothermic transitions that are observed after low-temperature incubation. Fig. 5 shows that the single heating endothermic transition centered at 27.6° (as observed by DSC) coincides with a sharp increase in the lamellar repeat spacing (≅53.5 Å-≅68.4 Å; see Fig. 4, *top*). In the initial cooling experiment (i.e., prior to low-temperature incubation), a comparable decrease in the lamellar repeat spacing coincides with the single exothermic phase transition centered near 23.1°C (see Fig. 5, *middle*). As was also observed with the DSC and FTIR spectroscopic studies, the temperature-dependent changes in the small-angle diffraction patterns observed after low-temperature incubation of the sample are consistent with the occurrence of a single, sharp phase transition near 26°C. Our X-ray diffraction experiments also showed evidence for a seemingly "irreversible" change in the cooling behavior of the samples after low-temperature incubation. The single diffraction pattern observed at temperatures above T_m (Fig. 6, *bottom*) indexes to a lamellar phase with a lamellar repeat near ≅68 Å and that observed at temperatures well below T_m (Fig. 6, *top*) indexes to a lamellar repeat near ≅54 Å. However, in marked contrast to the initial cooling experiment, there is a broad temperature range over which there appears to be a coexistence of two phases. As shown in Fig. 5 (*bottom*), there is evidence of phase coexistence beginning at the onset temperature of the higher-temperature cooling exotherm and ending at the completion temperature of the lower-temperature cooling exotherm. Moreover, throughout this entire temperature range the diffraction patterns observed are resolvable into patterns that are consistent with lamellar repeat spacings near 68 Å and 54 Å (see Fig. 5), even at temperatures at which DSC reports baseline separation of the two cooling exothermic peaks. Given that the lamellar repeat spacings resolved when there is baseline separation of the cooling exothermic peaks correspond to those of the liquid-crystalline and gel phases, respectively, the X-ray data are therefore consistent with the suggestion that the structural changes that occur at each of the two cooling exothermic events observed are similar to those that occur at the single cooling exotherm initially observed (i.e., both peaks correspond to liquid-crystalline to gel phase transitions).

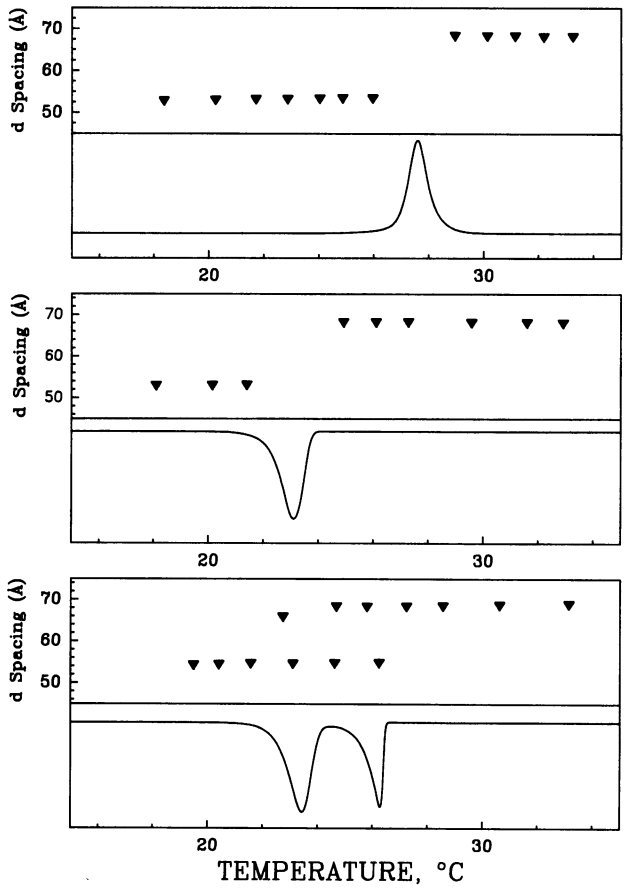

FIGURE 5 Temperature dependence of the d-spacings of dispersions of 10:20 PC in excess water. The corresponding DSC thermograms are also shown. *Top:* heating mode experiment. *Middle:* cooling mode experiment prior to low-temperature incubation. *Bottom:* cooling mode experiment after low-temperature incubation.

DISCUSSION

This study highlights some interesting aspects of the properties of asymmetric-chain PCs that form mixed-interdigitated gel phases. The main observation is that the heating endotherm, corresponding to the melting to the mixed-interdigitated gel phase to the liquid-crystalline state, is apparently insensitive to the thermal history of the sample, whereas the pattern of cooling exotherms observed is determined by the thermal history of the sample. In our studies of this phenomenon, we find that the changes in the cooling behavior of the sample upon low-temperature incubation are seemingly "irreversible" and are only restored after the lipids are extracted into an organic solvent, dried, and rehydrated. To date, we have examined the thermotropic phase behavior of 42 even-numbered mixed-chain PCs with varying degrees of chain asymmetry and with acyl chains ranging from 10 to

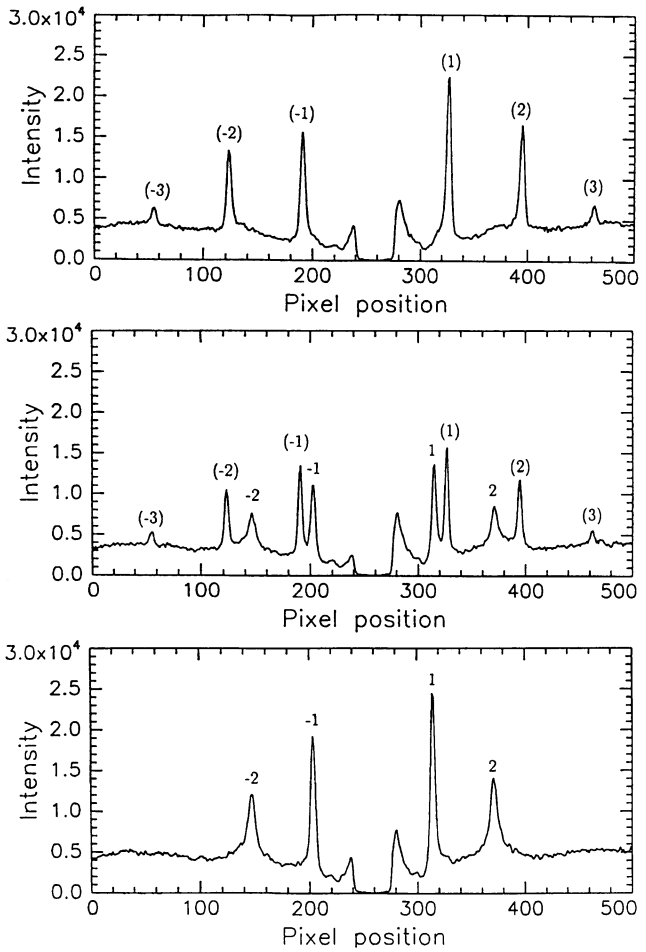

FIGURE 6 Azimuthally integrated X-ray powder diffraction patterns of 20:12 PC in excess water. The data shown were obtained in the cooling mode after incubation of the sample at low temperature. The bottom panel typifies data acquired at temperatures above the higher-temperature cooling exotherm, the middle panel typifies that data acquired at temperatures in between the two exothermic transitions, and the top panel typifies that data acquired at temperatures below that of the lower-temperature cooling exotherm.

22 carbon atoms (unpublished experiments from this laboratory). Of these lipids, the particular pattern of phase behavior reported here is only exhibited by the 12 lipids listed in Table 1. Ten of these exhibit properties consistent with the formation of such a mixed-interdigitated gel phase, have been proved to form such a structure, or meet the previously proposed structural criteria for the formation of mixed-interdigitated gel phases. However, our data clearly indicate that the two lipids that do not meet such criteria form mixed-interdigitated gel phases as well. Such behavior is not commonly observed in the overwhelming majority of symmetric-chain PCs that have been studied, nor is it typical of mixed-chain PCs that do not form mixed-interdigitated gel phases. This suggests that it may be a general property of lipids that form mixed-interdigitated gel phases. Although we are unaware of prior reports of the pattern of phase behavior reported here in previous studies of PCs that form mixed-interdigitated gel phases, it has been reported that the gel/liquid-crystalline phase transitions of these lipids appear as single endothermic transitions upon heating and as two exothermic transitions upon cooling (Mattai et al., 1987; Lin et al., 1990; Shah et al., 1990; Blutmann et al., 1991), with the distribution of the enthalpy change between the two cooling exotherms itself affected by the thermal history of the sample. We suspect that the particular pattern of phase behavior which we report here was probably missed in previous studies because the samples were subjected to various regimes of low-temperature incubation prior the study of their thermotropic phase properties (see Mattai et al., 1987; Lin et al., 1990; Shah et al., 1990; Blutmann et al., 1991).

At this time the molecular basis of the observed behavior remains unclear, as do the reasons why PCs that form mixed-interdigitated gel phases exhibit this behavior. Our infrared spectroscopic and X-ray diffraction data indicate that the two cooling exotherms observed with samples that have previously been incubated at low temperatures are each liquid-crystalline to gel phase transitions and are not attributable to the formation of some structural intermediate, as was previously speculated (Lin et al., 1990). Given our results, it seems unlikely that we were observing a system at thermodynamic equilibrium, and we suspect that a satisfactory explanation of this phenomenon may include the formation of one or more kinetically trapped, thermodynamically unstable states. Currently it is difficult to envisage any plausible mechanistic explanation of our observations without postulating that some property of the liquid-crystalline phase is altered when hydrated samples of these lipids are incubated at low temperatures. In these studies we do not find any evidence for significant thermodynamic, structural, or spectroscopic changes in the properties of these lipid bilayers that could explain the observed phenomenon. Also, in other ^2H nuclear magnetic resonance and FTIR spectroscopic studies of the liquid-crystalline phase of these PCs, we find no evidence that the thermal history of the sample causes any alteration in hydrocarbon chain order and dynamics or in conformer distribution (unpublished experiments from this laboratory). In the X-ray diffraction studies, however, it does appear that the lattice order of the sample increases after incubating the sample at low temperatures. Thus, although no discernible changes in the lamellar repeat spacings are observed, we find that the diffraction bands become sharper after low-temperature incubation of the samples. This observation suggests that our results may simply be due to an increase in the long-range order of the liquid-crystalline state after sample incubation at low temperatures and are not the result of any fundamental structural changes. Such a suggestion could explain why the two cooling exothermic transitions both appear to be liquid-crystalline/gel phase transitions. We speculate that the anomalous behavior reported here may be the result of changes in the distribution of domain sizes or degree of lamellar order when samples are incubated at low temperature. It is possible that these morphological features change markedly when the sample is incubated for prolonged periods at low temperatures. If such

is the case, then the higher-temperature and lower-temperature cooling exothermic transitions that are observed subsequently may be due to the differential freezing of domains in which the bilayers are in different local environments. One can also argue that the structure of the mixed-interdigitated phase is one that can promote a general change in domain morphology when samples are incubated for long periods at low temperatures. By definition, the two lipid monolayers forming mixed-interdigitated bilayers are strongly coupled to each other, and, as demonstrated recently, the mixed-interdigitated gel phase becomes progressively more compact as the temperature is lowered (Lewis and McElhaney, 1993). The latter process appears to be driven by the formation of an extended array of perpendicularly packed hydrocarbon chains with strong lateral interchain interactions. We suggest that the intrinsic stability of a large lipid assembly in which the monolayers are strongly coupled, and in which there are strong lateral interactions between the hydrocarbon chains, could promote changes in the domain morphology that may persist at higher temperatures. In principle, a systematic study of macroscopically oriented samples at various degrees of hydration could help to clarify these speculations about the possible effect of domain polydispersity on the thermotropic behavior of these lipids.

Our estimates of the thicknesses of the mixed-interdigitated gel phases of 20:10 PC and 22:10 PC, in combination with similar data obtained in studies of 18:10 PC (see Hui et al., 1984; McIntosh et al., 1984), provide some insight into the kinds of structural "adjustments" that presumably must occur with mixed-chain lipids for which the chain length inequivalence deviates from that required for the formation of an ideally packed, mixed-interdigitated PC bilayer. Listed in Table 2 are the experimentally determined thicknesses of the mixed-interdigitated gel phase bilayers of these three lipids along with their respective T_m's and ΔC/CL values, as well as our estimates of their hydrophobic thicknesses and the calculated hydrophobic lengths of their long chains and of their two end-opposed short chains. The effective hydrophobic lengths of their long chains and of their two end-opposed short chains were calculated by the formula described by Xu and Huang (1987), and the hydrophobic bilayer thicknesses listed was based on the assumption that two glycerophosphoryl moieties should account for approximately 13 Å of the measured bilayer thickness. The latter was deduced from previous studies of crystalline phospholipids (see Marsh, 1990, and references cited therein), from which we estimate a mean thickness of some 6–7 Å for each glycerophosphoryl moiety of a hydrated gel-state PC bilayer with untilted fatty acyl chains. The lipids 18:10 PC, 20:10 PC, and 22:10 PC form a series in which the length of the short *sn*2 acyl chains remains constant but ΔC/CL increases incrementally owing to increases in the length of the longer *sn*1 fatty acyl chains. The data show that for 18:10 PC, the effective lengths of the long chain and of the two end-opposed short chains are fairly well matched and that both values are in good agreement with our estimates of the actual hydrophobic thickness of that mixed-interdigitated gel phase bilayer. However, for 20:10 PC and 22:10 PC, the effective hydrophobic lengths of their long chains exceed those of their two end-opposed short chains, if close end-to-end packing of the latter is assumed. Moreover, the actual observed hydrophobic thicknesses of both 20:10 PC and 22:10 PC are significantly less than the effective lengths of their respective long chains, indicating that the hydrophobic termini of these chains must penetrate into the polar interfaces of their respective opposed monolayers. Also, the bilayer hydrophobic thickness of 20:10 PC is comparable to the effective hydrophobic length of its two end-opposed short chains if close end-to-end packing of the latter is assumed. With 22:10 PC, however, bilayer hydrophobic thickness significantly exceeds the close-packed effective length of its two end-opposed short fatty acyl chains, suggesting the formation of "voids" between the methyl termini of the end-opposed short fatty acyl chains. It thus appears that the structural adjustments necessary for the formation of mixed-interdigitated gel phase bilayers with lipids for which ΔC/CL deviates significantly from that required for the formation of ideally packed mixed-interdigitated bilayers (presumably a ΔC/CL value near 0.5) can involve penetration of hydrophobic termini into the polar interfaces of the bilayer and/or the formation of "voids" in the center of the bilayer. Clearly there is an energetic cost to such "adjustments" that would eventually define the outer limits of ΔC/CL values for which the formation of a mixed-interdigitated gel phase bilayer would be energetically feasible. Our studies suggest that a moderate penetration of the ends of the long chains into the polar interfaces of the opposing monolayer may be the less energetically costly of the two possibilities. A study of the bilayer thicknesses and one-dimensional electron density profiles of a series of lipids for which ΔC/CL is systematically varied, along with comparisons of Fourier transforms of strip models of the electron density profiles with the data so obtained, should be very useful in directly addressing this particular issue.

In conclusion, our demonstration that mixed-interdigitated gel phases can be formed by PCs that do not strictly conform

TABLE 2 Structural parameters of the mixed-interdigitated gel phases of fully hydrated phosphatidylcholines

Sample	T_m(°C)	ΔC/CL	d (Å)*	d_h (Å)‡	L_s (Å)§	L_1 (Å)¶
18:10 PC	19.3	0.559	33–34‖	20–21	20.3	20.6
20:10 PC	26.3	0.605	≅33	≅20	20.3	23.0
22:10 PC	29.4	0.643	≅35	≅22	20.3	25.5

* d, bilayer thickness as estimated by the distance between the maxima of the electron dense peaks in one-dimensional electron density profiles.

‡ d_h, bilayer hydrophobic thickness. Determinations were based on the assumption that two glycerophosphoryl moieties account for approximately 13Å of the measured bilayer thickness (see text).

§ L_s, calculated effective hydrophobic length of two ideally packed end-opposed short fatty acyl chains.

¶ L_1, calculated effective hydrophobic length of the long fatty acyl chain (see Xu and Huang, 1987).

‖ See McIntosh et al., 1984; Hui et al., 1984.

to previously proposed ΔC/CL criteria for the formation of such structures (see Huang, 1990) does bring into question the stringency of those criteria. It should be noted, however, that despite the intuitive logic of the arguments used to establish such criteria, they are nevertheless empirical and have been established from experimental observations of the relatively small number of PCs currently known to form mixed-interdigitated gel phases. Interestingly, the ΔC/CL values of two of the lipids that do not meet the criteria established both are greater than 0.6. This observation is significant because a survey of the literature indicates that very few of the saturated, mixed-chain PCs that have been studied so far have ΔC/CL values that are greater than 0.6. Given this, one can make the case that the currently available database is skewed toward the lower ranges of ΔC/CL and as a result there is probably insufficient data for one to empirically establish the upper limits of ΔC/CL that are compatible with the formation of mixed-interdigitated gel phases. Also, one should note that despite our evidence that mixed-interdigitated gel phases can be formed by PCs that do not conform to the ΔC/CL criteria, our data give no indication about the absolute or relative thermodynamic stabilities of those structures. This fact is significant because some of the PCs that form mixed-interdigitated gel phases will also form L_c phases with prolonged incubation at low temperatures. For example, our studies of 22:10 PC indicate that overnight incubation at temperatures well below 0°C and subsequent incubation at temperatures near 0–4°C results in the conversion of its mixed-interdigitated gel phase to a quasi-crystalline L_c phase. The formation of the latter results in the appearance of an additional thermotropic phase transition near 13°C and additional wide-angle reflections near 4.5 Å (data not presented). A similar conversion of a mixed-interdigitated gel phase to an L_c phase after low-temperature annealing was also observed in DSC and X-ray diffraction studies of 10:18 PC (Mattai et al., 1987). It is thus clear that some of the PCs listed in Table 1 form mixed-interdigitate gel phases that are thermodynamically unstable with respect to a quasi-crystalline L_c phase at most temperatures at which they are observed. Interestingly, however, the ΔC/CL values of those PCs are usually close to the outer limits of the range considered compatible with the formation of a mixed-interdigitated gel phase. Thus it is possible that the ΔC/CL criteria proposed by Huang and co-workers may be a better indicator of the structural requirements for the formation of thermodynamically stable mixed-interdigitated gel phases as opposed to being determinants of whether or not those phases can be formed. Our identification of an unusual and possibly distinctive characteristic of PCs that form mixed-interdigitated gel phases offers another means of quickly and nondestructively identifying those species for which currently established ΔC/CL criteria may not be very accurate. Such work could then serve as the starting point of more definitive structural studies.

This work was supported by operating and major equipment grants from the Medical Research Council of Canada (R. N. M.), major equipment grants from the Alberta Heritage Foundation for Medical Research (R. N. M.), NIH grant GM32614 (S. M. G.), and DOE grant (DE-FG0287ER60522) (S. M. G.).

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