Characterization of a Quasicrystalline Phase in Codispersions of Phosphatidylethanolamine and Glucocerebroside

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ABSTRACT Synchrotron x-ray diffraction, differential scanning calorimetry, and electron spin resonance spectroscopy have been employed to characterize a quasicrystalline phase formed in aqueous dispersions of binary mixtures of glucocerebroside and palmitoyloleoylphosphatidylethanolamine. Small- and wide-angle x-ray scattering intensity patterns were recorded during temperature scans between 20° and 90°C from mixtures of composition 2, 5, 10, 20, 30, and 40 mol glucocerebroside per 100 mol phospholipid. The quasicrystalline phase was characterized by a broad lamellar *d*-spacing of 6.06 nm at 40°C and a broad wide-angle x-ray scattering band centered at ~0.438 nm, close to the gel phase centered at ~0.425 nm and distinct from a broad peak centered at 0.457 nm observed for a liquid-crystal phase at 80°C. The quasicrystalline phase coexisted with gel and fluid phase of the pure phospholipid. An analysis of the small-angle x-ray scattering intensity profiles indicated a stoichiometry of one glucosphingolipid per two phospholipid molecules in the complex. Structural transitions monitored in cooling scans by synchrotron x-ray diffraction indicated that a cubic phase transforms initially into a lamellar gel. Thermal studies showed that the gel phase subsequently relaxes into the quasicrystalline phase in an exothermic transition. Electron spin resonance spectroscopy using spin labels located at positions 7, 12, and 16 carbons of phospholipid hydrocarbon chains indicated that order and motional constraints at the 7 and 12 positions were indistinguishable between gel and quasicrystalline phases but there was a significant decrease in order and increase in rate of motion at the 16 position on transformation to the quasicrystalline phase. The results are interpreted as an arrangement of polar groups of the complex in a crystalline array and a quasicrystalline packing of the hydrocarbon chains predicated by packing problems in the bilayer core requiring disordering of the highly asymmetric chains. The possible involvement of quasicrystalline phases in formation of membrane rafts is considered.

INTRODUCTION

Glycosphingolipids (GSLs) are important components of plasma membranes. There have been many reports implicating GSLs in a wide variety of biological processes such as cell growth, differentiation, development, aging, and apoptosis (Thompson and Tillack, 1985; Radin, 2001). Glycosphingolipids can act as antigenic determinants and mediators of immune responses as well as surface receptors for hormones, bacterial toxins, lectins, and other biomolecules (Thompson and Tillack, 1985; Ebara and Okahata, 1994; Berthelot et al., 1998; Marchell et al., 1998). In all of these processes, GSLs are involved in cell-cell and cell-ligand interactions and recognition. The glucose-containing GLS can accumulate in abnormally high amounts in membranes (Freire et al., 1980) where it can result in cell proliferation and growth (Radin, 2001). The association of glucocerebroside with lipid rafts has recently been reported in membranes of patients with lipid trafficking disorders (Harzer et al., 2003). The formation of ordered phases by the creation of complexes with membrane phospholipids may explain the physiological changes associated with high proportions of glucosphingolipids in membranes.

Many glycosphingolipids are confined to the outer leaflet of cell membranes where their particular functions are performed. Their presence in less conspicuous amounts on the inner leaflet, however, is also likely. There is convincing evidence, for example, that glucocerebroside, unlike galactocerebroside, is synthesized and located preferentially on the cytoplasmic surface of cell membranes (Coste et al., 1986). Of the aminophospholipids, phosphatidylethanolamines (PEs) are also known to be most abundant in the cytoplasmic leaflet of cell membranes (Fishman and Brady, 1976; Thompson and Tillack, 1985; Balasubramanian and Schroit, 2003; Daleke, 2003), although there is evidence that some PE is also present in the outer leaflet (Gurr and Harwood, 1991). Furthermore, certain biological processes might induce colocalization of both GSLs and PE on the same side of the membrane. Phosphatidylserine, like PE, is actively accumulated in the cytoplasmic leaflet by the action of aminophospholipid translocases and its presence in the outer leaflet triggers apoptosis.

The interactions between GSLs and phospholipids have been the subject of a number of investigations using a variety of biophysical techniques. Most of these have been devoted to studies of mixtures of GSLs and phosphatidylcholines (Bunow and Bunow, 1979; Correa-Freire et al., 1979; Bach et al., 1982; Ruocco et al., 1983; Maggio et al., 1985; Maulik and Shipley, 1996; Reed and Shipley, 1996; Hashizume

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et al., 1998). Few studies, however, have examined systems containing GSLs and PEs (Tsao et al., 1987; Perillo et al., 1994). In the study reported by Tsao et al. (1987), it was proposed that egg PE and ganglioside G_{D1a} formed a semifluid complex containing six PE molecules and one ganglioside molecule at temperatures above the gel-liquid crystalline phase transition as judged from calorimetric data. Nevertheless, no structural information was provided to characterize the semifluid complex.

Although neutral glycosphingolipids can be incorporated into lamellar phospholipid bilayers in relatively low proportions without disrupting the lamellar integrity, they have been found to phase-separate into enriched domains when present in higher proportions. One such domain can be isolated as detergent-resistant membranes prepared from intact cell membranes treated with nonionic detergents like Triton X-100 (Brown, 2002). These domains are believed to form lipid rafts that function in processes as diverse as protein sorting in secretory pathways and transmembrane signal transduction (Edidin, 1997; Simons and Ikonen, 1997; Rietveld and Simons, 1998). The structure of lipid rafts has been characterized as a liquid-ordered phase (L_o), a phase that is intermediate between gel and fluid phases. The hydrocarbon chains are said to be in an extended, ordered, and tightly packed arrangement as in gel phase, but with lateral mobility seen in liquid-disordered phase (Ipsen et al., 1987; Bloom et al., 1991; Almeida et al., 1992; Reinl et al., 1992; Ahmed et al., 1997; Brown and London, 1997, 1998; Brown, 2002).

GSLs are comprised of a preponderance of long, saturated fatty acyl chains, which pack tightly into gel or crystal phases. Interaction of GSLs and sphingomyelin with cholesterol is known to induce formation of L_o phases (Sankaram and Thompson, 1990a; Ahmed et al., 1997; Li et al., 2001). Cholesterol is believed to be an essential component in the creation of L_o phases and hitherto no L_o phases have been reported in lipid mixtures devoid of cholesterol (Fenske et al., 1994; Wang and Silvius, 2003). The enrichment of detergent-resistant membrane fractions in sphingomyelin and cholesterol represents the foundation upon which the raft hypothesis of protein segregation in membranes rests. Yet analyses of the lipid composition of detergent-resistant membrane fractions reveal that other phospholipids and glycolipids are prominent constituents.

This study was undertaken to examine whether condensed phases could be formed between lipid constituents of membrane rafts in the absence of cholesterol. Synchrotron x-ray diffraction, differential scanning calorimetry (DSC), and electron spin resonance (ESR) spectroscopic techniques have been used to characterize a lamellar quasicrystalline phase in binary mixtures of glucocerebroside and phosphatidylethanolamine. The phase is formed by a stoichiometric complex of two phospholipids per glucosphingolipid molecule and coexists with fluid bilayers of phospholipids at temperatures spanning the physiological range.

MATERIALS AND METHODS

Materials

Natural ceremide β-D glucoside (glucocerebroside; GlcCer) extracted from the spleen of patients with Gaucher's disease and 1-palmitoyl-2-oleoylphosphatidylethanolamine (POPE) were purchased from Matreya (Pleasant Gap, PA) and Sigma Chemical (St. Louis, MO), respectively. GlcCer was >98% pure as judged by thin layer chromatography and the mean molecular weight is 812 g/mol. Both GlcCer and POPE were used without further purification. Correa-Freire et al. (1979) reported that GlcCer extracted from the spleen of a patient with Gaucher's disease consisted mostly of a mixture of saturated, long acyl chains principally of lengths C_{22} and C_{24}. GlcCer dissolved in chloroform/methanol (2/1, v/v) and POPE dissolved in chloroform were mixed in the desired proportions and dried under a stream of oxygen-free dry N_2. Any remaining traces of solvent were removed by storage under vacuum for 16 h. The dry lipids were hydrated with buffer consisting of 10 mM Hepes buffer (pH 7.4), 0.1 mM $CaCl_2$, 0.1 mM $MnCl_2$, and 150 mM NaCl to give a dispersion of 25 wt% lipid. The hydrated lipid samples were thermally cycled several times between −20°C and 90°C and vortex mixed to ensure homogeneous dispersion. The samples were stored at −20°C and equilibrated at 4°C before transferring to the sample cells for x-ray diffraction experiments.

X-ray diffraction

Real-time synchrotron x-ray diffraction experiments were performed on Beamline 8.2 of the Daresbury Synchrotron Radiation Source (Warrington, UK). The real-time and simultaneous SAXS/WAXS measurement methods used to record diffraction intensity data during thermal scans have been described previously (Cunningham et al., 1994; Yu and Quinn, 2000). The SAXS quadrant detector was calibrated using wet rat tail collagen (67 nm; Bigi and Roveri, 1991) and the WAXS INEL detector was calibrated using high-density polyethylene (0.4166, 0.378 nm; Addink and Beintema, 1961). A camera length of 2.4 m allowed high resolution of SAXS reflections. Data analysis was performed using the OTOKO software program (Boulin et al, 1986).

Differential scanning calorimetry

Lipid mixtures containing the desired proportions of GlcCer and POPE were prepared in solvent and after removal of solvent the dry lipids were dispersed in buffer (75%, wt/wt) as in preparation of dispersions for x-ray diffraction experiments and transferred directly to aluminum sample pans. Enthalpy changes during heating and cooling scans at 2°/min between −5 and 90°C were recorded using a Mettler-Toledo DSC 821^e calorimeter (Columbus, OH).

Electron spin resonance spectroscopy

ESR spectra were recorded (continuous wave, X band, Bruker ER 200D spectrometer, Billerica, MA) and digitized with EPRWARE (Scientific Software Services, Plymouth, MI) from three spin-labeled phosphatidylcholine analogs, 1-palmitoyl-2-stearoyl-(n-DOXYL)-*sn*-glycero-3-phosphocholine (Avanti Polar Lipids, Alabaster, AL), where $n = 7$, 12, and 16 (referred to as 7-PC, 12-PC, and 16-PC hereafter). The spin label was added in a chloroform solution of the lipids to give a molar ratio of 0.25:100. The solvent was evaporated and remaining traces removed in vacuo for 48 h and 8.75 mg dry lipid dispersed in 500 μL buffer consisting of 10 mM HEPES (pH7.4), 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, and 150 mM NaCl. The suspension was sonicated for 1 h, centrifuged, and the pellet transferred into a flame-sealed capillary sample cell. The dispersions were stored at 4°C until examined; all spectra were recorded at 25°C. Simulation procedures were performed using an automated fitting of experimental data (Budil et al.,

1996; Chachaty and Soulie, 1995; Wolf and Chachaty, 2000) to estimate the molecular order parameter S_{mol} and the reorientation correlation time τ of the spin probes. S_{mol} denotes the mean orientation of the z axis of magnetic tensors, which is on the average parallel to the molecular axis of the spin probe, with respect to the director of the phase, perpendicular to the interface with water. τ corresponds to the mean lifetime of an orientation.

RESULTS

Synchrotron x-ray diffraction studies

To determine the effect of GlcCer on the thermotropic phase behavior and structure of POPE, mixed dispersions of the two lipids in molar ratios of 2:100, 5:100, 10:100, 20:100, 30:100, and 40:100 (GlcCer:POPE) were examined using real-time synchrotron x-ray diffraction methods. Small-angle (SAXS) and wide-angle x-ray scattering (WAXS) intensity profiles recorded from a mixed dispersion of GlcCer:POPE of 2:100 in molar ratio during an initial heating scan from 20° to 89°C is shown in Fig. 1. The initial phase is a lamellar-gel indexed by two orders of reflection with a d-spacing of 6.21 nm and a sharp wide-angle reflection at a spacing of ~0.43 nm. During the initial heating scan a transition to a lamellar liquid-crystal phase (L_α)is observed at ~25°C characterized by a sharp decrease of the repeat spacing from 6.11 nm to 5.46 nm. The change in lamellar d-spacing coincides with a disappearance of the sharp peak in the wide-angle region centered at ~0.43 nm ($S = 2.35\ nm^{-1}$) and its replacement by a broad band centered at ~0.46 nm ($S = 2.16\ nm^{-1}$). This signifies a transition in acyl chain packing from an ordered gel phase (L_β) to a disordered fluid state. The lamellar repeat spacing of the liquid-crystalline phase decreases progressively from 5.46 nm to 5.01 nm up to a temperature of ~65°C when reversed hexagonal phases (H_{II}) first appear. Two H_{II} phases can be distinguished throughout the remainder of the heating scan, both indexed by four orders of reflections with spacing ratios of $1:1/\sqrt{3}:1/\sqrt{4}:1/\sqrt{7}$ in the small-angle scattering region. The dominant H_{II} phase has a repeat spacing of 6.01 nm at 80°C and a minor H_{II} phase is seen as a shoulder on the high-angle side of the main H_{II} repeats. The most notable changes in structure and phase behavior of POPE due to the presence of ~2 mol GlcCer per 100 mol POPE, as judged from published data on the pure phospholipid (Katsaras et al., 1993; Lee et al., 1996; Yu and Quinn, 2000; Wang and Quinn, 2002), are a small decrease in d-spacing of the L_α phase and the presence of an additional H_{II} phase. The phase transitions observed during heating are completely reversible as seen in a subsequent cooling scan with a temperature hysteresis of 1–2°C (data not shown).

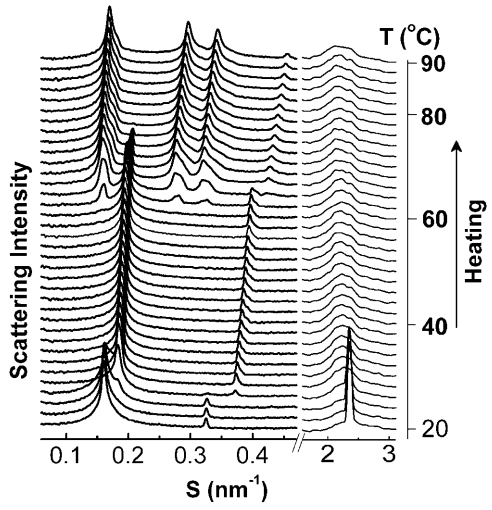

FIGURE 1 Small-angle (*left*) and wide-angle (*right*) x-ray scattering intensity patterns recorded from a GlcCer:POPE binary mixture (2:100, mol:mol) as a function of reciprocal spacing (S) recorded during an initial heating scan at 2°/min.

In dispersions containing increasing proportions of GlcCer, diffraction patterns in the SAXS region different from that of Fig. 1 are observed, suggesting that a GlcCer enriched phase separates from a phase that has features similar to POPE. This can be seen in Fig. 2 *A*, which shows a heating scan of a GlcCer:POPE codispersion (30:100, mol:mol). Sharp peaks in the SAXS and WAXS regions observed in the dispersion equilibrated at 12°C at the start of the heating scan are identical to those of a lamellar-gel phase of POPE. In the SAXS region this pattern is superimposed on two orders of a broad lamellar reflection, which is centered at approximately the same position as the d-spacings of the lamellar repeat of the POPE in gel phase. During the initial heating scan there is a gel to liquid-crystal phase transition of the assigned POPE domain at 25°C coinciding with a decrease in lamellar d-spacings from 6.13 nm to 5.39 nm and this is associated with disappearance of the sharp WAXS peak centered at ~0.43 nm. There is no change in the position or intensity of the broad SAXS peak, suggesting that this is a separate phase from that attributed to a POPE domain and that the hydrocarbon chains of this phase are not packed in a gel phase configuration. This phase is assigned as a GlcCer-enriched lamellar phase and it coexists with the L_α phase of POPE up to a temperature of ~70°C. Above this temperature multiple peaks begin to form from the GlcCer enriched phase and are indicative of a cubic phase (Q). The spacing of the cubic structure remains relatively constant until the lamellar liquid-crystal phase of POPE begins a transition into H_{II} phase at ~80°C. At this temperature the lattice constant of the cubic structure progressively decreases with increasing temperature. This suggests that the structure formed predominantly by the glycolipid is modified by the transfer of POPE molecules into the cubic phase resulting in a decrease in radii of curvature of the lipid-water interfaces present in this phase.

The phase transitions that take place during subsequent cooling reveal a different pathway with considerable hysteresis. This is illustrated by the diffraction patterns recorded during a cooling scan presented in Fig. 2 *B*. Cooling to temperatures below ~70°C converts the H_{II} phase to the L_α phase and this coexists with a cubic phase enriched in GlcCer. Cooling below 40°C results in a movement of POPE

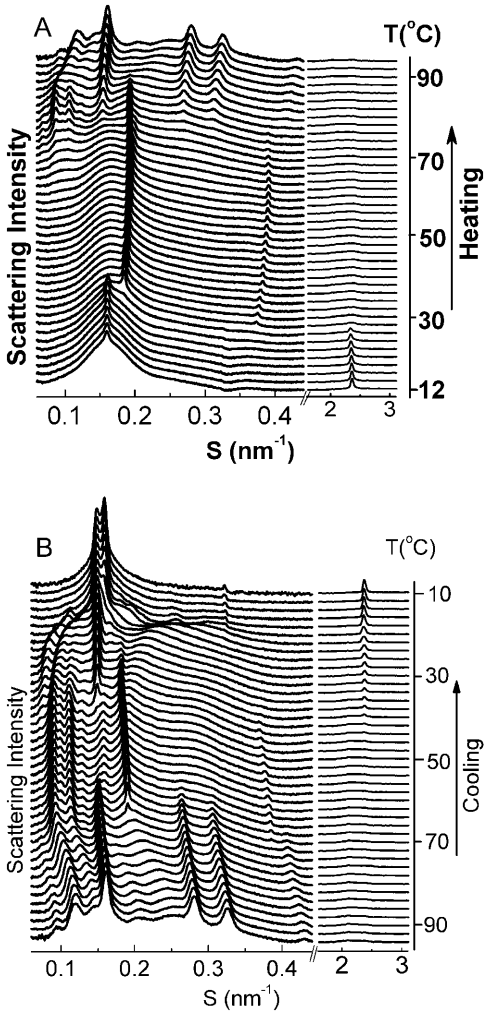

FIGURE 2 Small-angle and wide-angle x-ray scattering intensity patterns recorded from GlcCer:POPE binary mixture (30:100, mol:mol) recorded during initial heating (*A*) and subsequent cooling (*B*) scans at 2°/min as a function of reciprocal spacing.

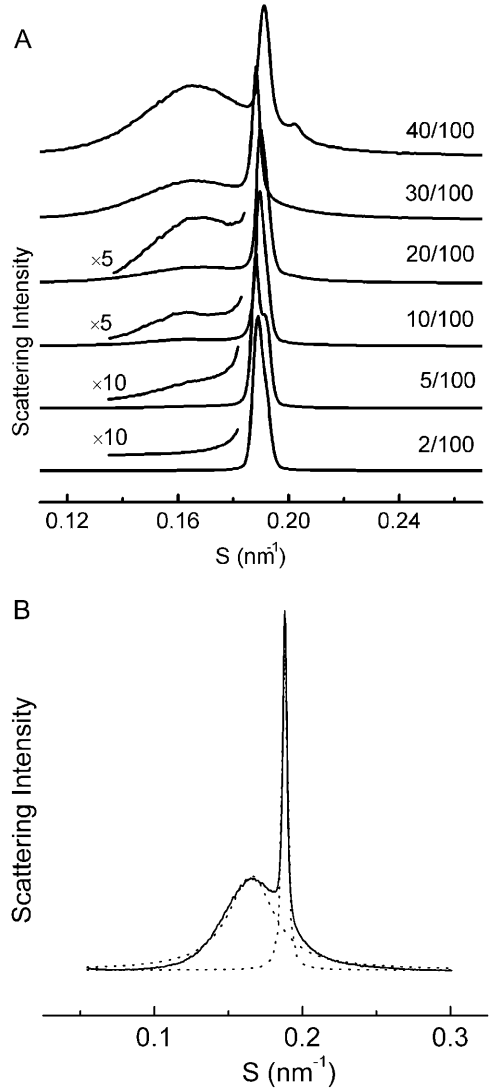

FIGURE 3 (*A*) Small-angle x-ray diffraction intensity plots of GlcCer:POPE binary mixtures at 40°C. Molar ratios of the lipids are indicated in the figure. The highest peaks were normalized to the same value for comparison. (*B*) Deconvoluted results of a mixture (30:100, mol:mol), showing two peaks centered at 6.06 and 5.24 nm, respectively.

from L_α phase into the Q phase with decreasing temperature, which is manifest as a decrease in lattice constant of the cubic phase as their radii of curvature decreases. At the same time an L_β phase of GlcCer:POPE appears (*d*-spacing 6.70 nm). When the sample is cooled below 25°C a new L_β phase (*d*-spacing 6.29 nm) forms from POPE, which is expelled from the Q phase of GlcCer:POPE. The removal of POPE from the Q phase results in conversion of the remaining Q phase into L_β of the appropriate stoichiometry.

Similar phase behavior was observed in heating and cooling scans of mixed dispersions of GlcCer:POPE at other molar ratios up to 40:100 (mol:mol). Representative SAXS intensity patterns recorded at 40°C during initial heating scans from binary mixtures of GlcCer:POPE at different molar ratios are shown in Fig. 3 *A*. The scattering intensities of the sharp peaks have been normalized for comparison. This shows that coexistence of L_α phase and the lamellar phase enriched in GlcCer is detectable when the content of the glycolipid is >5:100 (mol:mol) in the GlcCer:POPE codispersion. Furthermore, there is a direct relationship between the proportion of glycolipid in the mixture and the intensity of the broad SAXS diffraction band. To estimate the stoichiometry of the components of the GlcCer-enriched phase, deconvolution of the small-angle x-ray diffraction profiles was undertaken using the Microcal Origin program (v7.0) based on the Lorentzian model. The results of a fit to the SAXS intensity data of the mixture of GlcCer:POPE in 30:100 mol ratio is presented in Fig. 3 *B*. Two peaks centered at 6.06 and 5.24 nm, respectively, are clearly resolved. The sharp peak at 5.24 nm ($S = 0.191$ nm^{-1}) is the first-order diffraction of the L_α phase, assigned to POPE, whereas the broad peak with *d*-spacing of 6.06 nm ($S = 0.165$ nm^{-1}) can be attributed to the phase enriched in GlcCer. Deconvoluted

peak areas after baseline correction of the curves are summarized in Table 1. As the diffraction peaks occur at similar d-spacings it is reasonable to assume that the area of the scattering curves is directly related to the quantity of the respective lamellar phase in the mixture. If we further assume the two phases, GlcCer-enriched and L_α, possess fixed compositions with mol fractions of α and β, the number of total molecules in each phase can be described as:

$$n_1 = f_1 A_1, \quad n_2 = f_2 A_2, \tag{1}$$

where n_1 and n_2 are the mol of molecules in the GlcCer-enriched phase and the L_α phase; A_1 and A_2 are the integrated areas of the two phases; f_1 and f_2 are coefficients, respectively. The overall mol fraction of GlcCer in the mixed lipid preparation (x) is:

$$x = \frac{n_1\alpha + n_2\beta}{n_1 + n_2} = \frac{A_1 f_1 \alpha + A_2 f_2 \beta}{f_1 A_1 + f_2 A_2}. \tag{2}$$

By further defining $t = A_2/A_1$ as the relative scattering intensity and $f = f_2/f_1$, Eq. 2 can be rearranged into the following form:

$$x = f[t(\beta - x)] + \alpha. \tag{3}$$

The unknown parameters in Eq. 3 are f, α, and β. As phase separation is observed for the GlcCer:POPE binary mixture when the mol ratio is >5:100, it is reasonable to assume that the amount of GlcCer in the L_α phase is <5/105, i.e., $\beta <$ 0.048. This is also consistent with the fact that phase transitions of the low-content GlcCer mixtures are similar to that of pure POPE. By assigning a value between 0 and 0.04 to β, the remaining two parameters f and α in Eq. 3 can be obtained from least square linear regression when plotting x as a function of $t(\beta - x)$. Except for the mixture containing 5:100 GlcCer:POPE, where the broad peak was difficult to distinguish from the baseline, x and t values of 10:100, 20:100, 30:100, and 40:100 GlcCer:POPE mixtures were employed for calculation. The GlcCer-enriched phase composition α and other fitted parameters are summarized in Table 2. Similar values of α were obtained from all mixtures, i.e., 29.5 ± 0.5%. This suggests that the composition of the GlcCer-enriched phase consists of ~2 POPE molecules per GlcCer molecule.

Further insight into the structure and packing arrangements within the lamellar phase enriched in GlcCer was obtained by an analysis of the WAXS intensity profiles of the mixture GlcCer:POPE, 30:100 mol ratio. This is presented in

TABLE 1 Deconvolution results of SAXS peaks of the L_q and L_α phase fitted by Lorentzian function

GlcCer:POPE (mol:mol)	x (GlcCer)	A_1 (a.u.)	A_2 (a.u.)	$t = A_2/A_1$
10:100	0.0909	0.23519	1.18682	5.0462
20:100	0.1667	0.45754	0.75383	1.6476
30:100	0.2308	3.0375	0.91436	0.3010
40:100	0.2857	4.20533	0.98201	0.2304

TABLE 2 Evaluation of the composition (α) of the L_q phase at various assigned composition (β) of the liquid-crystal phase

β	f	α	R^2
0	0.430	28.7%	0.93
1%	0.487	29.0%	0.93
2%	0.558	29.5%	0.93
3%	0.649	29.9%	0.92
4%	0.767	30.5%	0.89
Average	0.58 ± 0.10	29.5% ± 0.5%	–

Fig. 4. WAXS intensity patterns recorded during an initial heating and subsequent cooling scan of the mixed dispersion at designated temperatures is shown in Fig. 4 *A*. The sharp diffraction peak at 0.425 nm assigned to L_β phase of POPE recorded at 20°C can be readily distinguished from the broad scattering bands observed at higher temperatures. The d-spacing of these broad bands recorded in the heating scan

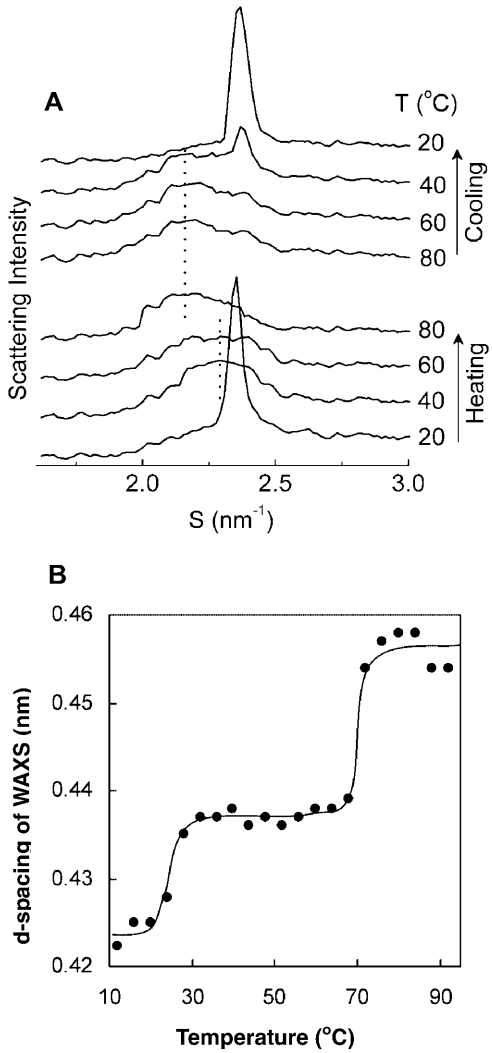

FIGURE 4 (*A*) WAXS intensity patterns recorded at the indicated temperatures from GlcCer:POPE (30:100, mol:mol) mixture during heating and subsequent cooling scans. (*B*) WAXS d-spacings of the mixture as a function of temperature upon heating.

in the temperature range corresponding to the lamellar phase of the GlcCer-enriched phase is 0.438 nm. This peak shifts to a *d*-spacing of 0.457 nm at temperatures above 60°C indicating disordered hydrocarbon chains. The relationship between *d*-spacing of the WAXS intensity maxima and temperature recorded during the initial heating scan of this mixture is shown in Fig. 4 *B*. The hydrocarbon chains remain disordered during cooling to 45°C whereupon a sharp peak signifying gel phase appears. This lamellar gel is formed from cubic phase enriched in GlcCer seen in the cooling scan (Fig. 2 *B*) and occurs 20° higher than formation of the L_β phase of POPE. The conclusions from analysis of the WAXS data are that the packing arrangement of the hydrocarbon chains of the GlcCer-enriched phase is identical to a gel phase on cooling from the disordered phase and that this gel phase is transformed into another phase with a chain packing density close to that of the gel phase upon equilibration at lower temperatures.

Differential scanning calorimetry

Enthalpy changes associated with the structural transitions observed by synchrotron x-ray diffraction were examined by differential scanning calorimetry. Thermograms from a binary mixture comprised of 20:100 of GlcCer:POPE (mol:mol) are presented in Fig. 5. A representative heating scan of a sample equilibrated at temperatures below 20°C (*a*) shows three endothermic transitions corresponding to the $L_\beta \rightarrow L_\alpha$ phase transition of pure POPE domain, the $L \rightarrow Q$ transition of the GlcCer-enriched domain and the $L_\alpha \rightarrow H_{II}$ transition of pure POPE domain. A cooling scan (*b*) shows three exotherms. Structural transitions associated with each transition enthalpy can be assigned from corresponding synchrotron x-ray diffraction analysis of the cooling scan (data not shown) and are $H_{II} \rightarrow L_\alpha$ transition of POPE, $Q \rightarrow L_\beta$ transition of the GlcCer-enriched domain and a simultaneous $L_\alpha \rightarrow Q$ and $Q \rightarrow L_\beta$ transition of POPE. It has already been noted that the two lamellar gel phases have distinctive *d*-spacings suggesting they coexist in separate domains in the mixture. An immediate rescan (*c*) shows that the endotherm of the $L_\beta \rightarrow L_\alpha$ transition of POPE is perturbed and an exothermic transition appears at ~37°C. Exotherms indicate transitions from disordered to ordered phases that are time and temperature-dependent relaxation processes (Yu et al., 1996; Chen et al., 2001). This was verified by calorimetry (Fig. 5, *d* and *e*). A heating scan recorded immediately after a cooling scan was held at 54.5°C (*d*) and subsequently cooled to −5°C (*e*). This shows a single exotherm corresponding to the $L_\alpha \rightarrow L_\beta$ transition of POPE. An immediate reheating of this sample produces a thermogram identical to the initial scan (*a*) recorded from the mixture equilibrated for several days at low temperature. It can be concluded from these results that stoichiometric complexes of GlcCer/POPE relax from an L_β structure to a more ordered lamellar phase.

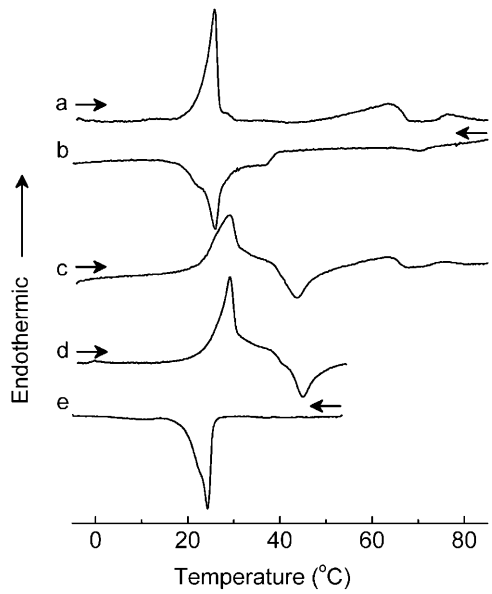

FIGURE 5 Differential scanning calorimetric curves recorded from a dispersion of GlcCer:POPE, 20:100 during temperature scans between −5 and 90°C at 2°/min. (*a*) Initial heating of an equilibrated sample; (*b*) a cooling scan; (*c*) a heating scan immediately after scan *b*; (*d*) a heating scan recorded immediately after a cooling scan; and (*e*) a cooling scan recorded immediately after scan *d*.

The thermal data taken together with the WAXS intensity profiles of equilibrated lamellar complex of GlcCer:POPE indicate an arrangement of molecules intermediate between a gel and a crystal phase. We designate this phase as a quasi-crystalline phase, L_q. The L_q phase coexists with fluid lipid bilayers and is the equilibrium structure at 37°C.

Electron spin resonance spectroscopy

The change in molecular arrangement associated with the formation of the more ordered phase of GlcCer:POPE was examined by spin-label methods. Mixed aqueous dispersions of GlcCer:POPE of 40:100 molar ratio containing spin-labeled probes 7-PC, 12-PC, or 16-PC were heated to 95°C and cooled to 25°C to record data in the L_β phase. The samples were then reheated to 42°C to induce relaxation to the ordered phase. After 5 min the samples were cooled to 25°C and spectra recorded from the ordered L_q phase. The order parameters and reorientation correlation times of the probes were determined using spectral simulation procedures. Fig. 6 *A* is an example of a least-squares fit of the spectrum of 16-PC in the L_q phase, whereas Fig. 6 *B* presents comparison of the ESR spectra of the probe in the L_q (*solid line*) and L_β (*dotted line*) phase. As can be seen, the anisotropy of the hyperfine coupling and therefore S_{mol} is clearly larger in L_β than in L_q. A summary of the results is presented in Table 3. The order parameters and correlation times recorded from the 7-PC and 12-PC spin labels in the L_q phase were indistinguishable from those obtained from lipid

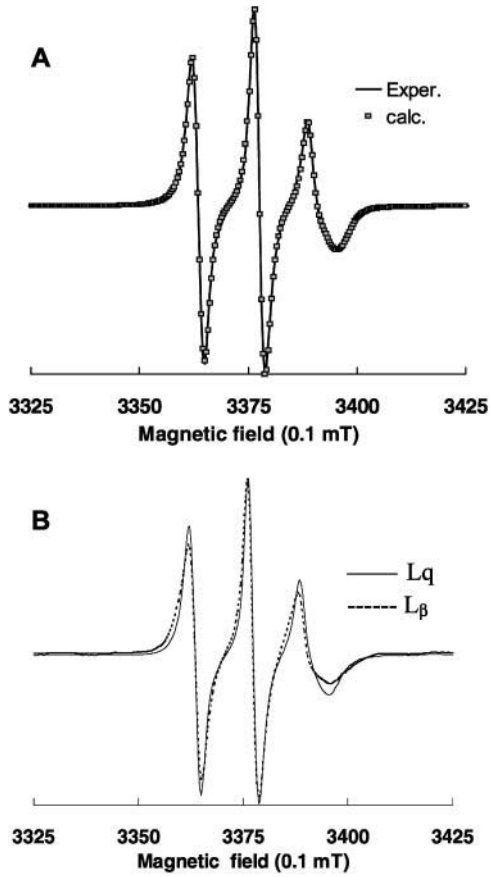

FIGURE 6 (*A*) Example of a least-squares fit of the spectrum of 16-PC in the L_q phase. The standard deviation between the experimental and computed spectra is 0.65%. The best fit is achieved with $S_{mol} = 0.148$ and $\tau = 0.493$ ns. (*B*) Comparison of the ESR spectra of the probe in the L_q (*solid line*) and L_β (*dotted line*) phase.

in gel phase, indicating that no significant change in probe environment occurs in the acyl chain region on relaxation of the L_β–L_q phase.

Fig. 6 shows that there is a marked difference between the spectra of the 16-PC probe in the L_q and L_β phases. There is indeed a significant decrease in order parameter (20%) and decrease in the reorientation correlation time (25%) of the 16-PC spin-label, sampling the central plane of the bilayer on transition from the gel to the L_q phase. Therefore the increase in order of the phase as evidenced from the DSC exotherm must result from reorientation of the molecules at the aqueous interface because the consequences in the hydrophobic core of the bilayer is a reduction in order and increase in molecular mobility. One interpretation of these results is that the complex of GlcCer:POPE is transformed into lamellar crystal phase such that rotation of the molecules about their axis perpendicular to the bilayer plane (i.e., the phase director) is restricted resulting in packing problems of the long, amide-linked fatty acids, which differ in length from the remaining hydrocarbon chains on average by six carbon atoms, in the center of the bilayer.

The packing problem is illustrated in the cartoon shown in Fig. 7. The disparity in length of amide-linked fatty acids to the GlcCer, which differ in length from the remaining hydrocarbon chains on average by six carbon atoms, are clearly evident in the model. The ratio of long and short chains in the complex does not permit an ordered interdigitation required for formation of a crystal phase and disordering occurs in this region to produce a quasicrystalline phase.

TABLE 3 Order parameters (S_{mol}) and reorientation correlation times (τ) of spin-labeled phosphatidylcholine analogs intercalated into dispersions of GlcCer:POPE (40:100, mol:mol)

Spin label	Phase	S_{mol}	τ (ns)
7-PC	L_β	0.58 ± 0.02	1.31 ± 0.10
	L_q	0.55 ± 0.02	1.40 ± 0.07
12-PC	L_β	0.33 ± 0.01	1.25 ± 0.04
	L_q	0.31 ± 0.01	1.18 ± 0.03
16-PC	L_β	0.180 ± 0.004	0.73 ± 0.11
	L_q	0.148 ± 0.004	0.55 ± 0.08

The spectra were recorded at 25°C after cooling from 95°C to form the L_q or gel phase.
The simulations were performed assuming one or two spectral components. The latter case, implying a mixture of phases, yielded unreliable values and was rejected. The values represent means of at least three simulations assuming a single site and using different methods.

DISCUSSION

There are several important features of the ordered quasicrystal phase (L_q) that are identified in this work. First, the stoichiometry of the phase was found to be ~1:2 in the molar ratio of GlcCer:POPE based on the analysis of the SAXS intensity profiles of different mixtures of GlcCer:POPE. By comparison, liquid-ordered phases (L_o) formed by cholesterol and dipalmitoylphosphatidylcholine exist as complexes in molar ratios closer to 1:3 or 1:4 (Vist and Davis, 1990; Reinl et al., 1992; McMullen and McElhaney, 1995). The higher proportion of GlcCer observed in the Lq phase must reflect differences in intermolecular interactions that take place in formation of the respective phases. The creation of a quasicrystalline arrangement between GlcCer and POPE implies that specific molecular interactions exist between the polar groups of the glycolipid and the PE. Such interactions are likely to be more specific than those between cholesterol and phospholipids in creation of L_o phases.

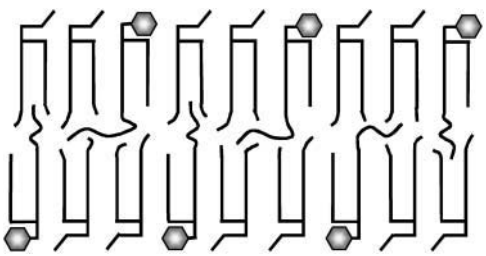

FIGURE 7 Molecular model of the L_q phase of GlcCer:POPE.

Second, L_q is a stable phase in the physiological range of temperatures where it coexists with L_α phase of POPE. Phase separation is evident from the distinction of the L_q domain, which has a longer *d*-spacing than that of the L_α phase. Moreover, the L_q phase is more stable than the lamellar-gel phase as evidenced by the exothermic transition associated with its formation (Fig. 5). Once formed, the phase is stable both in terms of stoichiometry and structure over a temperature range 28–70°C as evidenced by the position and intensity of SAXS peaks in mixtures where the L_q phase coexists with L_α phase (Fig. 2).

Third, the thermal and ESR spectroscopic studies infer that the L_q phase is more ordered in the headgroup region than in the hydrophobic core of the bilayer. The spin-label experiments indicate that the methylene groups of the hydrocarbon chains closer to the interface (probed by 7-PC) are packed as tightly as those of a gel phase, whereas the methyl and methylene groups in the central plane of the bilayer are less ordered than in gel phase (Table 3). It is presumably the ordering of the headgroups and associated hydration shells necessary for formation of an L_c phase that contributes to the transition exotherm from L_β to L_q phase. Specific interactions between the polar groups are likely to restrict long-axis rotation and lateral diffusion of the molecules, a factor that clearly distinguishes L_q from the L_o phase.

The L_q phase can be distinguished from an L_c phase because the hydrocarbon chains are not ordered into a regular lattice albeit they exhibit a packing density close to a gel phase. The ordered phase can also be distinguished from liquid-ordered (L_o) phases on these criteria. We have examined the WAXS intensity profiles of L_o phases formed by binary mixtures of cholesterol with sphingomyelin or phosphatidylcholines and these were invariably broad bands at a position expected of disordered liquid hydrocarbons. The order in such mixtures is believed to be imposed on the acyl chains by lateral interaction with the rigid sterol nucleus and ''condensation'' effects by a reduction in charge repulsion between the choline headgroups (Nielsen et al., 1999; Smondyrev and Berkowitz, 1999). Indeed there is evidence that the interaction of cholesterol with sphingomyelin is much stronger than with glycosylceramides containing equivalent hydrocarbon substituents (Smaby et al., 1996). PEs and GSLs have much less hydrated polar groups than choline phospholipids and a greater tendency to form L_c phases (Lewis and McElhaney, 1993; Saxena et al., 1999). It is reasonable to conclude that the order of the GlcCer:POPE phase is imposed by specific orientations of the polar groups and their associated hydration shells, leaving the hydrocarbon chains to pack into a quasi-crystalline arrangement. In this respect the phase is intermediate between L_β and L_c rather than between L_β and L_α, characteristic of the L_o phase.

Two factors need to be considered as to why complexes of GlcCer:POPE are prevented from forming a typical L_c phase. The first possibility is the lack of precise packing of the polar groups and their associated hydration shells at the bilayer-water interface. The second, and more probable reason, is the need to accommodate the mismatch in fatty acid chain lengths between the two lipids of the complex in the central region of the bilayer. This results in a decrease in order of chain packing and an increase in the rate of molecular mobility in the bilayer core. Such effects may contribute to loss of coordination of bilayer stacking evidenced by a broad SAXS lamellar repeat, which contrasts with regular bilayer stacking in typical L_c phases and emphasizes the importance of hydrocarbon chain packing in the bilayer core. In the context of formation of membrane rafts, the relative ordering of the hydrocarbon domains in the L_o and L_q phases combined with mismatch of chain lengths may serve as a mechanism coordinating ordered domains in opposite leaflets of the bilayer.

It is generally recognized that partitioning of cholesterol is correlated with domain formation (L_o or L_o-like phase) in sphingolipid or saturated molecular species of phospholipid (Sankaram and Thompson, 1990a; Vist and Davis, 1990; Bloom et al., 1991; Reinl et al., 1992; Mateo et al., 1995; Silvius et al., 1996; Ahmed et al., 1997; Brown and London, 1998; Wolf et al., 2001). Of the various lipids that form L_o phase with cholesterol in biomembranes, sphingolipid is an important component. On the other hand, previous studies have also shown that both sphingolipid and POPE can form L_o phase with cholesterol (Sankaram and Thompson, 1990b; Ahmed et al., 1997; Paré and Lafleur, 1998). Evidence from this study indicates an L_q phase is able to form at physiological temperatures from constituents expected to be present in biological membranes even when the proportion of GlcCer is relatively low.

There is now convincing evidence that L_o phases are of considerable biological importance (Simons and van Meer, 1988; Simons and Ikonen, 1997; Simons and Toomre, 2000; Brown, 2002; Silvius, 2003). Not all lipids recovered in detergent-resistant membrane fractions, however, are known to combine with cholesterol or to form L_o phases in proportions observed in such preparations. Sphingolipids, which are relatively rich in long and saturated fatty acyl chains, (for example, GlcCer extracted from Gaucher's patients, comprises saturated chains with C_{22}-C_{24} carbons) are likely to form ordered domains in biological membranes. The existence of such domains has long been sought (Brown and London, 1997) and some clustering of GSLs in membranes has been observed. One solid phase (crystalline) formed from lipid mixtures containing ceramide, cholesterol, and palmitic acid has been reported and this was said to have physiological roles in biomembrane functions (Fenske et al., 1994). Clearly, more work is needed to establish whether domains of L_q phase formed by GSLs and phospholipids are involved in dynamic membrane processes.

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