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ARTICLE

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Modulation of the phase heterogeneity of aminoglycerophospholipid mixtures by sphingomyelin and monovalent cations: maintenance of the lamellar arrangement in the biological membranes

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Abstract The phase behaviour of mixed molecular species of phosphatidylethanolamine, phosphatidylserine and sphingomyelin of biological origin were examined in aqueous co-dispersions using synchrotron X-ray diffraction. The co-dispersions of phospholipids studied were aimed to model the mixing of lipids populating the cytoplasmic and outer leaflets in the resting or "scrambled" activated cell membrane. Mixtures enriched with phosphatidylethanolamine and phosphatidylserine were characterized by a phase separation of non-lamellar phases (cubic and inverted hexagonal) with a lamellar gel phase comprising the most saturated molecular species. Inclusion of sphingomyelin in the mixture resulted in a suppression of the hexagonal-II phase in favour of lamellar phases at temperatures where a proportion of the phospholipid was fluid. The effect was also dependent on the total amount of sphingomyelin in ternary mixtures, and the lamellar phase dominated in mixtures containing more than 30 mol%, irrespective of the relative proportions of phosphatidylserine/sphingomyelin. A transition from gel to liquid-crystal phase was detected by wide-angle scattering during heating scans of ternary mixtures enriched in sphingomyelin and was shown by thermal cycling experiments to be coupled with a hexagonal-II phase to lamellar transition. In such samples there was evidence of a coexistence of non-lamellar phases with a lamellar gel phase. A transition of the gel phase to the fluid state on heating from 35 to 41 °C was evidenced by a progressive increase in the lamellar *d*-spacing. The presence of calcium enhanced the phase separation of a lamellar gel phase from a hexagonal-II phase in mixtures enriched in phosphatidylserine. This effect was counteracted by charge screening with 150 mM NaCl. The effect of sphingomyelin on stabilizing the lamellar phase is discussed in the context of an altered composition in the cytoplasmic/outer leaflets of the plasma membrane resulting from scrambling of the phospholipid distribution. The results suggest that a lamellar structure can be retained by the inward translocation of sphingomyelin in biological membranes. The presence of monovalent cations serves also to stabilize the bilayer in activated cells where a translocation of aminoglycerophospholipids and an influx of calcium occur simultaneously.

Keywords Bilayer stability · Phosphatidylethanolamine · Phosphatidylserine · Plasma membrane · Sphingomyelin

Abbreviations PC: phosphatidylcholine · PE: phosphatidylethanolamine · PS: phosphatidylserine · SAXS: small-angle X-ray scattering · SM: sphingomyelin · WAXS: wide-angle X-ray scattering · XRD: X-ray diffraction

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Introduction

The lipid matrix of biological membranes consists of a complex mixture comprising hundreds of molecular species each differing in polar head group, designating the lipid class, with fatty acids of varying length and number of unsaturated bonds. In living organisms the phase structure of membranes is dictated by biochemical remodelling responsible for insertion and removal of lipids from the structure in concert with the action of endogenous translocases which redistribute lipids from one side of the membrane to the other. The situation is

more complex in organisms with no physiological maintenance of internal temperature and where, in addition to the above influences, the structure and stability of the lipid phases must change in response to ambient temperature to preserve membrane integrity.

There is now accumulating evidence suggesting that phase separations of lipids in biological membranes and the creation of lipid domains play particular functional roles in cell physiology.

The phase structure of phospholipid classes present in cell membranes has been documented by X-ray diffraction over more than 40 years. Fewer studies have been reported on phase separation in mixtures of phospholipids extracted from biological tissues, which contain a variety of fatty acids attached at the *sn*-1 and *sn*-2 positions of the glycerol moiety. X-ray diffraction studies (Luzzati et al. 1968) have provided ample evidence of phase polymorphism. Such observations appeared to be at variance with the notion that the lipid matrix of biological membranes consisted of an apparently stable bilayer arrangement of the lipids. Nevertheless, there is convincing evidence that the lipids remain assembled into a bilayer configuration despite the preference for a significant proportion of them, especially the aminoglycerophospholipids populating the cytoplasmic monolayer, to form non-lamellar structures. It is commonly inferred that a bilayer arrangement is imposed on such lipids by their lateral and transverse interaction with other lipids and proteins in membranes. The present study highlights the particular role of sphingomyelin (SM) in influencing membrane lipid phase behaviour.

It is well established that the distribution of lipids within the membrane bilayer matrix is not symmetric. Active transport processes operate in plasma membranes to segregate the aminoglycerophopholipids, phosphatidylethanolamine (PE) and phosphatidylserine (PS), into the cytoplasmic leaflet and leave the choline phosphatides, phosphatidylcholine (PC) and SM, resident on the external leaflet (Zachowski 1993; Bevers et al. 1999; Pomorski et al. 2001). Scrambling of the asymmetric distribution of lipids across the membrane is associated with a number of physiological and pathological processes such as apoptosis, cell activation, ischemia (Musters et al. 1993; Zachowski 1993), sickle cell anemia (Franck et al. 1985) and aging (Diaz and Schroit 1996). A particular feature of these processes is that the activity of an ATP-dependent aminoglycerophospholipid translocase is inhibited. Furthermore, the activity of a scramblase is enhanced by an influx of calcium. As a consequence of these events, SM and aminoglycerophospholipids, which are largely segregated in the resting state on either side of the membrane, are mixed.

This study aims to determine whether phospholipid scrambling results in a disturbance of the lipid lamellar phase equilibrium. The study also takes into account the altered ionic environment (influx of calcium and sodium) resulting from the dissipation of ion gradients, an effect that is superimposed on lipid scrambling associated with cell activation or apoptosis. Phase diagrams of PE/PS binary mixtures and PE/PS/SM ternary mixtures have been constructed using synchrotron X-ray diffraction (XRD) methods to characterize the effect of the presence of SM on lipid mixtures modelling the cytoplasmic leaflet of the cell membrane. The underlying assumption in choosing the composition of these mixtures is that SM and aminoglycerophospholipids, with their strongly diverging phase preferences, dominate the final equilibrium.

Real-time synchrotron XRD methods have been used to characterize both the structure of the various phases that form at temperatures in the physiological range and the transitions that take place when aminoglycerophospholipids are mixed with SM. Of particular interest is the role of SM in maintaining a lamellar structure, considering that molecular species of SM are present in the membrane which exist in both gel and fluid phases at physiological temperatures (Barenholz and Thompson 1999). The influence of SM on the phase behaviour of the aminophospholipids will depend on the proportion of fluid SM that is able to mix with aminophospholipids as distinct from those molecular species that, because of their predominantly saturated hydrocarbon composition, are phase separated into a gel phase. The availability of SM to mix with aminophospholipids in biological membranes will also depend on the presence of cholesterol, which induces phase separation of a SM-cholesterol liquid-ordered phase at physiological temperatures. The properties of quaternary mixtures fall beyond the scope of the present study.

Materials and methods

Lipids (egg-yolk PE, SM, beef-brain PS) were purchased from Sigma-Aldrich (St Quentin-Fallavier, France). The fatty acid composition of each phospholipid class was examined by gas-liquid chromatography/mass spectrometry to check reproducibility of the samples and confirm the absence of degradation products after exposure to the synchrotron X-ray beam. For PE the values (C16:0, 5.7%; C16:1, 4.1%; C18:0, 25.9%; C18:1, 39.2%; C18:2, 13.4%; C20:4, 6.9%; C22:6, 4.7%) were typical of non-oxidized phospholipids before and after X-ray exposure. In brain PS the most abundant molecular species is C18:0 (42%)-C18:1 (34%). Egg SM is mostly amidified with C16:0 (84%) and C18:0 (6%) fatty acids.

Samples for XRD examination were prepared by dissolving dry lipids in chloroform/methanol (2:1, by vol) and mixing the solutions to obtain the desired proportions, designated as mole percent relative to the total moles of lipid. The solvent was subsequently evaporated under a stream of oxygen-free dry nitrogen at 45 °C and traces of solvent removed by storage under high vacuum for 2 days. The dry lipids were hydrated with an equal weight of buffer consisting of 100 mM Tris-HCl (pH 8). $CaCl_2$ was added as a concentrated aqueous solution to achieve a ratio of one cation per 270 molecules of phospholipid; in some experiments the buffer contained 150 mM NaCl to illustrate the charge screening effects of monovalent salts upon calcium. Because the activity of the divalent cation is expected to be well below 1 in the small volume of aqueous buffer (20–30 μL) used to hydrate the lipids, the concentration of calcium cannot be easily compared to the bulk concentration (10^{-7}–10^{-6} M) encountered in the cytoplasm of living cells. Indeed, preliminary tests have indicated that variations in a large range of concentrations produce similar X-ray diffractograms of a binary mixture of PE/PS = 8/2. The aqueous lipid dispersion was stirred and sealed under argon. The dispersed lipids were annealed by thermal cycling 50 times between 20 and 65 °C and equilibrated at

4 °C prior to examination. The samples were thoroughly mixed to prevent any possible macroscopic phase separation within the sample.

XRD measurements were performed at stations 8.2 and 16.1 of the Daresbury Synchrotron Radiation Laboratory (Daresbury, UK). Simultaneous small-angle (SAXS) and wide-angle (WAXS) X-ray scattering intensities were recorded so that a correlation could be established between the mesophase repeat spacings and the packing arrangement of the acyl chains. X-ray intensities were normalized to that of the incident beam monitored by an ionization chamber; the SAXS quadrant detector was corrected for channel response using a static radioactive iron source. The quadrant detector (sample to detector distance, 2 m or 2.5 m) was calibrated for *d*-spacing using hydrated rat tail collagen (*d*-spacing 66 nm). The WAXS intensity profiles were measured with a curved INEL detector (Instrumentation Electronique, France). The WAXS detector was spatially calibrated using the peaks from high-density polyethylene. The sample (20 μL) was sandwiched between thin mica windows and mounted on a programmable thermal stage (Linkam, UK). The temperature was monitored by a thermocouple (Quad Service, Poissy, France) inserted directly into the lipid. The setup, calibration and facilities on the beamlines are described on the web site (http://www.srs.dl.ac.uk/ncd/station82/). Data reduction and analysis were performed using the OTOKO for PC program kindly provided by M.H.J. Koch (Boulin et al. 1986).

Results

The phase behaviour of each of the phospholipids of biological origin subsequently included in complex mixtures was first examined as single dispersions by XRD methods. The individual phospholipid classes, each of which comprised a wide range of molecular species differing in hydrocarbon substituents, are represented at the apexes of the compositional triangle shown in Fig. 1. The composition of the mixtures examined and that of a number of biological membranes have been reported in terms of the relative proportions of PE, PS and SM. The values indicated in Fig. 1 represent the approximate composition of the cytoplasmic or exoplasmic leaflet of the membrane as it is maintained in the resting state.

X-ray scattering intensity profiles recorded during an initial heating scan of a dispersion of egg PE (data not shown) indicated that the phospholipid undergoes two successive transitions as a function of temperature. From around 9 °C a lamellar gel-to-fluid transition (Lβ-to-Lα) is observed in the SAXS region as a reduction in lamellar *d*-spacing from 5.50 to 5.05 nm associated with the disappearance of a sharp peak at 0.42 nm in the WAXS region, characteristic of a gel phase. This transition is completed at 18 °C for all molecular species of egg PE. At temperatures greater than 34 °C, a gradual transition of the fluid lamellar (Lα) to another phase with spacings of 6.15 nm, 3.55 nm ($d/\sqrt{3}$) and 3.05 nm ($d/2$) is observed, which can be assigned to the first three orders of an inverted hexagonal phase (H_{II}). The coexistence of phase-separated fluid lamellar and hexagonal domains is evident up to 45 °C.

X-ray scattering profiles of a dispersion of PS at temperatures around 37 °C (data not shown) consist of a broad SAXS peak centered at 6.43 nm, consistent with the diffuse scattering produced by poorly oriented bilayers. Despite the absence of higher-order reflections, a sharp WAXS peak at 0.42 nm over a wide temperature range of the scan is consistent with the presence of a gel phase which must be in a lamellar arrangement. As expected for a charged amphiphile, the formation of a coherent smectic phase by PS depends on the screening of the charged anionic groups by monovalent cations (data not shown).

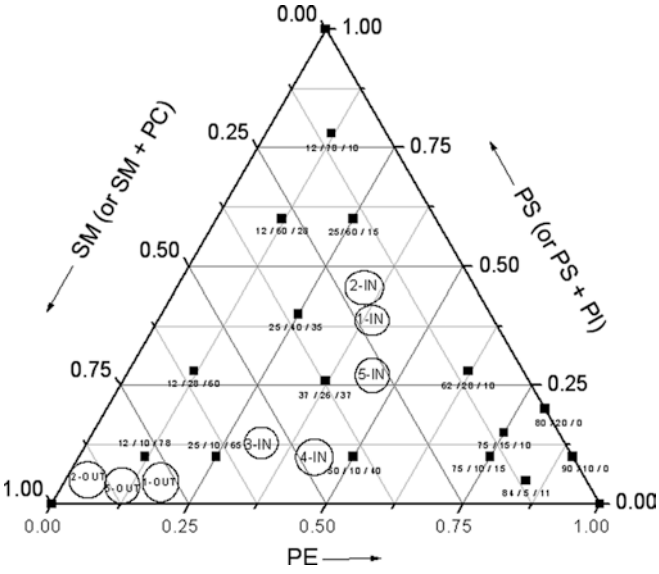


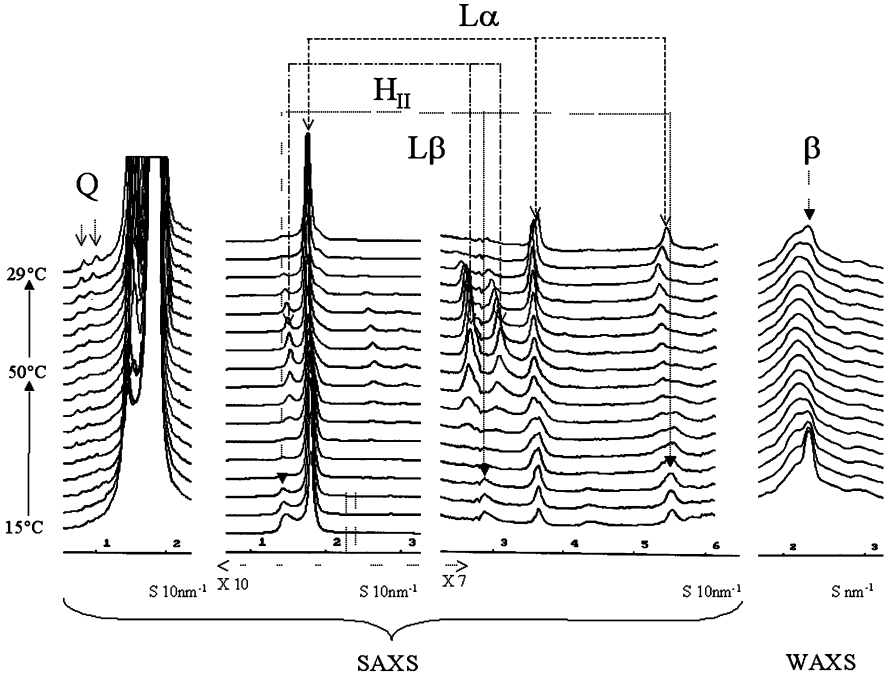
Fig. 1 Composition of the ternary mixtures PE/PS/SM investigated. Phosphatidylethanolamine (PE, from egg yolk), phosphatidylserine (PS, from beef brain) and sphingomyelin (SM, from egg yolk) were mixed in the proportions indicated as molar fractions. Three purified lipids and 15 mixtures were examined (*filled squares*). For the purpose of comparison, the composition characteristics of the inner or outer leaflets of biological membranes are also quoted (*open circles*, *numbered below*), where the values of choline-containing (PC + SM) and anionic (PS + PI) classes have been combined: *1*, IN and OUT for human platelets (Perret et al. 1979); *2*, IN and OUT for human red blood cells (Zachowski 1993); *3*, IN for cardiac sarcolemna (Post et al. 1988); *4*, IN for muscle sarcolemna (Herbette et al. 1984); and *5*, IN and OUT for Schwann cells (Calderon and DeVries 1997)

Finally, a dispersion of SM equilibrated at 5 °C (data not shown) displays gel phase immiscibility of two lamellar phases at low temperatures, one designated Lβ (*d*-spacing 6.70 nm) and the other Lβ* (*d*-spacing 5.93 nm). A sharp gel phase reflection is observed in the WAXS region. Heating the dispersion results firstly in the disappearance of the Lβ* phase followed by a progressive gel (Lβ)-to-fluid (Lα) transition at temperatures greater than 38 °C, in which the lamellar repeat centred at a *d*-spacing decreases from 6.70 nm at 30 °C to 6.15 nm at 45 °C. The disappearance of the sharp WAXS reflection is complete at 41 °C. A similar phase-transition sequence Lβ*-to-Lβ-to-Lα was previously reported in dispersions of sphingolipids (Boggs and Koshy 1994) or asymmetric diacyl-PC (Koumanov et al. 1995); the behaviour was ascribed to the acyl chain length inequivalence causing the interdigitation of asymmetric phospholipid molecular species of the opposing bilayer leaflets.

To examine how the phase behaviour of mixed aqueous dispersions was modulated by particular phospholipids, XRD studies were performed on two binary mixtures of PE/PS and 13 ternary mixtures of SM/PE/PS. The compositions of the mixtures investigated are distributed over the triangular composition diagram shown in Fig. 1 so as to delineate the compositional boundaries of the different phases. Figure 2 gives an example of the method applied for a diffractogram of the ternary mixture PE/PS/SM = 75/15/10. The recording selected illustrates the complexity of the multiple coexisting phases resolved by XRD in such a ternary mixture. As compared with synthetic lipids, such complexity for biological lipids reflects the numerous molecular species represented in each lipid class; for this reason it is suggested that the model has a greater biological significance than a single molecular species.

At any temperature the variety of coexisting phases can be understood as the result of distinct temperature transitions so that during a temperature scan the molecular species of each of the three phospholipid classes are likely to redistribute within the dispersion. It can be seen in Fig. 2 that a Lα (lamellar fluid phase) coexists with Lβ (lamellar gel) at low temperatures (< 34 °C); during heating, the H_{II} (inverted hexagonal) phase (> 35 °C) begins to phase separate from the Lα phase at temperatures greater than 35 °C. Furthermore, an additional Q phase could be detected after one temperature cycle. The indexing of the two additional peaks in the very small angle region to define the space grouping of the cubic phase was not possible, although the formation of *Pn*3*m* space-group symmetry has been reported previously in PE after temperature cycling (Tenchov et al. 1998). Such an assignment is based here on the observation that iterative heating/cooling throughout the temperature cycle increases the contribution of this presumptive Q phase. A visual inspection of the lipid mixtures after multiple temperature cycling showed also a vitreous-like appearance, consistent with an isotropic cubic phase. The lamellar gel-to-fluid transition as characterized by the simultaneous disappearance of the sharp WAXS gel peak centered at 0.43 nm with that of the lamellar *d*-spacing at 6.8 nm was found to be reversible, with a temperature hysteresis of a few degrees. The end-point and onset-transition temperatures for Lβ-to-Lα and Lα-to-H_{II} phase transitions are estimated from the data in Fig. 2 to be 34 °C and 35 °C, respectively. The subsequent transitions could be defined by temperature cycling coupled with XRD analysis, with an accuracy estimated at ± 0.5 °C with the data acquisition mode employed. In mixtures with higher enrichment in PS (> 20 mol%), the narrow temperature difference between Lβ-to-Lα and Lα-to-H_{II} transitions could not be resolved. Consequently, no intermediate fluid lamellar phase (Lα) was detected between the transition from Lβ to H_{II} during the heating scan. By contrast, the transition sequence shown in Fig. 2 can

Fig. 2 X-ray examination of the ternary mixture PE/PS/SM = 75/15/10 (mol/mol) hydrated in an equal weight of buffer (20 mg) consisting of 100 mM Tris-HCl (pH 8) in the presence of Ca (one cation per 270 molecules of phospholipid). The SAXS intensity (0.08–0.6 nm^{-1}) is plotted in different regions of the diffractogram, with expansion of the vertical axis to emphasize the weak scattering from higher-order reflections of the mesophases. The corresponding WAXS intensity (1.8–3.5 nm^{-1}) is shown in the panel on the *right*. Each diffractogram represents scattering collected during 30 s. The sample was heated from 15 to 50 °C and subsequently cooled to 30 °C at a scan rate of 1 °C min^{-1}. The interval of two adjacent patterns is 3.5 °C. The phase assignments are Q (cubic), H_{II} (inverted hexagonal), Lα (lamellar fluid), Lβ (lamellar gel) and β (hexagonally packed acyl chains forming the gel signature in the WAXS area)



clearly be resolved initially by a decrease in intensity of the Lβ peaks corresponding with an increase in intensity of Lα and subsequently by a decrease of Lα associated with the emergence of H_{II} as the temperature increases.

The *d*-spacings for Lβ, Lα and H_{II} structures in all the samples examined for temperatures above (40 °C) and below (35 °C) the gel-to-fluid transition of SM are presented in the phase diagrams shown in Fig. 3. The lower boundary for a non-lamellar component is shown tentatively by a dashed line in the figures. It should be noted that these phase diagrams are not greatly dependent upon temperature but are highly dependent on salt concentrations (see below). When the proportion of PS in the mixture was increased, the contribution assigned to the Lβstructure was also much greater, suggesting that the lamellar gel phase comprises a significant fraction of PS. The Lβ-to-Lα transition temperature seen in the WAXS profile was shifted up from about 33 to 39 °C, suggesting that PS favours the phase separation of lipids into domains of the gel phase. As indicated above, no fluid lamellar arrangement can be resolved between Lβ and H_{II} in the X-ray patterns recorded from mixtures highly enriched with PS. In a binary mixture containing 40 mol% PS (data not shown) the contribution to scattering from the inverted hexagonal phase dominates the entire temperature range of the scan (10–45 °C), with only weak non-hexagonal reflections detected which were ascribed to residual Lβ and Q contributions. The observation is consistent with a destabilizing influence of PS on the lamellar arrangement brought about by the anionic phospholipid in the presence of calcium.

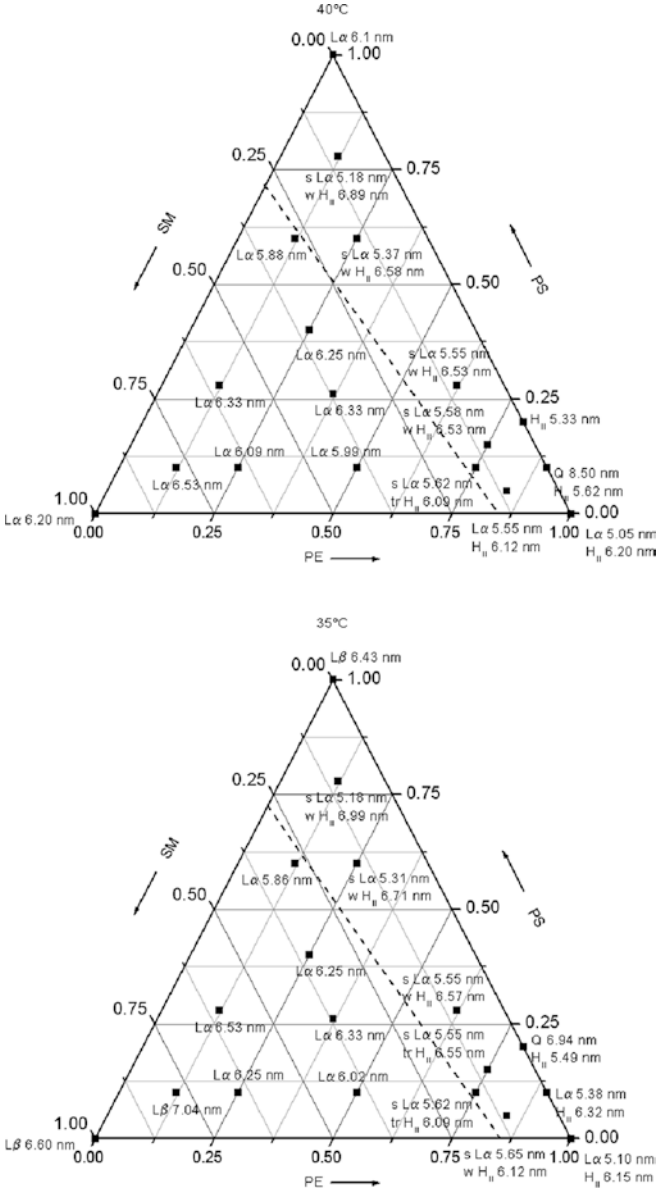


Fig. 3 Phase diagrams for the ternary mixture PE/PS/SM at 35 and 40 °C in the presence of calcium (one Ca^{2+} per 270 phospholipids) at pH 8. The phase and *d*-spacings used for phase assignments were obtained on the initial heating scan, when the Q phase contribution is reduced or absent. The Q contribution was found to increase upon repeated temperature cycling during the XRD measurements. The *dashed line* represents the lower boundary of the hexagonal phase separation disrupting the lamellar arrangement. No non-lamellar contribution was detected below this boundary. *Asterisks* (*) and *double asterisks* (**) refer to diffractograms detailed in Fig. 2 and Fig. 4, respectively; *s*, strong reflection; *w*, weak reflection

A particular insight into how the mixing and phase behaviour of PE/PS could be modulated by the presence of SM, which is believed to be enriched in the inner leaflet by lipid scrambling associated with cell activation, is presented in Fig. 4. One of the most conspicuous effects of increasing proportions of SM in mixtures of PE/PS is the suppression of the H_{II} phase up to 25 °C, which tends to dominate the binary PE/PS dispersion at high temperatures. It can be seen in Fig. 4 that the separation of a number of phases within the ternary mixture takes place, the assignment of which can be made on the basis of thermal scanning protocols. Firstly, a lamellar phase with a *d*-repeat of 6.73 nm can be assigned to Lβ because its disappearance at about 39 °C coincides with loss of a sharp peak in the WAXS region at 0.43 nm. It is noteworthy that at 39 °C the gel-to-fluid transition is accompanied by a shift in the spacing of the fluid lamellar peak from 5.88 nm to 6.07 nm. The simultaneous melting of the lamellar gel arrangement (peak at 6.73 nm) with the shift of the fluid lamellar peak to a longer *d*-spacing (5.88 to 6.07 nm) is plotted in Fig. 4B as a function of the temperature during three successive heating scans. This observation demonstrates how the influence of SM takes place after mixing with the aminoglycerophospholipids and constraining them into a lamellar configuration. Each minimum of the scattering intensity for the Lβ phase (melting of the gel phase) was observed at the highest temperature of the scan (lower panel) and correlated with a shift of the Lα phase *d*-spacing to a greater value (upper panel). It may be concluded that the fluid lamellar phase of the longer *d*-spacing is formed after melting of a domain enriched with molecular species of SM possessing the highest gel-to-fluid transition temperatures. When the high-melting-point molecular species of SM, which remain phase

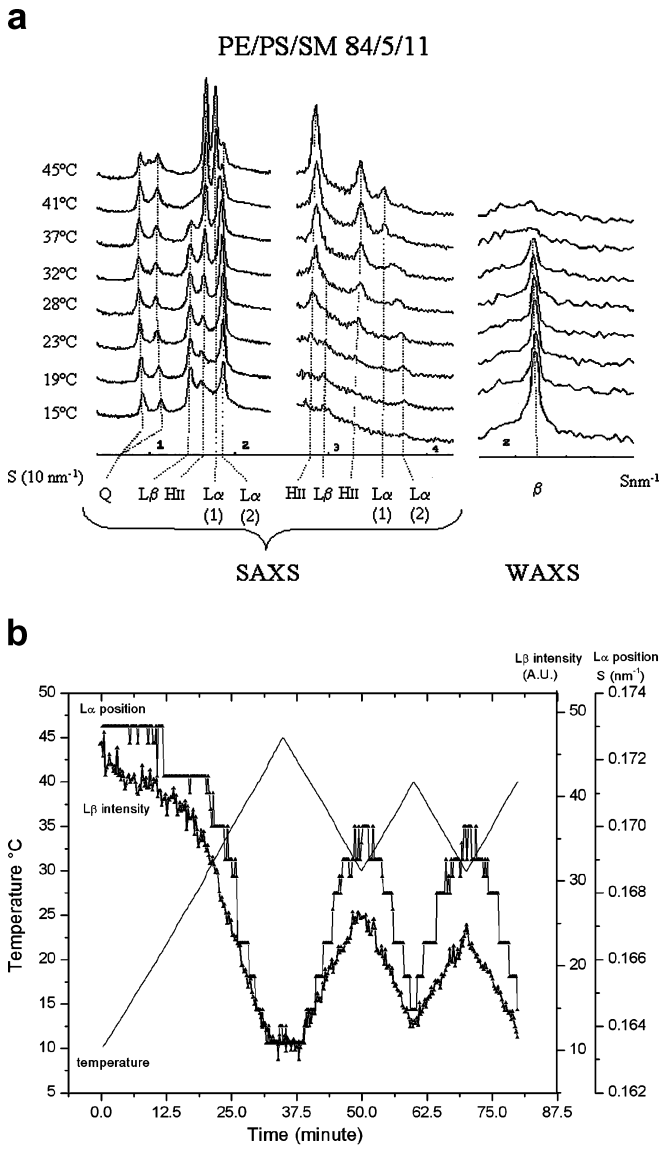


Fig. 4a, b Effect of SM on the thermotropic phase behaviour of PE/PS. (**a**) X-ray scattering intensity of a mixture of PE/PS/SM (84/5/11, mol/mol) recorded during a heating scan at 1 °C min^{-1}. Phase assignments are indicated under the profiles. The scale of scattering intensity of the centre panel has been expanded to illustrate the weak scattering of higher-order mesophase reflections. (**b**) Change in *d*-spacing of the L phase and relative scattering intensity of the first order of the Lβ phase as a function of temperature during thermal cycling at a scan rate of 1 °C min^{-1}

separated at low temperatures into gel domains, are fluidized at high temperature, they mix with the aminoglycerophospholipids to form a fluid lamellar structure with an expanded *d*-spacing. It should also be noted from the thermal cycling experiments that the phase transitions within the ternary mixture are rapidly and completely reversible. This indicates that there is rapid lateral diffusion and intermixing of fluidized lipids in the bilayers. Another phase present in the mixture (Fig. 4A) is H_{II} with a repeat spacing of 6.23–6.15 nm. H_{II} can be observed at 10 °C but the scattering intensity of the phase increases sharply above 39 °C, coincident with a gel-to-fluid transition seen in the WAXS region (right panel). Also evident throughout the temperature range investigated is a series of peaks at very small angles, which we interpret to be a cubic phase. Thus it is clear that the ternary mixture of aminoglycerophospholipids and SM may exhibit considerable heterogeneity, involving the coexistence of a cubic phase, hexagonal-II structure, lamellar gel and lamellar liquid-crystalline phases at different temperatures in the physiological range. This suggests there is extensive phase separation of molecular species of lipid forming the respective structural phases.

The mechanism of phase separation in mixtures of PE and PS has also been characterized as a function of ionic strength of the dispersion buffer and the results are presented in Fig. 5. The results show that the presence of NaCl causes a shift in the phase equilibrium, which is dominated by a single lamella phase. In this case, no non-lamellar phases can be detected. The diffractograms shown in Fig. 5A indicate that screening the negatively charged headgroups by addition of 150 mM NaCl results in a single and stable fluid lamellar phase in the binary mixture containing 20 mol% PS. The effect of this high ionic strength is reversed if the concentration of calcium is increased from one Ca^{2+} per 270 phospholipid molecules to four Ca^{2+} per PS molecule, since this results in reappearance of the hexagonal phase in the mixture from the initial temperature of the scan, 10 °C (Fig. 5B). Taken together, these results suggest that the sequestration of the anionic headgroups of PS into restricted domains induced by calcium may be balanced by charge screening. The result can be understood if the mixing of PS and PE maintains the phase preference for a lamellar structure in a manner similar to the mixing of PE and SM in maintaining a lamellar structure.

Discussion

The present study demonstrates the remarkable heterogeneity in structure and complexity of thermotropic phase behaviour exhibited by phospholipids extracted from biological tissues. From previous studies it has been shown that polyunsaturated molecular species of hydrated PE tend to form an inverted hexagonal phase around physiological temperatures (Caffrey 1985; Tate and Gruner 1987). Under similar conditions, SM prefers a lamellar arrangement (Koynova and Caffrey 1995). The structure of aqueous dispersions of PS depends markedly on the pH and salt concentration, especially of divalent cations, which influence the arrangement as a lamellar or non-lamellar phase (Newton et al. 1978; Holwerda et al. 1981; Kroon et al. 1990). Sequestration of the negatively charged headgroup of PS by divalent ions has been evidenced from numerous NMR studies (Arnold et al. 1981; Bally et al. 1983). Clearly, the characterization of complex mixtures in terms of the effects of temperature and solutes represents a considerable challenge.

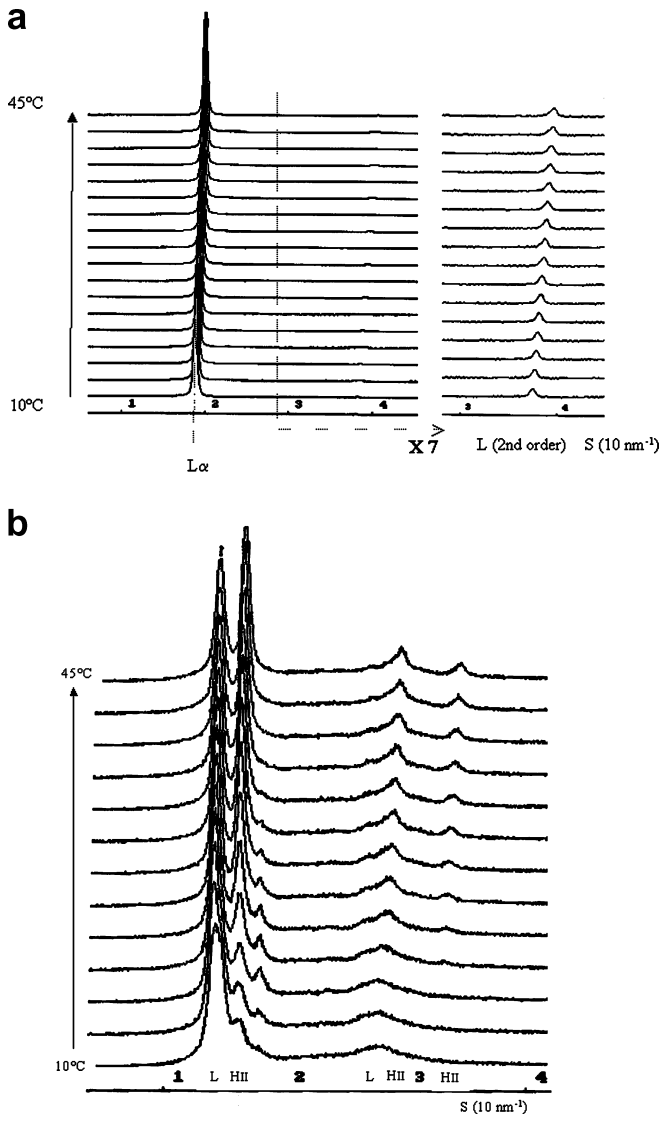


Fig. 5a, b Effect of sodium and calcium ions on the thermotropic phase behaviour of PE/PS co-dispersions. X-ray scattering intensity profiles were recorded during an initial heating scan at 1 °C min^{-1} of a co-dispersion of PE/PS (80/20, mol/mol) hydrated with an equal weight of buffer containing (**a**) $CaCl_2$ (one Ca^{2+} per 270 phospholipid molecules) and 150 mM NaCl; (**b**) $CaCl_2$ (four Ca^{2+} per PS molecule) and 150 mM NaCl. The interval of two adjacent patterns is 2.06 °C and 2.9 °C in (**a**) and (**b**), respectively

One of the principle aims of the present study was to define the factors that govern the arrangement of lipids in complex mixtures that more accurately represent the lipid matrix of biological membranes. The phase state of lipids in mixed aqueous dispersions depends markedly on temperature and, in many instances, on the thermal history of the mixture. Depending on the mixture, it is likely that phase separations of lipid classes and even different molecular species within particular lipid classes will occur to create domains or complexes with characteristic properties. The structure and properties of such domains can be assessed conveniently by synchrotron XRD methods. Structural rearrangements that take place during temperature scans can be identified and when combined with appropriate scanning protocols, such as thermal cycling, miscibility of particular components exhibiting characteristic phase preferences within complex mixtures can be assessed. Of particular interest in the present work has been the mixing properties of the aminoglycerophospholipids populating the cytoplasmic leaflet of membranes, which has been represented by binary mixtures of PE/PS. Biophysical studies clearly indicate phase heterogeneity with naturally derived lipids to be widely influenced acyl chain unsaturation (Tilcock and Cullis 1981). The present observations suggest a role for SM which might reconcile the potentially unstable inner leaflet composition with the need to maintain a "physiological" barrier in the form of a stable bilayer under physiological conditions (Hill and Zeidel 2000).

The studies of dispersions of PE and PS extracted from biological sources and binary mixtures of the two phospholipids have given some insight into the basic features governing their overall thermotropic phase behaviour. These show that the presence of PS in the binary mixture favours the formation of an inverted hexagonal phase. Such a structure can also be observed in a dispersion of PE, but at higher temperatures. The phase separation of PS in the presence of divalent cations to form a lamellar phase was previously deduced from ^{31}P NMR evidence, leaving the polyunsaturated PE to form the hexagonal phase. Wide-angle XRD measurements confirm this conclusion since a gel phase, which is invariably lamellar, was detected in the presence of calcium at temperatures up to 39 °C in mixtures containing 20 mol% PS. The composition of the gel phase is likely to consist of calcium-bound PS and the most saturated molecular species of PE. The influence of the unsaturation of acyl chains on PE/PS co-miscibility in the presence of calcium has already been acknowledged as "exquisitely sensitive" by ^{31}P NMR methods (Tilcock et al. 1984). It is therefore suggested that only the most saturated molecular species of PE associate with the saturated molecular species of PS clustered by calcium to form the lamellar gel phase. This would leave the unsaturated species of aminoglycerophospholipids free to form a coexisting inverted hexagonal arrangement.

It can be also inferred from observations of binary mixtures that in the presence of physiological saline (150 mM NaCl) the heterogeneity of the binary PE/PS mixture is suppressed in favour of formation of a single fluid lamellar phase. A possible explanation for the effect of ionic strength is that screening of the charges of PS by monovalent cations releases the anionic phospholipid from calcium-sequestered domains and allows the mixing of the saturated and unsaturated molecular species in the dispersion. Accordingly, the completion of the intermixing is detected in X-ray scattering measurements by the disappearance of a sharp gel reflection in the WAXS region. This emphasizes a critical role played by the association of the acyl chains of the most saturated

species. Calcium in excess (four Ca^{2+}/PS) re-establishes the clustering of PS in the presence of 150 mM NaCl, because it also segregates the more unsaturated species when present at higher concentrations. Indeed, the concentrations used in these experiments cannot be directly compared with concentrations in the cytoplasm, which are typically less than micromolar. However, in the experimental conditions used for X-ray studies, the low activity of ions in the interbilayer hydration layers of the smectic mesophase are expected to modulate calcium binding. Therefore it is likely that the ionic balance serves to maintain a stable lamellar arrangement, an influence superimposed on the variation of lipid composition of the inner leaflet associated with cell activation.

The phase behaviour of ternary mixtures was used to examine the influence of SM on lipid mixtures modeling the cytoplasmic membrane leaflet. These observations showed that SM is a major modulator for the maintenance of the bilayer structure and that SM can gradually impose a lamellar arrangement on mixtures of aminoglycerophospholipids, PE and PS. Proportions of SM below 30 mol% likely span the range of concentrations expected in the inner leaflet of membranes during scrambling of the membrane phospholipid distribution. Although lower SM levels can be expected in membranes of particular cell types [<7 mol% in human platelet (Perret et al. 1979) or rat cardiac sarcolemna (Post et al. 1988)], it may be expected in reference to Fig. 1 that the composition of the scrambled state of the membrane will lie between the positions of the IN and OUT leaflets in the triangular compositional diagram. It must also be emphasized that the particular fatty acid composition is an important factor in the way SM exerts its lamellar influence on mixtures of aminoglycerophospholipids. In this respect, high molecular weight SM species amidified with very long, saturated fatty acids partition together with saturated molecular species of PS and PE to a create gel phase bilayer. However, low molecular weight species of SM, on the other hand, are assumed to mix with the fluid components such as polyunsaturated molecular species of PE, acting to reduce their tendency to form the H_{II} structure. Indeed, egg SM is known to be enriched in palmitic acid ($>70\%$) at the expense of very long acyl chains ($<8\%$). The partitioning of SM molecular species in this way is consistent with the XRD data, which reveal a fluid lamellar arrangement Lα coexisting with Lβ in the ternary mixture PE/PS/SM, but no hexagonal contribution in the presence of relatively high proportions of SM. In samples containing lower proportions of SM, the detection of a shift in the periodicity of the fluid lamellar phase Lα strongly supports the possibility that the fluid phase may be gradually enriched in fluidized SM. It is noteworthy that the gel-to-fluid transition of SM detected by WAXS intensity peaks is associated with an abrupt increase of spacing of this fluid lamellar component, whereas a decreased spacing is usually observed due to increased disorder of the acyl chains. A possible explanation is that an enrichment of the Lα phase with fluidized long-chain species of SM, assuming that the hydration layer is constant, would increase the bilayer thickness in the order of 0.2–0.3 nm. This interpretation would provide a mechanism whereby partition of fluid SM brings about the stabilization into the lamellar arrangement of polyunsaturated co-dispersed compounds.

The phase heterogeneity in a mixture of biological lipids is a property consistent with its chemical complexity. The purpose of such complexity is not yet fully understood, but it is likely to give rise to localized domains each with distinctive lipid composition. When subjected to a temperature scan, mixed phospholipids dispersions will undergo a sequence of thermotropic phase transitions which drive the formation of phase-separated domains that are believed to differ widely in composition. For instance, the XRD data suggest an association of high molecular species of SM with the most saturated species of calcium-bound PS to form a gel. Alternatively, the mixing of low molecular weight SM with unsaturated PS and PE results in the formation of a stable fluid lamellar phase. The formation of multiple membrane microdomains in vivo by association of distinct phospholipids classes is conjectural on the basis of present evidence and in any case would be modified by the presence of other membrane constituents.

The results of the present study underline the importance of molecular species of membrane lipids that tend to form a lamellar gel phase at physiological temperatures. Saturated molecular species of PS, for example, can be clustered by interaction of the polar group with calcium ions and associated in the form of a lamellar gel with SM. The presence of saturated and mono-unsaturated molecular species of PS has already been demonstrated in phospholipid extracts of biological membranes (Fridriksson et al. 1999). Possibly greater abundance of the saturated molecular species of PS can be observed in the membrane fractions identified as "detergent resistant membrane" domains where it co-localizes with SM.

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