Biochimica et Biophysica Acta 1788 (2009) 169–177

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Review

# Effects of ceramide and other simple sphingolipids on membrane lateral structure

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ARTICLE INFO

*Article history:*
Received 30 July 2008
Received in revised form 9 September 2008
Accepted 10 September 2008
Available online 20 September 2008

*Keywords:*
Ceramide
Sphingolipids
Membrane domains
Lateral phase separation
Lipid phases

ABSTRACT

The available data concerning the ability of ceramide and other simple sphingolipids to segregate laterally into rigid, gel-like domains in a fluid bilayer has been reviewed. Ceramides give rise to rigid ceramide-enriched domains when their N-acyl chain is longer than C12. The high melting temperature of hydrated ceramides, revealing a tight intermolecular interaction, is probably responsible for their lateral segregation. Ceramides compete with cholesterol for the formation of domains with lipids such as sphingomyelin or saturated phosphatidylcholines; under these conditions displacement of cholesterol by ceramide involves a transition from a liquid-ordered to a gel-like phase in the domains involved. When ceramide is generated *in situ* by a sphingomyelinase, instead of being premixed with the other lipids, gel-like domain formation occurs as well, although the topology of the domains may not be the same, the enzyme causing clustering of domains that is not detected with premixed ceramide. Ceramide-1-phosphate is not likely to form domains in fluid bilayers, and the same is true of sphingosine and of sphingosine-1-phosphate. However, sphingosine does rigidify pre-existing gel domains in mixed bilayers.



**Contents**



## 1. Introduction

Sphingolipids are lipids structurally based on sphingosine, (2S, 3R, 4E)-2-amino-4-octadecene-1,3-diol. They are known to exist in nature with varying degrees of complexity. Sphingosine itself is a biomolecule, thus the simplest of sphingolipids. At the other end of the scale, the more complex gangliosides contain a bulky oligosaccharide moiety. In addition, some sphingolipids are known that contain sphingosine analogues, e.g. the saturated sphinganine, the 20 C-atom eicosasphingosine, or the 4-hydroxylated and saturated phytosphingosine, among others. This review deals with four molecules, namely sphingosine (Sph), N-acylsphingosines or ceramides (Cer), and their phosphorylated derivatives sphingosine-1-phosphate (SphP) and ceramide-1-phosphate (CerP). These four have been called "the simple sphingolipids", in opposition to the more complex sphingomyelin, glycosphingolipids, etc. The present paper deals only with the lateral separation, or domain formation, properties of the simple sphingolipids. The reader is referred to our recent review [1] for the other biophysical properties of these lipids, and to a previous one [2] for the relationship between physical properties and biological effects. Historical aspects and general reviews

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doi:10.1016/j.bbamem.2008.09.002

on sphingolipids can be found in [1,2]. Hardly 2 years have elapsed since our last review covering, among others, this topic [1]. The fact that enough novel data have been published in this period to warrant a new review gives an idea of the liveliness of the field.

## 2. Conceptual and methodological aspects

The idea that cell membranes are not homogeneous at the 0.1–1 μm scale is not new. It was proposed by Jain and White [3] over 30 years ago, mainly on the basis of physical studies in the early seventies of the last century, that showed inhomogenous mixing of lipids in model membranes [4–7]. Now it is widely accepted that such inhomogeneities do exist, so that different regions or "domains" are seen to coexist in the same membrane. These domains differ from each other in composition and physical properties. Sometimes, domains are said to exist in a given "phase". Terminological use is not always accurate in this respect. "Phases" are thermodynamic idealisations defined under equilibrium conditions, characterized by a set of physical parameters, while "domains" are real objects with the physical properties of a given phase [8–10].

For the purposes of this review, only lamellar phases need be considered and, among these, only the gel, or solid-ordered phase ($L_\beta$ in Luzzati's nomenclature), the fluid, or liquid-crystalline, or liquid-disordered phase ($L_\alpha$), and the liquid-ordered ($L_o$) phase [11–13[.

In the gel phase the lipids are virtually immobile ("frozen") with the acyl chains in the highest-order conformation (all-*trans*). In the fluid phase the lipids are free to diffuse, laterally and rotationally, and acyl chains are disordered (low proportion of *trans* C-C conformers). The liquid-ordered phase represents an intermediate situation, in which lipid diffusion is allowed, but the acyl chains exist in a highly ordered conformation (high proportion of *trans* conformers).

Also relevant in this context is the concept of "raft", a particular kind of microdomain. The lipid raft hypothesis was first proposed by Simons and Ikonen [14]. A currently accepted definition of rafts, which is in good agreement with the original proposal, is "Small (10–200 nm), heterogeneous, highly dynamic, sterol-and sphingolipid-enriched domains that compartmentalize cellular processes." [15]. Rafts have been proposed to exist in the $L_o$ phase [8,16].

Methodology is as important in this as in any other area of science. In principle, membrane domains can be either seen under a fluorescence or electronic microscope, or detected using spectroscopic (fluorescence, ESR, NMR…) or calorimetric (differential scanning calorimetry, DSC), or other methods. One disturbing factor of current research on membrane domains is the occasional lack of correlation between results obtained on similar samples using different methods. This occurs most often when results obtained from imaging and from spectroscopic techniques are compared, and the discrepancy may be due to the presence of nanodomains, detected by the spectroscopic techniques, but not by the fluorescence microscope. Methodological matters and apparent discrepancies in the field of membrane domains have been discussed in a recent review [9].

In contrast with the variety of methods that have been used in the study of membrane domain heterogeneity, lipid phases were first described on the basis of X-ray scattering methods [17] and these techniques are still considered as essential in the diagnosis of a lipid phase [12,18].

## 3. Formation of ceramide-enriched domains

In mixtures with phospholipids, ceramides have two main effects, they increase the molecular order of phospholipids, and they give rise to lateral phase separation and domain formation [1].

### 3.1. Ceramide/glycerophospholipid mixtures

Separation into ceramide-rich domains was first observed by Huang et al. [19], who examined the structure of bilayers composed of fully deuterated DPPC and bovine brain ceramide using $^2$H-NMR spectroscopy. These authors observed that addition of ceramide induced lateral phase separation of fluid phospholipid bilayers into regions of gel and liquid-crystalline (fluid) phases, ceramide partitioning largely into the gel phase of $d_{62}$-DPPC. Further $^2$H-NMR studies by Hsueh et al. [20], of mixtures of C16:0 ceramide and $d_{31}$-POPC found that $L_\beta$ and $L_\alpha$ phases coexisted over a wide range of temperatures and compositions, with domains of different composition and physical state being present at physiological temperatures. However, no evidence of liquid–liquid (e.g. $L_\alpha$ and $L_o$) phase separation in the fluid phase was found. A similar behavior of long-chain ceramides had been detected in phosphatidylcholine/phosphatidylserine mixtures [21]. The use of a pyrene-labeled phospholipid, a fluorescent probe that is sensitive to lateral mobility and to the local concentration of fluorophore in the membrane, allowed Holopainen et al. [22] to detect ceramide-enriched microdomains in fluid phosphatidylcholine membranes.

Domain formation by ceramides was also described by a combination of differential scanning calorimetry and IR spectroscopy, using natural ceramides (brain, egg) and several synthetic phospholipids [23]. Calorimetry was used to detect gel-fluid transitions. Different domains, when formed, "melt" at different temperatures, so that they can be easily detected. Veiga et al. [23] found lateral separation of ceramide-rich domains with as little as 5 mol% ceramide. IR spectroscopy, together with the use of deuterated lipids, can be applied to the resolution of the gel-fluid transition in ceramide-rich and -poor domains. Veiga et al. [23] prepared mixtures of deuterated phospholipid ($d_{54}$-DPPC) with natural ceramide. IR spectroscopy clearly reveals a gradual melting of DPPC along a wide range of temperatures (covering the various domains), while ceramide exhibits a much sharper transition, corresponding to the high-temperature melting, ceramide-enriched domains [23]. Carrer and Maggio [24] have studied mixtures of bovine brain ceramide, which contains mostly C18:0 and C24:1 fatty acids, with DPPC, both by differential scanning calorimetry and in lipid monolayers extended at the air–water interface. The calorimetric results are essentially coincident with those by Veiga et al. [23], with in-plane phase separation being hinted at with only 1% ceramide.

Langmuir balance studies have revealed interesting aspects of domain formation by ceramide in phospholipid monomolecular films. Holopainen et al. [25] studied the mixing behaviour of dimyristoyl phosphatidylcholine (DMPC) and either N-palmitoylsphingosine (C16: ceramide) or N-nervonoylsphingosine (C24:1 ceramide). C16:0 ceramide appears to be immiscible with DMPC, while C24:1 ceramide and DMPC are miscible, albeit non-ideally. In both cases fluorescence microscopy reveals the segregation of ceramide-rich and -poor domains, but with very different morphologies. For C16:0 ceramide the dark, ceramide-enriched domains seen under the microscope exhibit a complex, interconnected network with some round domains entrapped into the bright continuum. C24:1 ceramide/DMPC monolayers exhibit flower-like ceramide-enriched, solid domains that do not fuse together even at high surface pressures. Domain shape is the result of a balance between line tension and dipole–dipole repulsion. When the former dominates, round domains with minimum domain boundaries arise, while flower-shaped domains and networks are the result of predominant dipole–dipole repulsion [26]. The different domain morphologies found for the two ceramides in mixtures with DMPC are probably reflecting the different interaction between the film constituents. In spite of the fact that observations in lipid monolayers cannot be directly translated into phenomena occurring in membrane bilayers, the results by Holopainen et al. [25] suggest that different ceramide species may serve very different biological functions, determined by their different impact on the membrane physical properties.

Further study of C16 ceramide/POPC interaction in fluid bilayers [27] shed new light on the ability of ceramides to give rise to rigid, ordered domains under these conditions. These authors have combined fluorescence spectroscopy and transmission electron microscopy to construct a detailed phase diagram for the binary mixtures of the above lipids. The phase diagram is dominated by spherical vesicles in the fluid phase at low ceramide concentrations and high temperatures. Lowering temperature leads to coexisting fluid and ceramide-rich gel phases, and eventually to coexisting POPC-rich and ceramide-rich gel phases. At ceramide concentrations above 50 mol% a new highly ordered ceramide-rich phase appears. Also at these high ceramide concentrations cylindrical membranous structures with round extremities are visible under the electron microscope, while crystalline ceramide structures are only seen above 92 mol% ceramide. In agreement with several previous studies, Silva et al. [27] find significant changes in membrane properties already at 2 mol% ceramide. This, together with their observation that ceramide-rich domains are large in size, support the biological relevance of these studies, considering that ceramide-driven formation of large liquid-protein "platforms" in cell membranes has been proposed to have a key role in viral and bacterial internalization, and in the induction of cell apoptosis by death receptors and stress stimuli [28–30].

The most recent contribution to date by Prieto and co-workers [30bis] describes the behaviour of the very long-chain N-nervonoyl (C24:1) ceramide (NCer), both pure and in mixtures with POPC. The asymmetric length of both NCer hydrocarbon chains leads to transbilayer interdigitation of the short and long chains. NCer exhibits at least two interdigitated gel phases, and a broad gel-fluid transition at ≈52 °C. The solubility of NCer in fluid POPC is low, and the phase diagram is characterized by large regions of coexistence between the interdigitated gel phases and the fluid phase.

The origin of lateral phase separation of ceramide-enriched domains is probably the much higher gel-fluid transition temperature $T_m$ of most Cer species (≈90 °C) [31] compared to that of the phospholipids. Of all the glycero- and sphingophospholipids used in these studies, DPPC exhibits probably the highest $T_m$, and this is only 41 °C [32]. Thus even at temperatures when the phospholipids exist in the $L_\alpha$ phase, ceramide "freezes" into rigid, gel domains, separated from the phospholipid-enriched fluid domains. The high melting temperature is in turn revealing a high intermolecular interaction of ceramides, perhaps favoured by a dense network of H-bonding, and by the relatively small head group. When a particular Cer species has a lower $T_m$, as in the case of NCer mentioned above [30bis], the region of gel-fluid phase coexistence is correspondingly shifted to lower temperatures, always below $T_m$ of the pure ceramide (i.e. below 52 °C in the example above). It has been suggested that molecular dipole potentials would play an important role in lateral phase separation, with unfavourable ceramide-PC dipolar matching explaining domain segregation [24]. A molecular dynamics simulation of a hydrated Cer bilayer at 95 °C [33] shows that, compared to more complex sphingolipids, the lack of a large ceramide polar group leads to a different electron density, thus a different electrostatic potential, but not to a different overall dipole potential. Perhaps it would be appropriate to repeat the simulation at a temperature when the Cer is in the gel phase, and then compare its dipole potential with that of a phospholipid in fluid phase.

### 3.2. Ceramide/sphingophospholipid mixtures

Ceramide-enriched domains in sphingomyelin/ceramide mixtures were described by Sot et al. [34], using mainly differential scanning calorimetry (DSC) and fluorescence microscopy. DSC data showed for pure egg sphingomyelin a rather narrow transition centered at 39 °C. Egg ceramide, even at low proportions (5 mol%) had the effect of widening the phase transition, shifting it to higher temperatures. More important, the endotherms of ceramide-containing samples had a clearly asymmetric shape, indicating formation of high-$T$ melting ceramide/sphingomyelin domains. In fact the observed overall endotherms could be fitted to three-component endotherms, corresponding to as many coexisting domains with different compositions. The

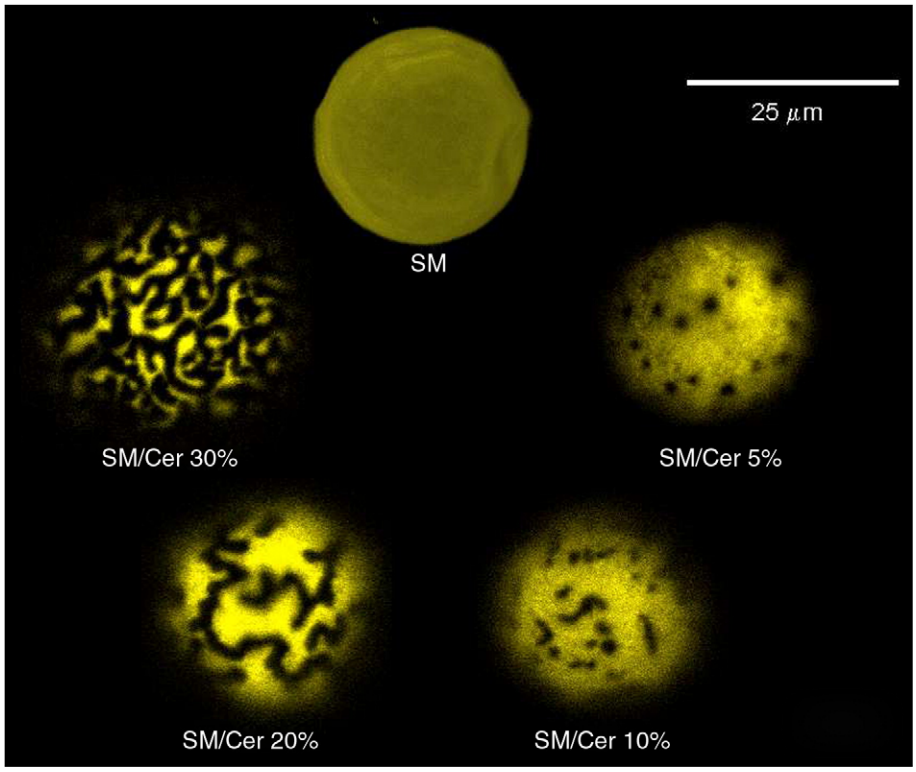


**Fig. 1.** Cer-rich domains in GUV composed of SM/Cer. Images obtained by fluorescence microscopy of DiI-stained vesicles. DiI partitions preferentially into the less ordered domains. Room temperature. Taken from [34], with permission.

corresponding phase diagram includes a wide temperature/composition region of coexisting domains [34]. Essentially similar results were obtained for DPPC/brain Cer mixtures [24], an example of chemically different molecules behaving in the same way due to structural similarities. Furthermore, a series of observations were carried out on giant unilamellar vesicles (GUV) composed of SM and egg Cer, using fluorescence microscopy. The vesicles were doped with the fluorescent probe $DiI_{C18}$, that was excluded from the Cer-enriched, gel membrane regions (Fig. 1). Pure sphingomyelin vesicles appeared uniformly stained, whereas those containing egg ceramide displayed dark areas, corresponding to rigid, ceramide-rich domains. Increasing ceramide concentrations caused a parallel increase in dark areas, that changed their shape from circular to elongated or worm-like. The percolation point for the ceramide-rich domains appeared to occur between 20 and 30 mol% ceramide, at the latter concentration the dark domain being already continuous [34]. Interestingly, these authors found that sphingomyelin/ceramide bilayers were resistant to solubilization by Triton X-100 at 4 °C, the non-solubilized residue being enriched in ceramide.

### 3.3. Ceramides in ternary or complex mixtures

The physical properties of the ternary system PSM/POPC/Cer at 24 °C were systematically explored by Castro et al. [35] using two fluorescent membrane probes, namely DPH and t-PnA. DPH partitions approximately equally between the gel and fluid phases in the PSM/POPC binary mixture [36] but is excluded from the Cer-enriched domains [27]. t-PnA shows a high preference for the Cer-rich gel phase [27]. In the above mixture, the authors find one fluid phase (POPC-rich) and two-gel phases (Cer-rich and PSM-rich), with regions of two-phase coexistence and a region of three-phase coexistence in the triangular phase diagram [35]. Most of the phase diagram is occupied by the Cer-rich gel phase, either alone or in coexistence with the other two. The fluid and Cer-rich gel phases coexist in a broad region that occupied about half the surface of the diagram. In the 10–20 mol% PSM range, 2 mol% Cer is enough to induce gel phase formation. Higher PSM concentrations lead to a region of three-phase coexistence, and eventually to a two-gel phase coexistence region. The work by Castro et al. [35], is a good example of the rigourous application of a quantitative methodology to provide a thorough phase characterisation of a relatively complex system. Even if conducted at 24 °C, there are reasons to believe that the behaviour of that lipid mixture at 37 °C will be qualitatively similar. The fact that gel domain formation by low Cer concentrations is enhanced by PSM at physiological levels (e.g. 20 mol%) suggests that in mammalian plasma membranes SM concentration is such that Cer-rich gel formation is optimised, in order to provide a better response to stress stimuli.

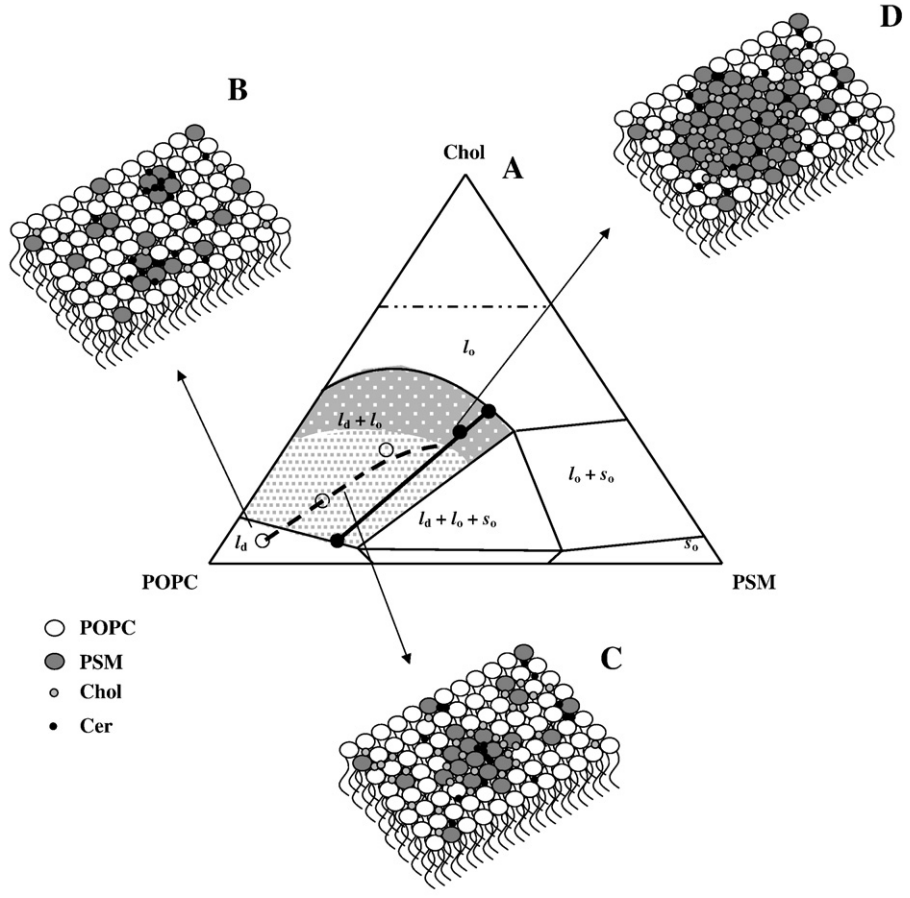


**Fig. 2.** Schematic representation of the effect of PCer in lipid rafts biophysical properties and organization. (A) By recruiting PSM, PCer changes the composition of the mixtures and consequently the position of the tie line of the remaining fluid phase. The dashed black line and the white dots correspond to the estimated composition of the fluid phase that remains after the sequestering of PSM for gel-domain formation (see [36] for details). When increasing Chol content, the effect of PCer is opposed and less PSM is recruited for domain formation until 25%–33% Chol is reached and gel-domain formation completely abolished. Thus, the line that defines fluid phase composition should connect with the tie line in this region. According to this model the $L_o$ mole fraction $X_{lo}$ should decrease from 26% and 58% to 21% and 54%, respectively, when 4% PCer is present. (B) Starting from the 100% $L_\alpha$ phase one PCer molecule recruits up to three PSM molecules and forms highly ordered PCer/PSM-gel domains. The amount of gel formed is considerable, $X_{gel} \approx 15\%$, but the size of the domains is small, ≈4 nm. Panels B, C, and D are a pictorial top view of the bilayer because ≈250 molecules should be involved in the formation of a nanodomain of this dimension. (C) In the low-to-intermediate $X_{Chol}$, i.e. in the range of small sized rafts, PCer/PSM-gel domains are still present and are surrounded by lipid rafts ($L_o$ phase). FRET experiments show that PCer is not forming platforms or promoting the coalescence of the small rafts into large ones. (D) In the high Chol, large sized rafts range, PCer ability to form gel domains with PSM is abolished by the presence of Chol that competes for the association with PSM. In this situation, lipid rafts are governing membrane properties. Taken from [36], with permission.

Cholesterol introduces a further degree of complexity in these mixtures. Fidorra et al. [37] provide a fine example of this assertion, in their work in which binary (POPC/Cer) and ternary (POPC/Cer/Chol) mixtures are studied with a combination of calorimetric, spectroscopic and microscopic techniques. The binary mixture exhibits gel-fluid phase coexistence over a broad composition and temperature span. Addition of Chol causes a gradual ordering effect of the fluid phase, with smaller changes in the Cer-rich gel phase, up to a certain Chol concentration. Above that point (e.g. 22 mol% Chol in POPC/Cer 5:1 mol ratio), a complex situation occurs in which three regions coexist in the same GUV, that the authors assign tentatively to one gel and two ordered phases. Interestingly, no coexistence of $L_o$ and $L_\alpha$ phases was observed in the POPC/Cer/Chol mixture, at variance with systems in which SM (i.e. ceramide phosphorylcholine) was used instead of Cer [38,39].

Mixtures containing all four lipids, i.e. POPC, SM, Chol, and Cer were explored by Silva et al. [40] using the fluorescence spectroscopy methods applied to the construction of phase diagrams by Prieto and co-workers in a number of systems. Their results can be summarized in Fig. 2. As in the case of Fidorra et al. [37] two situations can be considered, above and below a range of Chol concentrations that for the quaternary mixture of Silva et al. occurs at 25–33 mol% [40]. At low Chol concentrations, Cer sequesters PSM for gel formation, thus the proportion of fluid phase decreases. At this stage the size of the gel domains is small, ≈4 nm (Fig. 2B). In the low-to-intermediate range of Chol concentrations, 10–25 mol%, in which de Almeida et al. [36, 39] had observed "small sized rafts", Cer/PSM-enriched gel ($L_\beta$) domains are still present, surrounded by lipid rafts, in the $L_o$ phase (Fig. 2C). Finally in the high-Chol region,>25 mol% characterized by the presence of large rafts [36, 39], Cer ability to form gel domains with PSM is abolished by the presence of Chol that competes for the association with PSM. In this situation, large $L_o$ and $L_d$ domains coexist, and, in the words of Silva et al., "lipid rafts are governing membrane properties" [40]. This study has some important implications, namely: (i) in lipid mixtures mimicking the outer leaflet of mammalian plasma membranes, <25 mol% Chol, the large "Cer-platforms" proposed by several authors [41,42] and indeed found in binary mixtures [27,34] do not appear to exist in the more complex systems, small "islands" of $L_\beta$ phase surrounded by small $L_o$ domains being found instead (but see Section 5 below). (ii) According to Silva et al. [40] each Cer molecule recruits more than one PSM molecule in $L_\beta$-domain formation. This would constitute an amplification mechanism for a sphingomyelinase activity elicited in response to a stimulus. (More on sphingomyelinases in Section 5). Finally, some of the results of Silva et al. [40] are explained in terms of a competition between Chol and Cer for association with SM. This interesting subject is dealt with in the following section.

Montes et al. [42bis] have recently provided an example of Cer-enriched domain formation in a highly complex mixture, namely the erythrocyte membrane. It has been known for a long time that SM-ase activity caused the so-called "hot–cold" haemolysis, i.e. that red blood cells incubated at 37 °C with the enzyme underwent only slow haemolysis, while, upon transferring the cells to 4 °C, rapid and extensive haemolysis took place. Montes et al. have shown that, under these conditions, Cer-enriched domains are formed and that, at 4 °C, they constitute rigid membrane regions that confer a high fragility to the normally elastic membrane. As a result of this fragility the erythrocytes break down massively [42bis].

Before leaving the subject of Cer-rich domain formation in complex mixtures, we would like to note that some lessons can be learned from the behaviour of Cer in a rather different system, namely the *stratum corneum* of the skin. In fact, an important observation related to domain formation by ceramides in phospholipid bilayers comes from the studies by ten Grotenhuis et al. [43] involving ceramides, cholesterol, and free fatty acids, that is, the lipids believed to constitute the stratum corneum matrix. These authors examined an extensive series of binary and ternary mixtures of the above lipids in the form of supported Langmuir–Blodgett monolayers. The monolayers were probed by atomic force microscopy. C16-ceramide mixed well with cholesterol, but the longer-chain ceramides did not, and lateral phase separation was observed under a variety of conditions. In a more recent paper [44] neutron diffraction has been applied to a mixture of Chol, Chol sulphate, free fatty acids, and a Cer [N-($_\alpha$-hydroxioctadecanoyl)-phyto-sphingosine]. It is interesting that, in this system, free fatty acids are forced by ceramide to occupy a bilayer of a given thickness, so that fatty acid interdigitation occurs when chain length is >C16. Also, long-chain free fatty acids are less miscible with Cer, and tend to form a separate "fatty acid-rich phase". To what extent these observations can be translated to lipid mixtures containing sphingomyelin and phospholipids remains an open, and interesting, question.

## 4. Ceramide displacement by cholesterol

Some basic aspects of Cer–Chol interactions, as studied by Schefler et al. [45] and by Massey [46] were discussed in our previous review [1]. The observation by London and co-workers [47] that Cer displaces Chol from $L_o$ domains containing PC, or SM, and Chol is very interesting, and has had implications for our understanding of these systems. Recent work from our laboratory [47bis] has provided morphological evidence of the generation of Cer-enriched gel domains within $L_o$ domains in GUV composed of PC:SM:PE:Chol upon addition of Cer. Cholesterol displacement by ceramides has also been described by Slotte and co-workers [48,49], and by Chiantia et al. [50]. Tight lipid packing is critical for sterol displacement by ceramide. The authors suggest that the driving force of the event is the hydrophobic effect that tends to minimise unfavourable contact between the hydrocarbon groups of the small headgroup lipids (ceramides, sterols) and the surrounding aqueous environment. Hydrocarbon-water contact would be prevented in ordered domains because of the capacity of certain lipids with large headgroups (sphingomyelin, phosphatidylcholines) to accommodate small headgroup lipids in the ordered domain lattice. Ceramide would replace sterols with advantage from this point of view. (See in this respect the "umbrella model" proposed for phospholipid/cholesterol mixtures by Huang and Feigenson [50bis], and the "molecular cavity" concept developed for ganglioside/ceramide mixtures by Carrer and Maggio, [51]).

Further work on this subject by Megha et al. [52,53] has consolidated the idea that Cer and Chol compete for the SM-based $L_o$ domains. These authors studied the effect of a number of Chol precursors on a SM/Cer mixture from the points of view of (i) thermal stabilization of the Cer-rich complexes, and (ii) displacement of Chol precursors from SM/sterol mixtures by Cer. There was a reciprocal correlation between both effects, precursor displacement by Cer and Cer displacement by Chol precursors. Precursor displacement by Cer was greatest for the precursors displaying highest $L_o$-phase stabilizing properties. The latter study was complemented by one [53] in which the effect of Cer N-acyl chain and polar headgroup structure on the properties of SM-rich domains was examined. For this purpose, SM/DOPC/Cer mixtures (32:50:18 mol ratio) were prepared with different ceramides. The thermal stability of the SM-rich domains increased with Cer N-acyl chain length, in the order C2≈C6≈C8<no Cer<C12<C16. This is in agreement with other studies on the properties of different chain length ceramides [54–56]. In the presence of Chol, i.e. mixtures SM/DOPC/Chol/Cer (20:18:37:25 mol ratio), Megha et al. [53] found that stability increased in the order C8≈C6<C2<C12<no Cer<C16. Nybond et al [49], studying the ability of ceramides to displace sterols from liquid-ordered domains, found that an N-acyl chain with at least 8 methylene units was required for a ceramide to displace cholesterol. Variations in sphingoid base structure had in general little effect on the stability of the SM-rich

domains [53]. These results reinforce the hypothesis (see above section on Ceramide/glycerophospholipid mixtures) that the high-melting temperatures of ceramides explain the phenomenon of lateral phase separation. In addition, it should be noted that, as expected, only those (long-chain) Cer, that stabilize the bilayer can segregate laterally. Short-chain Cer, that promote inverted hexagonal phase formation [32] cannot stabilize SM-rich domains, as observed by Megha et al. [53]. A word of caution should be given here on the nomenclature of the SM-rich domains in the presence of Cer. Some authors have referred to them as "ordered domains". This should not be necessarily understood as "domains in the $L_o$ phase". SM/Chol domains will certainly be in the $L_o$ phase, but as soon as some Cer is introduced, SM/Chol/Cer domains will exist in the gel, or $L_\beta$ phase, as witnessed by the appearance of a cooperative endotherm in DSC thermograms, and by a variety of other spectroscopic and microscopic data [35,36,50]. An additional, relevant comment in this context is that certain spectroscopic techniques can show Chol displacement by Cer but they cannot demonstrate formation of Cer-enriched domains, e.g. [49,53], while these domains are clearly detected by morphological/ultrastructural techniques e.g. [40, 47bis, 50]. On the methodological problems of domain detection by spectroscopic and microscopic techniques, see [9].

In a related, interesting work, Ali et al. [57] studied POPC/Chol/Cer mixtures at Chol concentrations approaching the solubility limit of the sterol, at the $L_o$-crystal phase boundary. Under those conditions, Cer was observed to drive Chol from the $L_o$ phase to Chol crystals. Interestingly, at least under the conditions of this experiment, the displacement of Chol by Cer follows a 1:1 ratio [57]. In the same context of Cer-Chol competition, Pandit et al. [58] simulated POPC/Chol/Cer bilayers at varying Cer concentrations. Molecular dynamics calculations suggest that Chol and Cer have a very similar effect on the POPC bilayer, although, somewhat surprisingly, the authors find Cer less effective than Chol in inducing order in the bilayer.

A methodological note to close this section: Bakht et al. [59] have described the properties of a novel fluorescence anisotropy probe, the phenyltetraene lysophospholipid analogue PTE-ET-18-OMe, that has the interesting property that, while having a moderate affinity for both $L_\beta$ and $L_o$ domains , it is not displaced from $L_o$ domains by Cer, at variance with fluorescent sterols or DPH. The ability of PTE-ET-18-OMe to be associated to Cer-rich domains to a much greater degree than DPH is likely to find useful applications in the field.

## 5. *In situ* generated ceramide by sphingomyelinase

Sphingomyelinases (SMases) hydrolyse SM yielding Cer and water-soluble phosphorylcholine. SM degradation by SMases is accepted as the main source of Cer in the early stages of the sphingolipid signalling pathway (see reviews in [2,40,50,60]). Thus it is relevant to the cellular situation to what extent Cer generated *in situ* by SMases segregates laterally in the same way as Cer added as one of the components during vesicle preparation. The first evidence of SMase-induced formation of Cer-rich domains was provided by Holopainen et al. [61]. A parallel kinetic study of ceramide generation and formation of ceramide-rich domains [61] revealed that, under conditions allowing fast ceramide synthesis, changes in the membrane lateral organization took significantly longer time to occur (one order of magnitude, under the conditions of the experiment).

The experiments by Holopainen et al. were performed on SM-containing vesicles. Maggio and co-workers have provided careful data on a similar system, except that the lipid was organized in monolayers at the air–water interface and domain formation was studied by epifluorescence [62,63]. The details of these elegant studies have been summarized in our previous review [1]. Suffice it to remember here that, also in this system, formation of ceramide alters the surface topography by inducing phase separation into condensed (Cer-enriched) and expanded (SM-enriched) domains [62]. Also significant is the observation by Fanani et al. [62] that the surface topography derived from the sphingomyelinase-driven reaction is different from that obtained by premixing sphingomyelin and ceramide in the same proportion, indicating that the information contents depends on the manner in which the surface was generated. The same difference between lateral phase segregation induced by externally-added or by SMase-generated Cer was noted in a systematic study of domain shapes [63]. SMase gives rise to a series of well-defined patterns, that evolve with time and with Cer concentration. These patterns are not seen when Cer and SM are mixed and extended onto the air–water interface. The observed differences between pre-mixed and *in situ* generated Cer can be simply due to the fact that in the former case the system would be in a quasi-equilibrium, while in the latter it would clearly be out of equilibrium. However this matter requires further study for a convincing explanation to be attained.

*In situ* generation of Cer in supported bilayers consisting of ternary mixtures (DOPC/SM/Chol) has been monitored by a combination of atomic force (AFM), and fluorescence microscopy [50,64,65]. Chiantia et al. [50], starting with an equimolar DOPC/SM/Chol mixture, find that ceramide formation due to SMase activity causes changes in the height and shape of the lipid domains, similar to those found with pre-mixed Cer, only higher Cer proportions are required in the latter case. Ira and Johnston [64,65] describe that AFM of a DOPC/SM/Chol 2:2:1 mixture shows separation of SM/Chol-rich $L_o$ domains, about 1.1 nm higher than the surrounding fluid phase. SMase activity produces heterogeneous domains with many raised subdomains (about 2 nm high) and coalescence of domains leading to heterogeneous clusters with diameters 1 μm and beyond. The increased height of these Cer-containing domains has been attributed to ceramide flip–flop motion to the lower leaflet with formation of a raised subdomain due to negative monolayer curvature [65]. Bilayers containing premixed ceramide give rise to very similar heterogeneous domains, but no domain clusters are observed [64] in agreement with the spectroscopic observations of Silva et al. [40]. The observation of SMase-induced domain clusters is therefore interesting since it can be related to the proposed Cer-platform formation upon cell activation [41,42]. However, the authors themselves [64,65] point out that the observed clusters contain probably more Cer and less Chol than would the proposed "platforms".

As a consequence, the clusters in [64,65] would probably be in a physical state more akin to a $L_\beta$ than to a $L_o$ phase, the latter being the one predicted for the "platforms". Nevertheless it could also be argued that no direct evidence exists about the physical state of the "platforms". As pointed out by Carrer et al. [66] gel phases induced by natural ceramides would be more fluid-like than an ordinary phospholipid $L_\beta$ domain.

SMase activity in Chol-containing bilayers has been applied to the study of Chol displacement by Cer. Taniguchi et al. [66bis] used Cer generation by SMase to displace Chol from $L_o$ domains and observed the subsequent formation of gel regions in GUV.

## 6. Short-chain ceramides

Ceramides with an N-acyl chain shorter than 8–10 C atoms are usually called "short-chain ceramides". Since most naturally occurring ceramides cannot be dispersed in water because of their hydrophobicity, short-chain ceramides, and particularly N-acetylceramide, or C2-ceramide, have been extensively used as agonists when ceramide effects had to be elicited, both in intact cells and in cell-free systems. The advantages and drawbacks of the use of short-chain ceramides in experimentation, and their different physical properties, have been discussed elsewhere [1,2]. From the point of view of lateral domain segregation, the above-mentioned (section 4) studies by Megha et al. [53] are relevant. These authors find that in SM/DOPC/Cer mixtures, both in the presence and in the absence of Chol, short-chain

ceramides, i.e. C8 and shorter, fail to stabilize the SM-rich domains. In fact, in a DMPC/C8-Cer mixture [66], far from detecting a high-melting compound, Carrer et al. observe that small amounts of C8-Cer cause a decrease in the transition temperature of DMPC, that they interpret (also on the basis of AFM and ESR spectroscopy measurements) as an indication of hydrocarbon chain interdigitation. At 30 mol% C8-Cer the mixture behaves as a stoichiometric compound, with a single gel-fluid transition centred at ca. 26 °C. Chiantia et al. [67] have combined AFM and fluorescence correlation spectroscopy to study formation of Cer-enriched gel-like domains in membranes containing coexisting $L_d$ and $L_o$ regions, using ceramides with different N-acyl chains. They found that only C16-and C18-ceramides were able to generate Cer-enriched domains segregated from the $L_o$ phase. From these and other studies, including our own [55, 67bis], on the behaviour of C2-, C6-, and C16-ceramides in phospholipid bilayers, we conclude that only long-chain ceramides, i.e. those with "symmetric" N-acyl and sphingosine chain lengths, give rise to lateral segregation of gel-like Cer-enriched domains.

## 7. Transmembrane ceramide channels

A very special mode of lateral segregation of ceramides has been proposed by Colombini and co-workers [55,68,69], according to which spontaneous formation of transmembrane channels would occur, composed of pure ceramide, with ceramide chains parallel to the plane of the membrane. Short-chain ceramides, e.g. C2, would also be able to form such channels. A recent molecular model [70] describes the channel as consisting of columns of four to six Cer H-bonded via amide groups and arranged as staves in either a parallel or antiparallel manner. However, such structures would be highly unfavourable from a thermodynamic point of view. For the time being, direct structural evidence is lacking.

## 8. Other simple sphingolipids

Although ceramides are by far the "simple sphingolipids" that have been studied in more detail, some data from the other less complex sphingolipids are also available. Results for ceramide-1-phosphate (CerP) have been recently published [71]. CerP can form bilayers and closed vesicles by itself. At low *T*, CerP forms a highly organised subgel (crystalline) bilayer phase ($L_c$), which transforms into a regular gel phase ($L_\beta$) at 45 °C, with the gel-to-fluid phase transition ($L_\beta$–$L_\alpha$) occurring at ≈65 °C. When incorporated into dielaidoylphosphatidylethanolamine (DEPE) bilayers, that exhibit a $L_\beta$–$L_\alpha$ transition at 37 °C, CerP mixes well with the lipid in fluid phase, although below the DEPE transition temperature CerP in $L_c$ phase may coexist with almost pure DEPE in $L_\beta$ [71]. These properties suggest that no CerP-rich domains are likely to be found under physiological conditions.

Sphingosine (Sph) constitutes yet a different case. According to Contreras et al. [72], Sph mixes well with glycerol-and sphingophospholipids, no lateral phase separation being detected. However, in mixed bilayers in which $L_\beta$ and $L_o$ domains coexist, e.g. DPPC/Chol (80:20 mol ratio), SM/Chol (80:20 mol ratio), or SM/PE/Chol (50:25:25 mol ratio), Sph increases the transition temperature, and transition enthalpy, and narrows the transition width of the $L_\beta$ phase (Fig. 3). This is interpreted in terms of Sph mixing with the lipids in the gel phase and giving rise to more rigid, more cooperative domains, coexisting with $L_o$ phase domains. The rigidifying effect of Sph was further demonstrated as an increase in DPH polarisation upon addition of Sph to lipid bilayers [72]. Thus Sph does not form gel domains by itself, but reinforces pre-existing ones.

Finally recent data from our laboratory on sphingosine-1-phosphate (SphP) (J. Sot, unpublished) show in general good mixing of SphP with DEPE, even at equimolar ratios. At 10 mol% SphP and above, small shoulders are seen in the DSC thermograms, but they are too small to allow (or even justify) undergoing a signal decomposition procedure. IR spectroscopy also fails to reveal formation of SphP-enriched domains.

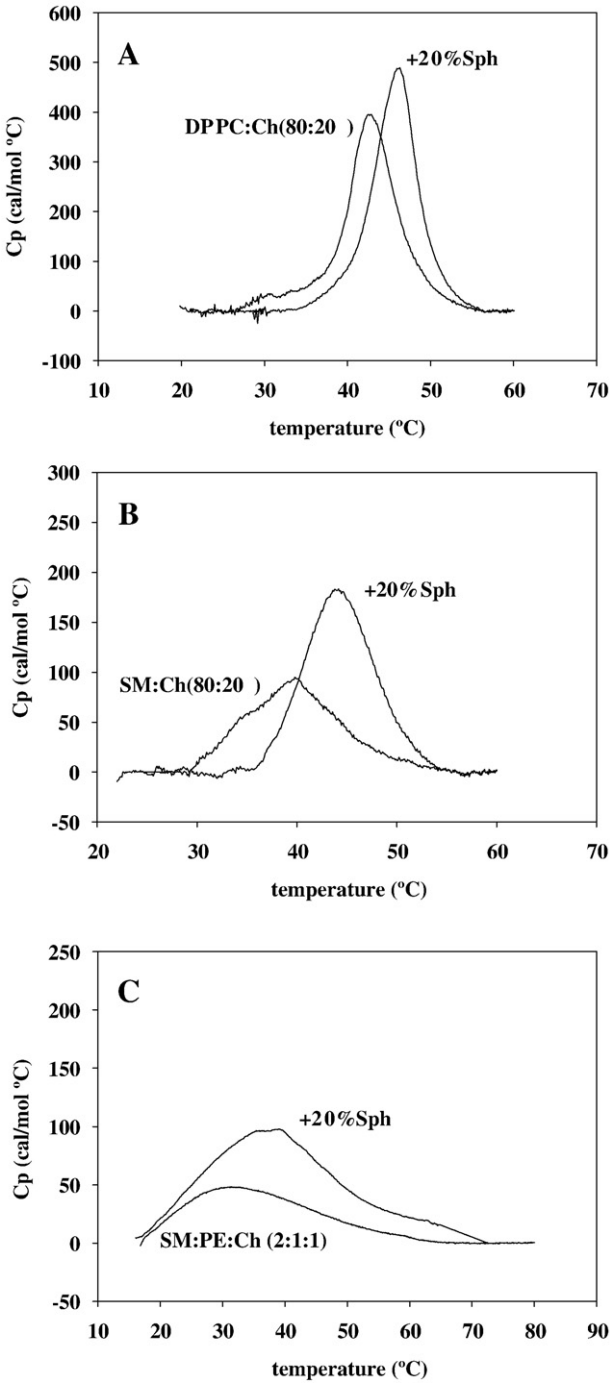


**Fig. 3.** Effect of sphingosine on the gel-fluid transition of different lipid mixtures. (A) DPPC/Chol (80:20, mol ratio)±20 mol% sphingosine. (B) SM/Chol (80:20, mol ratio)± 20 mol% sphingosine. (C) SM/PE/Chol (50:25:25, mol ratio)±20 mol% sphingosine. Representative thermograms of the second or third scans. Taken from [72], with permission.

## Acknowledgments

The authors are indebted to Fundación Areces (contract No. 06/01) for generous financial help. The experimental work in the authors'

laboratory described in this review was supported by funds from the Spanish Ministerio de Educación y Ciencia, and from the Basque Government.

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