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Review

The liquid-ordered phase in membranes

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ABSTRACT

A range of physiological processes has been imputed to lateral domain formation in biological membranes. However the molecular mechanisms of these functions and the details of how domain structures mediate these processes remain largely speculative. That domains exist in biomembranes and can be modeled in relatively simple lipid systems has contributed to our understanding of the principles governing phase behaviour in membranes. A presentation of these principles is the subject of this review. The condensing effect of sterols on phospholipids spread as monomolecular films at the air–water interface is described in terms of the dependence of the effect on sterol and phospholipid structure. The thermodynamics of sphingomyelin–cholesterol interactions are considered from calorimetric, densitometry and equilibrium cholesterol exchange measurements. Biophysical characterisation of the structure of liquid-ordered phase and its relationship with liquid-disordered phase is described from spectroscopic and X-ray scattering studies. Finally, the properties of liquid-ordered phase in the context of membrane physiology and permeability barrier properties are considered.

Contents

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1. Introduction

One of the enduring sources of scientific intrigue with regard to the properties of cell membranes is the highly complex assortment of polar lipids that make up the bilayer matrix. Why does such diversity exist when most membrane functions, like the permeability barrier properties and catalytic activity of intrinsic membrane proteins, can be reconstituted using one or a mixture of a few molecularly-defined lipids? Likewise, our knowledge of the biochemical pathways that operate to create and preserve the particular lipid composition of each morphologically-distinct membrane in living cells within relatively narrow limits is incomplete.

Despite our ignorance of the reasons for lipid diversity in biological membranes the arrangement of particular lipid classes on either side of the bilayer structure and the enzymic processes responsible for maintaining lipid asymmetry are known in some detail [1,2]. There is also a great deal known about how this asymmetry is exploited in a range of different physiological responses [3]. Currently interest is turning to the lateral asymmetry of lipid distribution fueled by the concept of membrane rafts and speculation about their possible role in trans-membrane signal transduction [4].

Given the observed diversity of membrane lipids there is obviously scope for specific interactions between different molecular species of lipid in biological membranes. The interaction between sterols and phospholipids to create ordered domains in monolayer and bilayer membranes is of particular interest in this context. We aim to review current knowledge of factors leading to formation of ordered lipid structures in membranes.

2. Molecular dynamics simulations of lipid mixtures

Molecular dynamics simulations have been greatly refined over recent years to include realistic ensembles of molecules and water in bilayer configuration. This has been driven largely by considerable strides in computing power allowing simulations to extend to time domains approaching equilibrium. Studies of lipid–water systems have been performed to provide a strong theoretical basis upon which the results of experiments may be tested.

Molecular dynamics simulation studies have been undertaken to derive interaction energies and mutual orientations between cholesterol and phospholipids. Early studies of binary mixtures were unable to identify complex formation between cholesterol in bilayers of phospholipids until the introduction of hydrogen bonding between the lipids was invoked to explain structures formed by cholesterol and diacylglycerophosphatides [5,6]. A molecular dynamics study has been reported to investigate the hydrogen bonding propensity of sphingomyelin (SM) in aqueous systems [7]. In a series of 3ns simulations of C16:0-SM in water it was found that the OH and NH groups of the sphingosine can form hydrogen bonds with the phosphate oxygens of their own polar headgroup. Simulations of C16:0-SM in bilayers, on the other hand, showed that the OH group of the sphingosine was predominantly involved in intramolecular hydrogen bonds while the amide NH moiety was almost exclusively involved in intermolecular hydrogen bonding. It is in this context that the interpolation of cholesterol into the network of sphingomyelin must be considered.

Comparisons of binary mixtures of cholesterol with glycerophospholipids and sphingomyelin have concluded that the mode of interaction of the sterol is different in the respective phospholipids and this is attributed to their particular structures [8]. In a series of molecular dynamics simulations of hydrated bilayers of binary mixtures of cholesterol with stearoyl and oleoyl derivatives of sphingomyelin and 1-palmitoyl-2-oleoyl-*sn*-glycerophosphocholine (POPC) it was found that energies of interactions between cholesterol and all three phospholipids were not significantly different [9]. This is rather reassuring in the case of the formation of ordered domains in membranes because a preferential interaction with one or other phospholipid would be antithetical to the preservation of their dynamic properties. A difference in the hydrocarbon chain ordering and structures formed between cholesterol and sphingomyelin on the one hand and phosphatidylcholine on the other must distinguish the two cases.

Attempts to clarify the relative interaction affinities between cholesterol and glycerophospholipid and sphingomyelin have been reported by molecular dynamics simulations of tertiary mixtures of the three lipids [10,11]. In a mixture comprised of palmitoyl-oleoyl-PC/C16:0-SM/cholesterol, 62:1:1 a snapshot of one monolayer of a simulated array showed half of the cholesterol formed dimers with the sphingomyelin distributed in a fluid phase of glycerophospholipid. It was concluded that direct hydrogen bonding alone between sphingomyelin and cholesterol could not explain the preferential interaction observed between the two lipids. Instead a combination of hydrophobic entropic forces, charge pairing and van der Waals interactions results in a lower tilt angle in the sphingomyelin adjacent to cholesterol compared to that in cholesterol with only palmitoyl-oleoyl-PC neighbours. This inferred that the hydrocarbon chains of sphingomyelin were ordered more efficiently by cholesterol than they were in the glycerophospholipid. This particular effect of the tilt angle of cholesterol in the structure is apparently due to the two methyl groups on the steroid ring system because the tilt angle increases on removing these methyl groups [12].

3. Monomolecular films

Monolayers spread at the air–water or oil–water interface have long been used to characterise the surfactant properties of membrane lipids and their synthetic analogues. The method has proved particularly valuable for investigating the interactions between lipids because, unlike assemblies of lipids in aqueous systems, the lateral pressure can be varied over a wide range up to the collapse pressure. While the relationship between the properties of monolayers and bilayers may be a moot point the principles governing lipid–lipid interactions can be clearly defined by monomolecular film studies. Where contention arises is in the fact that the density of lipids in a bilayer is constrained within narrow limits equivalent to surface pressures in monolayers in the region of 32–34 mN/m. Moreover, there is evidence to suggest that domains within bilayers are tightly coupled so that lateral phase separations can be mediated by contacts between molecules on either side of the structure. Coupling effects are absent in monomolecular films and domain formation is solely dependent on lateral interactions between the molecules of the film.

Molecular dynamics simulations have therefore been useful in characterising the nature of hydrogen bonding in pure sphingomyelin systems particularly intermolecular as opposed to lipid–water hydrogen bonds as well as hydrogen bonds between cholesterol and phospholipids. The fact that interaction energies between diacyl phospholipids and sphingomyelins with comparable hydrocarbon chain structure are similar emphasizes the importance of the differences in hydrocarbon chain substituents of the different phospholipid classes observed in biological membranes on how the membrane cholesterol is distributed in the bilayer. Finally, cholesterol has been shown to have a particular effect on the orientation of sphingomyelin in the bilayer that distinguishes the structure of Lo phase in bilayers.

3.1. Characterisation of mixed monolayer properties

Monolayer techniques have evolved steadily with improvements in sensitivity and reliability. The automatic balance for applying slow compression rates allows films such as mixed monolayers of sphingomyelin and cholesterol to achieve a steady-state equilibrium which is certainly relevant for the highly viscous lipid at temperatures

lower than Tm. Repeated compression–expansion cycles can be used to monitor hysteresis effects and check out the formation of extended gel state SM domain from which cholesterol is excluded. Experimental conditions involving an inert atmosphere of nitrogen or argon in the gas phase protects the lipid from oxidation. However little is known of the "solvent-effect" of the intercalated inert gas around the acyl chains. Specific methods have been applied to investigate the interaction between sphingomyelin and cholesterol including enzyme intercalation (sphingomyelinase, cholesterol oxidase) and cholesterol desorption by β-methyl-cyclodextrin.

3.1.1. Enzyme intercalation: sphingomyelinase and cholesterol oxidase assays

The interaction of bacterial sphingomyelinase with substrate monolayers has been investigated to define how the quality of the buffer/membrane interface influences the activity of the enzyme acting at the interface [13]. The enzyme reaction was carried out in a zero-order trough using a surface barostat, an approach that enabled precise control of the physico-chemical properties of the substrate monolayer. Since the molecular area of the ceramide product at the interface is smaller than that of the substrate sphingomyelin the hydrolysis reaction could be monitored from the decrease in area of the monolayer at constant surface pressure. The hydrolysis reaction could be divided into two distinct phases, an initial lag-phase, measured as the time between addition of the enzyme to the subphase and commencement of the change in monolayer area, and the subsequent phase representing the actual hydrolysis reaction from which a maximal degradation rate could be determined. The activity of sphingomyelinase (*Staphylococcus aureus*) toward bovine-brain sphingomyelin (bb-SM) was found to be markedly enhanced by Mg^{2+} with maximal activation at 5 mM. Mg^{2+} also influenced the length of the lag-phase of the reaction such that the lag-time increased markedly when Mg^{2+} concentrations decreased below 1 mM. Saturated sphingomyelins (bb-SM and C16:0-SM) were digested more slowly than the mono-unsaturated C18:1-SM. Both bb-SM and C16:0-SM monolayers underwent a phase transition at about 20 °C, whereas C18:1-SM monolayers remained in a liquid-expanded phase over a wide range of temperatures. Substrate monolayers in the liquid-condensed phase have significantly longer lag-times than did monolayers in the liquid-expanded state. This is consistent with the need for the enzyme to penetrate between the substrate monolayers at the interface and to orient in a favourable way for hydrolysis to take place. This is also consistent with the observation that sphingomyelinase activity is also sensitive to the lateral surface pressure of the substrate monolayer. Maximal degradation rates are achieved with surface pressures in monolayers of bb-SM of 20 mN/m at 30 °C; above this pressure the lag-time of the reaction increases sharply. As regard to the formation of liquid-ordered domains it is interesting to note that inclusion of as little as 4mol% of cholesterol into a [^{3}H]-sphingomyelin monolayer markedly increased the extent of [^{3}H]-sphingomyelin degradation, and shortened the lag-time of the reaction. The inclusion of 10mol% of zwitterionic or negatively-charged phospholipids to the [^{3}H]-sphingomyelin monolayer did not affect the sphingomyelinase reaction significantly [13]. How such low concentrations of cholesterol affect the overall activity of sphingomyelinase can only be conceived if the diffusion of cholesterol in the sphingomyelin monolayer is extremely fast. This is also consistent with the view of a liquid-ordered phase formed by the assembly of very small metastable domains fluctuating rapidly between the liquid-ordered and disordered states. This view has been supported further on by the ESR measurements consistent with liquid-ordered nanodomains (see Section 5.1).

The action of cholesterol oxidase on mixed monolayers of sphingomyelin and cholesterol has provided complementary information on the structure of these films independent enzyme specific requirement [14]. Mixed monolayers, containing a single phospholipid class and cholesterol at differing cholesterol/phospholipid molar ratios, were exposed to cholesterol oxidase at a lateral surface pressure of 20 mN/m at 22 °C. With monolayers comprised of equimolar ratios of cholesterol and phospholipid, the rate of cholesterol oxidation was found to be greatest with admixture with unsaturated phosphatidylcholines (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine and egg-yolk phosphatidylcholine), intermediate with 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine, and slowest with sphingomyelin monolayers (egg-yolk or bovine-brain sphingomyelin). The rate of oxidation in mixed monolayers was not only dependent on packing density of the lipids in the monolayer since cholesterol in egg-yolk and bovine-brain sphingomyelin were oxidized at different rates despite the fact that the two phospholipids occupy similar mean molecular areas. This infers that the phospholipid acyl chain composition influences the positioning of cholesterol to protect oxidation. Variation in the proportions of the cholesterol and phospholipid in the monolayer showed discontinuities in the otherwise linear rate of hydrolysis at a 1/1 stoichiometry with phosphatidylcholine mixed monolayers and at a 2/1 cholesterol/sphingomyelin stoichiometry with sphingomyelin mixed monolayers. This difference between SM and PC is reminiscent of molecular dynamics simulations showing that the strong hydrogen bonding properties characteristic of C16:0-SM results in significant structural changes in the polar headgroup and interface regions [15]. The existence of intermolecular hydrogen bonds is manifest in the differences in area/molecule at comparable surface pressures. Thus the area per molecule of C16:0-SM is 0.52 nm^2 at 30 °C and 30 mN/m [16] but is 0.65 nm^2 per molecule for dipalmitoylphosphatidylcholine (DPPC).

Molecular simulations show that the headgroups of DPPC are on average lying almost precisely along the surface of the bilayer (0° relative to the interface) but there is a distinct orientation of the phosphorus–nitrogen alignment (P–N orientation) in C16:0-SM. The most frequent orientation is + 15° toward the interior of the bilayer and a significant number of SM molecules are oriented toward the aqueous phase (− 35° relative to the interface) [15].

3.1.2. Surface potential measurements

Surface potential measurements that represent the sum of vertically-oriented dipoles *in the area investigated (ca mm^2) by the measurement* confirm that the changing orientation assumed for the dipole P^-–N^+ in DPPC but constant orientation for sphingomyelin is in agreement with molecular simulations [17]. A reorientation of molecular dipoles takes place upon compression of the monolayers which leads to changes in the hydration of the polar headgroups [18] and reorganization of the vicinal water layers [19]. The relatively flat slopes of surface potentials in monolayers consisting of palmitoyl-oleoyl-PC mixed with high proportions of egg-sphingomyelin (e.g. 90 and 100mol%) indicate few dipole reorientations take place during compression of the films. This is in agreement with an initial alignment of the molecules upon domain formation that does not change upon further compression due to the strong intermolecular interactions. In POPC enriched domains a progressive decrease in interfacial junction potential is observed. It is noteworthy that in monolayers comprised predominantly of palmitoyl-oleoyl-PC there is no significant domain formation in the film. A plot of dipole moments as a function of the proportion of egg-sphingomyelin in monolayers at a constant area/molecule of 0.75 nm^2/molecule is presented in Fig. 1. The films with this density are at a surface pressure of about 35 mN/m, which is close to the proposed lateral pressure in biological membranes [20] but less than the collapse pressure of the monolayers. In monolayers with low proportions of SM (10 and 20mol% SM) the dipole moment decreases as compared to additivity and increases at higher SM contents, particularly at 80mol% SM. It was suggested that the differences in proportions of SM and PC in mammalian cell membranes may also result in significant differences in dipole

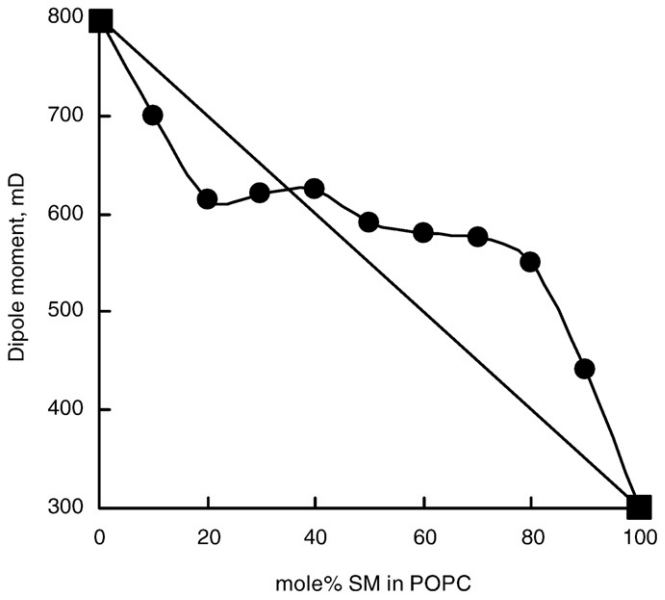

Fig. 1. Dipole moment in mixed monolayers of POPC and egg-SM at surface areas per molecule of 0.75 nm^2 plotted as a function of the proportion of egg-SM in the film. Data calculated on the assumption of ideal additivity in the binary mixtures are shown on the line joining the pure phospholipid monolayers (■); experimental values are plotted (●). Data obtained from [19].

moments that may impact binding or insertion affinities of ligands or proteins[17].

3.1.3. Cyclodextrin uptake of cholesterol from the interface

Cyclodextrins are cyclic oligosaccharides consisting of *-(1-4)-linked D-glycopyranose sugars. The crown structures are water soluble and their use in lipid exchange studies resides in possession of a hydrophobic cavity that binds lipids. The inward-facing non-polar surfaces of the cyclodextrin monosaccharide subunits form a hydrophobic cavity whose size depends on the number of monosaccharide units. The size of the pocket confers selectivity for the type of lipid that can be accommodated and β-cyclodextrins have a particular affinity for sterols like cholesterol. The stacking of two cyclodextrin molecules is required to form a large enough hydrophobic pocket to solubilize one cholesterol molecule and solubility and specificity of the complex in water can be improved by using methylated or other derivatives of β-cyclodextrin. The use of cyclodextrin to determine physical parameters associated with the interaction of cholesterol with phospholipid membranes, however, needs to be regarded with circumspection [21]. Not least amongst the problems is the fact that cyclodextrins bind phospholipids [22] and translocate them between membranes [23]. Moreover, the rates of cyclodextrin-mediated removal of cholesterol from a monolayer, while often interpreted to reflect the activity of cholesterol or its affinity for the other lipids in the monolayer, i.e., thermodynamic qualities, could also in principle reflect purely kinetic effects. Indeed, the effects of sphingolipid inclusion on the kinetics of cholesterol desorption from monolayers and bilayers are often quantitatively much larger than might be predicted from thermodynamic effects (modulation of cholesterol) alone. Notwithstanding these problems, cyclodextrins have provided useful information on the interaction of cholesterol with membrane lipids.

A number of studies have focused on the use of cyclodextrin to examine desorption of cholesterol from mixed monomolecular films at the air–water interface. At a given surface pressure the intercalation rate of the methyl-β-cyclodextrin is dependent on the interaction with cholesterol as compared with the interaction of cholesterol with other components present in the mixed film. Studies of desorption rates of cholesterol from mixed monolayers of cholesterol and DPPC or C16:0-SM with β-cyclodextrin in the subphase have been reported [24]. It was found that at a constant surface pressure of 20 mN/m (30 °C), 0.9 mM β-cyclodextrin caused a desorption of about 13pmol of cholesterol/min/cm^2 of monolayer of pure cholesterol. The rate of cholesterol desorption increased as the monolayer surface pressure increased (3–> 35 mN/m) but decreased slightly with increasing temperature (15–> 30 °C). Cholesterol desorption from mixed monolayers of both cholesterol and a phospholipid was much slower than from a pure cholesterol monolayer and considerably slower when the phospholipid was C16:0-SM compared to DPPC. This can be interpreted as a stronger affinity of cholesterol for C16:0-SM compared with DPPC. Taken together, the results suggest that β-cyclodextrin-enhanced desorption of cholesterol (and other sterols) from monolayer membranes is influenced by the polarity of the desorbing molecules, as well as by lipid/lipid interactions in the membranes. The use of β-cyclodextrins in catalyzing cholesterol exchange between vesicles to obtain parameters such as partition coefficients and partition free energies is discussed in the next Section.

3.2. Condensing effect of sterols

The condensing effect of cholesterol on polar lipids was first reported in the Croonian Lectures of J.B. Leathes published in 1925 [25] who noted from studies of mixed monolayers at the air–water interface that "it (the condensing effect) is observed with fatty acids even more than lecithine, though still observable with this, tempts one to suppose that the action is between cholesterol and the paraffin chains rather than the complex glyceryl cholyl phosphoric acid, and therefore is again a change in physical behaviour, an alteration in the force of cohesion between these chains, that depends upon chemical characteristics not capable of resulting in actual chemical union in the ordinary sense". Since this early report there has been a considerable body of work aimed to explain how the interaction between these two lipid types generates the observed physical effect. The magnitude of the condensing effect is determined by the reduction of the average molecular area of the phospholipid spread as a monolayer at the air–water interface together with cholesterol. Of the phospholipids found in cell membranes the greatest condensing effects are seen with the choline phosphatides, sphingomyelin (SM) and phosphatidylcholine (PC); any condensing effects on other membrane lipids are trivial in comparison with the choline-containing phospholipids.

The interaction between the choline-containing phospholipids and cholesterol in monolayers leads to a "condensed" or more compacted phase consisting of the complexes formed between the two lipids. The complexes formed by sphingomyelin and cholesterol may eventually form a separated condensed phase at the interface. Despite representing only half a bilayer simple monolayers spread at the air/aqueous phase interface are claimed to model some of the features of detergent resistant domains of bilayer membranes.

In the case of monomolecular films of cholesterol the abrupt increase of surface pressure versus mean molecular area at values greater than 43$Å^2$ indicates that the film is in a solid-phase during the compression, in relation with the planar and rigid structure of the molecule. The orientation and the mean molecular area do not change significantly during the compression. For instance, mean molecular areas are estimated around 41$Å^2$ and 38$Å^2$at 10 mN/m and 30 mN/m, respectively [26]. The mean molecular areas at both these surface pressures were compared with the areas of ideal mixtures according to the equation:

$$A_{\text{ideal}} = (A_{\text{Sterol}} \times X_{\text{Sterol}}) + (\text{ASM} \times (1 - X_{\text{Sterol}})) \quad (1)$$

where A_{Sterol} and ASM are the mean molecular area Sterol and SM at a given pressure and X_{Sterol} the molar fraction of Sterol in the monolayer.

If the two molecular species present in the monolayer are immiscible experimental molecular areas will conform to Eq. (1). Any deviation provides evidence of miscibility of the two molecular species (because of attractive or repulsive forces, leading to lower or higher mean molecular areas, respectively).

The relatively simple monolayer methods are sensitive to the subtle chemical characteristics of both the acyl moiety and the headgroup structure and orientation which are complicit in the formation of complexes. The similarities of monolayer films to bilayers seems contrary to the large differences expected for the positioning of acyl chains extending into the air above the interface in the monolayer and for the emergence of the condensed phase after intercalation of the sterol polycycle. A number of key studies during the '90s detailed the structural requirements to observe the condensing effect which could be assigned as "building blocks" of the liquid-ordered phase representing detergent resistant membrane domains [27,28]. More specifically these studies which employed measurement of intercalation of enzyme and β-methyl-cyclodextrin into the monolayer or direct microscopic imaging of fluorescence probes that partition in/out of separated phases [8] have paved the way for the quantitative description of complexes detected by model-dependent spectroscopic and XRD methods. The measurements of affinity and diffusion coefficient of lipids in monolayers have also contributed to the calculation of parameters to fit the condensing effect.

3.3. Variation of sphingomyelin structure

The structural requirements for the molecular interactions between cholesterol and sphingomyelins in model membranes have been investigated by the effects of changing the structure of sphingomyelins [29]. In these studies derivatives of sphingomyelin were synthesized in which (a) the 3-hydroxy group was replaced with a hydrogen atom or various bulky groups (b) the *N*-acyl chain length was varied, and (c) the *N*-acyl chain length contained an alpha-hydroxy group to serve as a model of hydroxylated SMs found in the central nervous system. The synthetic sphingomyelin derivatives are epimerized at C-3 following the preparation of the lyso-SM. The mean molecular area of pure sphingomyelin derivatives in monolayer at the air/water interface was found to be between 0.52 nm^2 for C16:0-SM and 0.60 nm^2 for C18:1-SM (Π = 35 mN/m, 22 °C). Replacement of the 3-hydroxy group of sphingomyelin with a hydrogen atom or its substitution with a methoxy or ethoxy group did not affect the ability of cholesterol to condense the molecular packing in monolayers. The amide group at C-2 in sphingomyelin was shown to be essential for the strong interaction between sphingomyelin and cholesterol. Replacement of this amide by an ester bond increases the availability of cholesterol in mixed vesicles for oxidation with cholesterol oxidase [30] revealing its looser association. In agreement the condensing effect of cholesterol on derivatives of amide chain-linked sphingomyelins is significantly larger than observed with disaturated PCs.

The stereochemistry of the long chain base influences the compactness of the film as it is revealed with a high sensitivity by cyclodextrin extraction of cholesterol and SM sphingomyelinase hydrolysis. In a study with D-*erythro*-sphingomyelins (the natural isomer of SM) with 16:0 as the N-linked acyl chain [31] (see Fig. 2) it was reported that the D-*erythro* N-16:0-SM in monolayers packs more densely than the corresponding racemic sphingomyelin. More condensed domains were seen in cholesterol/D-*erythro*-N-16:0-SM monolayers than in the corresponding racemic monolayer when NBD-PC was used as a probe of fluorescence microscopy. In contrast to monolayers of N-16:0-SMs, both the lateral packing densities and the rates of cholesterol desorption were similar for D-*erythro* and racemic SM. Finally, D-*erythro*-sphingomyelins in monolayers (both N-16:0 and N-18:1 species) were not as easily degraded at 37 °C by sphingomyelinase (*S. aureus*) as the corresponding racemic sphingomyelins. The overall conclusion from these studies was that the association with cholesterol of racemic sphingomyelins may differ significantly from the physiologically relevant D-*erythro* sphingomyelins. Moreover these studies have shown the reliability and sensitivity of ensemble monolayer measurements. The resolution of spectroscopic methods is not sufficient to detect the subtle differences of molecular association of SM with cholesterol. Indeed the multiple molecular species of biological SMs is considered to play a role in the association with cholesterol.

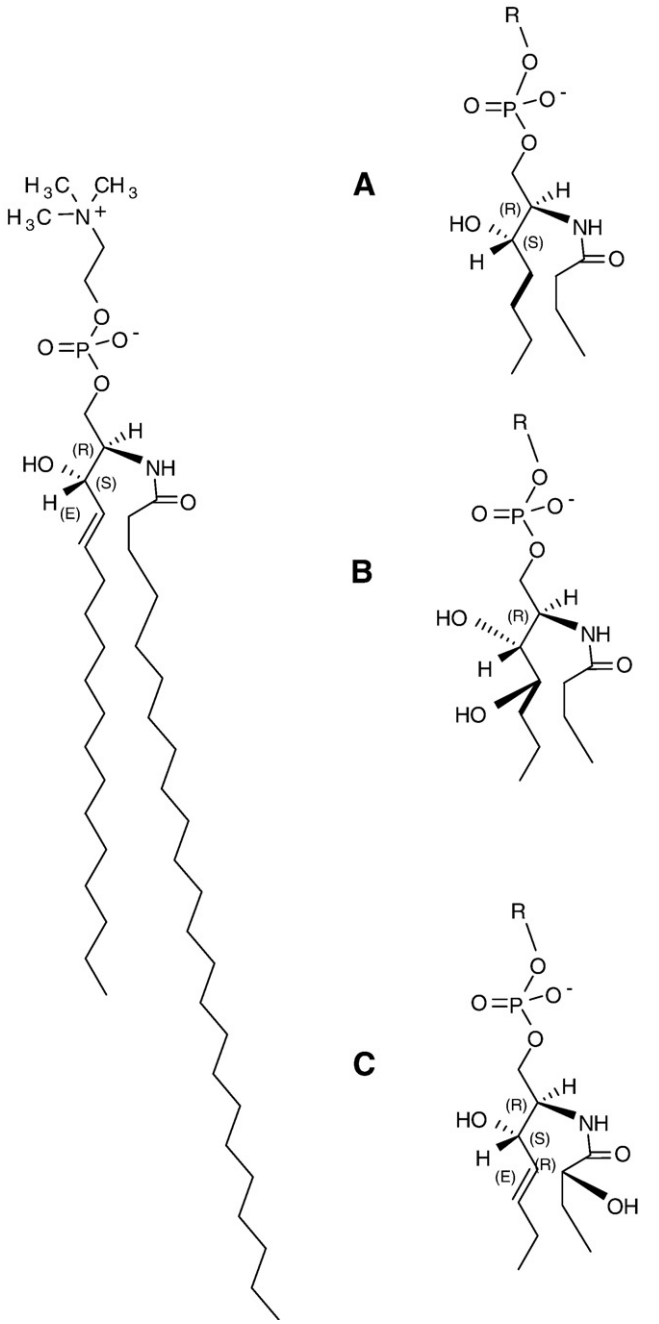

Fig. 2. *N*-lignoceryl (C24)-sphingomyelin and the by-derivatives with modified interfacial region. Sphingosine, the long chain base of sphingomyelin (left), has the natural structure of (*E*)-sphing-4-enine.The very long fatty acyl C-24 residue is responsible for the wide large chain inequivalence which, in the absence of cholesterol, may produce bilayer halves coupling by interdigitation. In some tissues and in various sphingolipids, the △4-double bond is reduced to sphinganine (A) or substituted by a C4-hydroxy group (B). In the central nervous system abundant C2-hydroxy fatty acids are also found (C). Any alteration in the interfacial region has potential consequences on the hydrogen networking of the molecule including H-bonding to cholesterol and water participating in the building up of the lamellar liquid-ordered phase Lo.

3.4. Variations of sterol structure

Sterols represent a large group of molecules yet only cholesterol, stigmasterol and ergosterol are found in substantial amounts in nature, cholesterol in animals, stigmasterol in plants and ergosterol in fungal membranes. An approach to investigate why these particular sterols are found in membranes of living organisms with different acclimatization temperatures has been to compare their effects on phospholipid structure and dynamics with different sterol analogues. Cholesterol consists of a planar tetracyclic ring system with the 3β-hydroxyl (OH-Cholesterol) group and a short eight-carbon chain (*iso*-octyl tail) attached to C_{17}. The ring system is asymmetric about the ring plane and has a flat side with no substituents (-face) as well as a rough side with two methyl groups (β-face).

Dihydrocholesterol (DChol) has been favoured for monolayer studies because it is resistant to oxidation [32]. However recent and detailed studies of monolayers of egg-SM and POPC [26] have shown that at relatively high surface pressure (30 mN/m) the condensing effect of DChol is only observed when the proportion of sterol in the monolayer approaches 20–30mol% and reaches a condensation percentage of − 26%. In the other mixed monolayers (< 20mol% and > 30mol%) the experimental points were on the A_{ideal} straight line. These changes could be related to a reorganization of molecules induced by compression. For comparison, in the case of SM-cholesterol monolayers, a strong condensation effect of cholesterol on SM molecules was observed at a surface pressure of 30 mN/m, with condensation percentages increasing progressively until 17% and saturating at cholesterol percentages greater than 50mol%. Moreover, even if the effect of cholesterol seems to be maximal at 30mol%, there is no detectible inflection point.

In addition to the cholesterol ring, the side chain of sterol interacting with the acyl chains of sphingomyelin was found to be influential in the structure formed. These effects have been examined using epifluorescence examination of mixed monolayers of sterol with sphingomyelin or dipalmitoylphosphatidylcholine to reveal the condensed phase domains in the film. Variations in the side chain of the sterol were compared with the natural *iso*-octyl side chain of cholesterol [27]. Domain formation was detected using NBD-cholesterol as the probe. The sterols had either unbranched (*n*-series) or single methyl-branched (*iso*-series) side chains (see Fig. 2) with the length varying between 3 and 10 carbons (C3–C10). A side chain length of at least 5 carbons was necessary for the unbranched sterols to form visible sterol/phospholipid domains in DPPC or N-16-SPM mixed monolayers. With the *iso*-analogues, a side chain of at least 6 carbons was needed for domains to form. The condensed arrangement formed by SM and sterol was also studied as a function of surface pressure. It was reported that macroscopic domains were stable up to a certain surface pressure ranging from 1 to 12 mN/m. At this onset phase transformation pressure, the domain line boundary dissipated, and the monolayer became homogeneous with no clearly visible lateral domains. However, with mixed monolayers of DPPC containing short chain sterols (*n*-C3, *n*-C4, *n*-C5, and *iso*-C5), a new condensed phase appeared to form when the proportion of sterol reached 20mol% and when the monolayer was compressed to values above the phase transformation pressure. In this case precipitates which formed at surface pressures between 6 and 8.3 mN/m were clearly seen at pressures at least up to 30 mN/m suggesting that they may be present in bilayers. When the monolayers containing these four sterols were allowed to expand, the condensed precipitates dissipated at the same pressure at which they were formed during monolayer compression demonstrating that the process is completely reversible. No condensed precipitates were observed with these sterols in mixed monolayers with *N*-palmitoyl-D-sphingomyelin. These results imply that the sterol side chain fulfils a specific role possibly in augmenting interaction of the steroid ring system through van der Waals interactions in the hydrophobic region of the structure and strengthening H-bonding at the interface. The observation is relevant for all sterols sharing the same 3β OH-cholest-Δ5-ene nucleus but side chains that vary in sterols found in plants, yeasts or mammals. It is noteworthy that the sphingomyelin content of these organisms varies widely [33] and that disaturated phosphatidylcholine does not mimic SM in every aspect, such as packing induced by the lateral pressure, which leads to the Lo condensed phase.

Monolayer techniques have proved particularly useful in characterising the interactions between cholesterol and phospholipids not least because the density of molecules can be varied to reveal subtleties in force-area relationships dependent on the precise composition of the film. Correlations between the density of substrate molecules for sphingomyelinase and cholesterol oxidase and the hydrolytic activity of the enzymes have been demonstrated providing information about the structure of the interfacial region of monolayers. A reduced lag-phase, for example on introducing unsaturated *N*-acyl substituents into sphingomyelin, indicates that sphingomyelinase is able to orient more favourably at the interface of liquid-expanded monolayers. The acyl chain composition of sphingomyelin is also a factor in cholesterol oxidase activity in mixed monolayers in addition to the density of the substrate but the precise mechanism of this modulation is not clearly understood. Monolayer studies also indicate differences in orientation of sphingomyelin and diacyl phospholipids in the film which has been attributed to intermolecular hydrogen bonds forming a network in sphingomyelin thus complementing conclusions based on molecular dynamics simulations. The affinity of cholesterol for sphingomyelin has been shown to be greater than that for diacyl phospholipids as judged by β-cyclodextrin desorption measurements from monolayers. Finally, the condensing effect of cholesterol is most convincingly demonstrated in mixed monomolecular films at the air–water interface and the technique has been most useful in characterising the structural features of both phospholipid and sterol that contribute to the condensing effect.

4. Thermodynamics of sphingomyelin–cholesterol interactions

The interaction between cholesterol and phospholipids has been examined by observing the perturbation of thermotropic phase transition behaviour of the phospholipid using calorimetry and the measurement of the relative affinity of cholesterol manifest as the partition coefficient between different phospholipids. Both methods have their inherent practical limitations but considerable insight into the specificity of cholesterol–phospholipid interactions has been achieved by such studies.

4.1. Calorimetric studies

A number of calorimetric studies of sphingomyelin–cholesterol bilayer dispersions have been reported [34–37]. Universally, it was found that the gel to liquid-crystal phase transition was broadened progressively by the presence of increasing proportions of cholesterol. The transitions are completely reversible on heating and cooling and can no longer be detected when the proportion of cholesterol exceeds about 50mol%. This is manifest as a progressive decrease in the transition enthalpy inferring that cholesterol prevents the sphingomyelin from phase separating into a gel phase at temperatures below the main phase transition temperature. In addition to thermotropic phase behaviour, sphingomyelin exhibits lyotropic mesomorphism and hydration forces would be expected to play an important role in the interaction of sphingomyelin with cholesterol.

Calorimetric studies of binary mixtures of cholesterol with synthetic sphingomyelins differing in the length of the N-linked saturated fatty acid show the presence of two main endothermic peaks, one sharp indicating a cooperative transition and another broad component signifying a domain of different composition from that contributing to the sharp component [37,38]. A detailed examination of the profiles of the two peaks was reported where it was concluded that cholesterol does not distribute randomly within

the bilayer when cooled below the phase transition temperature of the phospholipid. Thus during the cooling process cholesterol tends to be excluded from domains forming gel phase and becomes enriched in the remaining fluid phase. This was consistent with the progressive disappearance of the sharp endothermic transition with increasing proportions of cholesterol in the mixture. Conversely, the enthalpy of the broad component tended to increase with mixtures up to about 20mol% cholesterol and thereupon decreased as cholesterol removed the remaining sphingomyelin from an endothermic phase transition. The calorimetric method cannot be used to give information on the distribution of cholesterol in fluid bilayers but this is most likely to be random. It is clear, nevertheless, that the affinity of binding of cholesterol to sphingomyelin is weaker than the van der Waals forces and intermolecular hydrogen bonding that come into play during formation of gel phase.

Calorimetric studies of ternary mixtures of SM/PC/cholesterol have been reported. In mixtures containing equimolar proportions of porcine brain-SM and cholesterol in POPC no endothermic transitions was observed between 3 and 80 °C [39]. A complex heat capacity profile was seen with lower proportions of cholesterol down to 8:1, SM/cholesterol the most noteworthy of which was that the melting profile extended to temperatures higher than either SM in POPC or pure SM. This feature has also been observed in ternary mixtures of C16:0-SM/dimyristoylphosphatidylcholine/cholesterol where a relatively broad endothermic peak appears when the mole fraction of cholesterol exceeds 0.25 and on increasing the cholesterol up to 0.33 mole fraction has a peak temperature of greater than 50 °C [40].

4.2. Equilibrium exchange of cholesterol

As described above for desorption of cholesterol from monomolecular films, β-cyclodextrin can also mediate cholesterol transfer between vesicle populations. If vesicle populations comprised of different phospholipid composition are used the relative affinities of the particular phospholipids for cholesterol can be obtained from the partition coefficient. The experimental system is limited to phospholipid–cholesterol mixtures that can form large stable unilamellar vesicles but in practice it is difficult to separate vesicle populations to determine the partition coefficient directly. Instead, partition coefficients have been determined between β-cyclodextrin and vesicles of different composition because it is easy to separate the vesicles from β-cyclodextrin by filtration due to the large difference in their size. The method has been successfully applied to determine the partition coefficients of cholesterol between large unilamellar vesicles of POPC and DOPC on the one hand and vesicles comprised of different phospholipids to assess how acyl chains and polar headgroups influence the affinity of the phospholipid for cholesterol [41]. A summary of the results is presented in Table 1. This shows that the relative affinity of cholesterol for sphingomyelin is more than 6-fold greater than for POPC. Vesicles of phosphatidylserine (PS) and phosphatidylethanolamine (PE) of identical acyl chain composition had higher and lower affinity for cholesterol than PC, respectively. Partition coefficients between dioleoylphosphatidylcholine (DOPC) and phosphatidylcholines of different acyl chain composition indicate that molecular species with saturated chains have a higher affinity for cholesterol than those with unsaturated chains and the affinity decreases with increasing number of unsaturated residues in the chains. Relative partition coefficients between POPC and vesicles consisting of mixtures of POPC and PS, PE or 16:0-SM were shown to have intermediate values compared with those between the pure phospholipids suggesting that the affinity was not influenced by the presence of other phospholipids in the bilayer.

Isothermal titration calorimetry has been used more recently to measure the affinity of cholesterol for POPC bilayers referenced to β-cyclodextrin–cholesterol complexes [42]. The method not only provides a measure of partition coefficients but also gives enthalpic and entropic contributions to the interaction of cholesterol with phospholipids. It was reported that the interaction of cholesterol with POPC bilayers are exothermic resulting in an increase in heat capacity of the system. This is associated with a significant decrease in entropy which was apparently a result of an ordering of the acyl chains by interaction with the sterol.

Table 1
Relative cholesterol partition coefficients $K_{p,rel}$, and partition free energy, ΔG between vesicles of different phospholipid composition

Vesicle composition	$K_{p,rel}$	ΔG, kJ/mol
Relative to di18:1-PC		
18:0,18:1-PC	1.4	− 0.87
di18:2-PC	0.55	1.54
di18:3-PC	0.51	1.74
di22:6-PC	0.33	2.95
16:0,22:6-PC	0.54	1.58
Relative to 16:0,18:1-PC		
16:0,18:-PE	0.33	2.85
16:0,18:1-PS	1.91	− 1.67
16)-SM	6.78	− 4.94

Data from Ref. [41].

The most conspicuous effect from these thermodynamic studies is the relatively high affinity of cholesterol for sphingomyelin compared with phosphatidylcholines which is favourable even with comparable saturated chains. It should be noted that while partitioning measurements indeed suggest that 'saturated' sphingomyelins indeed have a markedly higher affinity for cholesterol than do unsaturated phospholipids, the ternary phase diagrams reported to date for SM/unsaturated PC/cholesterol systems generally suggest only a modest enrichment of cholesterol in the liquid-ordered (sphingomyelin-rich) domains compared to the liquid-disordered domains. Considering the molecular species of phospholipids in biological membranes where sphingomyelin is invariably more saturated than the phosphatidylcholines the sterol is likely to be enriched in sphingomyelin domains and depleted from domains of other phospholipids. This may explain why the cholesterol content of biological membranes is broadly related to the proportion of sphingomyelin in the membrane [43].

4.3. Density measurements

Volumetric measurements based on neutral flotation in solvent mixtures of D_2O/H_2O have been used to examine the properties of phospholipid–cholesterol mixtures [43,44]. The density of mixed aqueous dispersions of cholesterol with phospholipids above and below the gel to liquid-crystal phase transition temperature has provided evidence for the condensing effect of cholesterol. These showed that partial molecular volumes of lipids such as DPPC, DMPC and bovine-brain-SM at temperatures above their respective Tm decrease with increasing cholesterol up to mole fractions of about 0.25 but remained unchanged on further increases in the proportion of cholesterol. At a mole fraction of 0.25 it was stated that each of the phospholipids was condensed to a maximum extent presumably by direct contact with a cholesterol molecule. Interestingly, unsaturated phospholipids (POPC and DOPC) at 30 °C do not undergo any volume condensation for any mixtures with cholesterol up to equimolar proportions of cholesterol.

In summary, the detection of more than one transition enthalpy in binary mixtures of phospholipid and cholesterol is evidence that the sterol is not randomly distributed in phospholipid bilayers. The fact that the transition temperature of some of these events is greater than that of the gel to liquid-crystal phase transition temperature of the pure phospholipid suggests that relatively stable complexes are formed. Higher affinities of cholesterol for bilayers of sphingomyelin compared with bilayers of diacyl phosphatidylcholines with

comparable hydrocarbon chain composition have been reported on the basis of partition coefficients with β-cyclodextrin. In confirmation of monolayer studies and volumetric measurements partition coefficients of cholesterol are greatest with saturated compared with unsaturated molecular species of phospholipid.

5. Structure of sphingomyelin–cholesterol mixtures

The structure and dynamics of sphingomyelin and cholesterol mixtures in aqueous dispersions have been examined using a range of biophysical methods. Indeed one of the first characterisations of liquid-ordered phase was based on the physical properties of the phase by ESR probe techniques [45–47]. This section gives a review of results of studies of liquid-ordered phases particularly with regard to the structure and dynamics of sphingomyelin–cholesterol mixtures.

5.1. ESR spectroscopy

The archetypal Lo phase is comprised of a mixture of SM and cholesterol. The lamellar ordered arrangement can be characterised by 2 properties assessed by ESR spectral parameters: a liquid state characterised by a fast motional rate of a spin-labeled probe embedded in the lipid mixture and a relatively high degree of molecular order judged by the orientation of the rotational axis of probe relative to the bilayer interface. The spectral parameters are referred to as the rotational correlation time (τ) and the molecular order parameter (Szz). How these parameters are defined and derived from the experimental spectrum by spectral simulation methods can be found in [48,49] (www.esr-spectsim-softw.fr).

The spin-labeled probe commonly used in these investigations are spin-labeled fatty acids such as stearic acid (SLFA) which comprises a C18 acyl chain with the polar carboxyl group spontaneously positioned at the water interface. The rod-shaped probe is labeled by a stable nitroxide group of a reporter doxyl or proxyl ring attached at position 5, 7, 10, 12, 14 or 16 of the fatty acid chain. The probe can also be a molecule of phosphatidylcholine with the spin-labeled fatty acid ester-bound at position *sn*-2 (SLPC). The bulky *N*-doxyl group produces a bending of *sn*-2 acyl chain which results in the fluid state of SLPC in a wide temperature range. Spin-labeled fatty acids have an advantage over SLPC to derive the spectral parameters because they have a faster rotational motion which produces narrower resonance lines. The position and linewidth are used to monitor the dynamic motion of phospholipids arranged around their equilibrium position. However the positioning of SLPC in the bilayer is predicated by the phosphorylcholine polar headgroup while the spectrum of SLFA reveals a minor component (at acidic pH) which is misaligned with the local director of the lamellar phase. This minor component is presumably fatty acid with undissociated functional group that is not anchored at the lipid–water interface. Thus the motional regime of SLPC may be regarded as a more reliable index of the regime of fluid biological phospholipids. On the other hand the rod-shaped SLFA partitions more randomly between gel and fluid states in mixture where the phases coexist providing a more reliable measure of the state of complex phase separated mixtures.

Spin-labeled SM spectra have been compared with that of SLPC in ternary SM/PC/cholesterol mixtures to detect phase separation [49]. Both of the spin labels bearing the nitroxide free radical group on the 5 or 14 C-atom in either the *sn*-2 stearoyl chain of phosphatidylcholine (predominantly 1-palmitoyl) or the *N*-stearoyl chain of sphingomyelin produce heterogeneous spectra. Two-component ESR spectra of the probe located in the 14-position indicate the coexistence of gel and liquid-ordered phases at low temperatures which are then transformed into a mixture of liquid-ordered and liquid-disordered phases with increasing temperature. Phase coexistence is observed. An alteration of ESR lineshape in one or other of the two probes is able to detect gel > fluid, fluid > ordered and interdigitated > non-interdigitated transitions in mixtures containing different amounts of cholesterol with increasing temperature. Because the distinct phases forming in the lipid mixtures can be resolved from the ESR spectra it was concluded that the inter-site exchange of spin labels is slow. In this context fast and slow motion using ESR in the frequency X-band (9.5GHz) means rotational correlation time < 2ns and 2ns< rotational correlation time< 10ns, respectively. Indeed, if the rotational correlation time is longer than 10ns the spectral lineshape does not change anymore. Therefore the ESR method in the frequency domain of X-band is informative for rotational correlation time shorter than 10ns. Under this condition the slow spectral component of the lineshape is dependent on microviscosity. This is especially relevant in the case of experiments where cholesterol increases in the environment of spin labels. By contrast in the gel component (in which the cholesterol is excluded) the motion of many different spin labels appears to be “blocked” as detected by ESR.

In an investigation of binary mixtures of egg-SM and cholesterol ESR spectral simulation methods were used to provide a quantitative measure of changes in lineshape [50]. The study was conducted with 16-SLPC, a probe with a motional regime able to provide an accurate spectral representation of Lo phase with ESR in the X-band. Using this approach the exchange rate of SLPC with a doxyl group attached at position16 between different environments has been obtained in the binary mixture at around physiological temperatures. The molecular and intramolecular order parameters arising from segmental motions of the probe produce a complex spectrum that can be resolved into one highly ordered site assigned as Lo structure which coexists with another structure that is less ordered than the fluid phase of pure sphingomyelin. In quantitative terms the domains of Lo structure have dimensions in the range of a nanometer and have a transient lifetime in the order of a nanosecond. The exchange rate of the probe between the two sites indicates a mean lifetime of the sites of about 100ns during which the displacement of the lipid is approximately 1 nm. The short lifetime of the sites probed by ESR in binary mixtures of egg-SM and cholesterol in which only a single phase can be detected by X-ray diffraction suggests that Lo phase is formed by associations between randomly distributed sphingomyelin–cholesterol condensed complexes.

The absence of extended domains coherently arranged in binary mixtures which are detectable by X-ray diffraction methods contrasts with Lo/Ld domain coexistence found in ternary and quaternary mixtures containing glycerophospholipids [51]. In the quaternary mixture of SM/cholesterol/ phosphatidylethanolamine/phosphatidylserine two fluid lamellar phases of periodicities of 6.3 and 5.6 nm, respectively, at 20 °C are observed. The lamellar phase of periodicity 5.6 nm is assigned to a phase enriched in amino-glycerophospholipids and the other of periodicity 6.3 nm to a liquid-ordered phase formed from cholesterol and high melting point molecular species of sphingomyelin. Examination of the same mixture by ESR probe methods shows liquid-ordered microdomains separated from the surrounding fluid lamellar phase.

ESR spectroscopy has been used to examine the effect of sterol structure in formation of Lo phase with sphingomyelin [52]. Both cholesterol and 7-dehydrocholesterol give a phase assigned to Lo in the form of a highly anisotropic spectral component. At temperatures above 36 °C, the contribution to the ESR spectrum of the Lo phase formed by 7-dehydrocholesterol and sphingomyelin is reduced whereas the Lo phase formed by sphingomyelin and cholesterol is stable at temperatures up to 42 °C. These results indicate that the B-ring of the sterol plays an important role in the association of SM with sterol to form Lo structure.

5.2. X-ray diffraction studies

X-ray diffraction methods have provided information on the mesophase structure of multilamellar arrangements of binary mixtures of sphingomyelin and cholesterol and tertiary mixtures

including phosphatidylcholines. This is illustrated in Fig. 3 which shows small-angle scattering peaks recorded at 50 °C from binary mixtures of cholesterol with egg-sphingomyelin and egg-phosphatidylcholine and compares this with equimolar mixtures of the two phospholipids co-dispersed with increasing proportions of cholesterol. Firstly, it is clear that because the Bragg peaks are not symmetrical that the two phospholipids do not mix homogeneously and there is fluid–fluid immiscibility between sphingomyelin and phosphatidylcholine. Secondly, binary mixtures of cholesterol with sphingomyelin and with phosphatidylcholine form smectic mesophases with different lamellar d-spacings. Thirdly, the characteristic d-spacings are retained in the ternary mixture. Finally, the amount of Lo phase formed from sphingomyelin and cholesterol (peak 1) increases with increasing amounts of cholesterol (inset to the figure) but in the presence of higher proportions of cholesterol the sterol partitions into the phosphatidylcholine-enriched phase (peak 2) to cause an ordering of this phospholipid.

Coexisting Lo and fluid phase in ternary mixtures of phospholipids and cholesterol has also been observed in an analysis of the wide-angle X-ray scattering patterns of stacked multilayers [53]. While this study did not include sphingomyelin it was reported that liquid-ordered phase could be detected in the appearance of two chain ordering profiles that coincided with order parameters derived from ^{2}H-NMR measurements. Cholesterol was found to have a greater effect on chain ordering than phospholipids with unsaturated fatty acyl residues [53]. A coexistence of two fluid phases judged from the wide-angle X-ray scattering intensity profile under conditions in which only a single peak in the small-angle scattering region was observed highlighted a potential difficulty in defining liquid–liquid immiscibility by small-angle scattering measurements.

5.3. Dynamic and structural studies

Examination of mixed aqueous dispersions of lipids using a combination of ESR spectroscopy and a structural method like X-ray or neutron scattering gives a powerful insight into the molecular dynamics of these mesophase systems. Spectral simulation of spin-label probe signals provides parameters such as the lamellar, hexagonal or cubic geometry of the lipid phase which also have unique signatures in X-ray or neutron scattering patterns. In addition, X-band continuous wave ESR spectroscopy only accesses a narrow time frame (0.1–10ns) where the spectra are altered by changes in the motional regimes of the spin-labeled probe. Therefore in the binary SM/cholesterol mixture the transient molecular associations which may eventually evolve into a condensed phase separation are detectable by ESR in the ns–nm range in circumstances where no phase separation is apparent by X-ray diffraction. Accordingly, differences observed between binary SM/cholesterol and ternary SM/cholesterol/fluid PC mixtures can be interpreted in terms of a rapid diffusion of SM/cholesterol complexes in a more fluid matrix of phosphatidylcholine (also containing cholesterol) to produce a condensed Lo phase transiently separated from the fluid lamellar phase (Fig. 4).

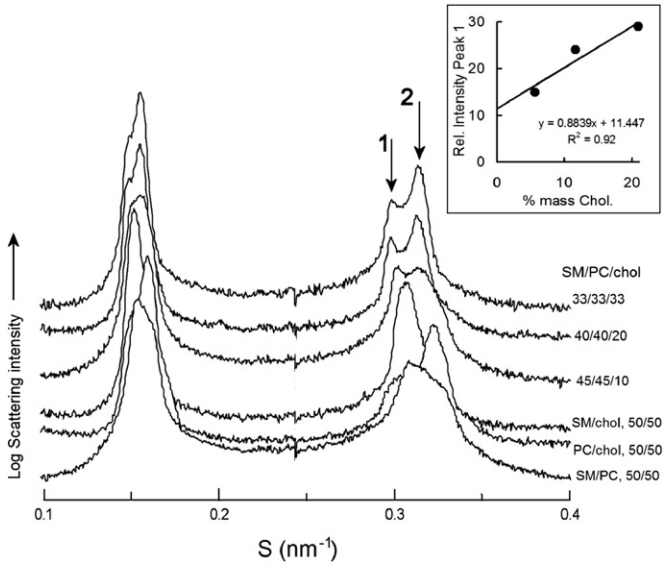

Fig. 3. Static SAXS intensity patterns recorded at 50 °C from aqueous dispersions of egg-SM and eggPC dispersed with equimolar proportions of cholesterol and ternary mixtures of equimolar proportions of the two phospholipids with varying molar proportions of cholesterol. Arrows indicate the position of peaks #1 and #2 on the second-order reflection. Inset shows the relationship between the relative intensity of peak #1 and the % mass of cholesterol in ternary mixtures. (Tessier, Wolf and Quinn, unpublished).

5.4. NMR spectroscopy

The motion and conformation of the polar headgroup of sphingomyelin has been investigated using ^{31}P-NMR methods [54]. These studies showed that the phosphocholine group undergoes similar motions to that of the headgroup of phosphatidylcholines. However, the interfacial region of sphingomyelin bilayers is more rigid due to intermolecular hydrogen bonding between the amide groups and intramolecular H-bonding between the − OH group and the phosphate group [55,56]. The gel phase is characterised by restricted mobility of both the headgroup, as revealed by ^{31}P-NMR, and hydrocarbon chains, as seen by ^{13}C-NMR, and several gel phases can be recognized [57]. Addition of cholesterol to sphingomyelin bilayers resulted in an increased mobility of the headgroup and the motional characteristics were similar to that observed in liquid-crystal phase of the phospholipid [58,59].

Studies of ternary mixtures of synthetic C16:0-SM/POPC/cholesterol by ^{2}H-NMR showed that at temperatures above the gel to liquid-crystal phase transition temperature of the sphingomyelin (40 °C) cholesterol distributes into coexisting liquid-ordered and liquid-disordered phases [60]. The chain order parameter was different for the two phospholipids and it was concluded that while cholesterol is distributed in both phases it redistributes into the liquid-ordered phase on cooling. The presence of microdomains of liquid-ordered structure was inferred from the data which had a minimal diameter of 80–140 nm.

The structure of thermotropic phases in egg-SM–cholesterol mixtures has also been investigated by ^{31}P magic-angle spinning NMR methods [61,62]. Linewidth analysis of sphingomyelin-cholesterol mixtures indicate that structural transitions take place between different gel phases of sphingomyelin and inclusion of cholesterol attenuates this transition. The origin of the structural transition appears to be a realignment of hydrogen bonds in the interfacial region. The presence of cholesterol causes a loss of intermolecular hydrogen bonds between sphingomyelin molecules and this is reflected in increased motion of the phosphocholine residue.

Pulsed field gradient NMR methods have provided information about lateral diffusion of phospholipids in the plane of the bilayer and how the diffusion coefficient is affected by the presence of cholesterol and other sterols [63–67]. Cholesterol interacts with sphingomyelin to form a phase which is characterised by a significantly lower diffusion coefficient compared with the fluid phase and with a higher apparent energy of activation. The diffusion coefficient, unlike that seen when cholesterol interacts with glycerophospholipids, is independent of the proportion of cholesterol in the bilayer. This phase is consistent with a liquid-ordered phase. In bilayers of sphingomyelin and cholesterol under conditions where Lo/Ld phase coexistence occurs a fast exchange takes place between the two phases on a time scale of about 100ms. From this it was concluded that domains of liquid-

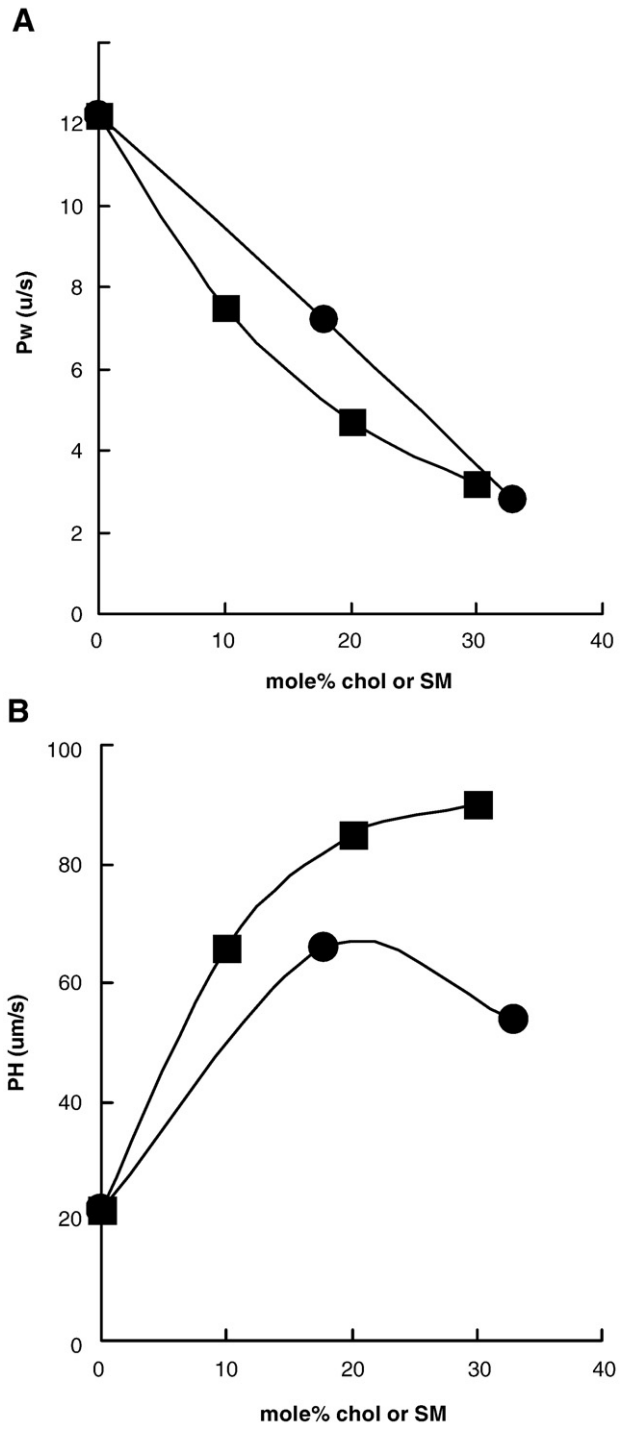

Fig. 4. Permeability of liposomes of palmitoyl-oleoyl-phosphatidylcholine to (A) water and (B) protons recorded at 25 °C as a function of the mol% of cholesterol (●) or (■) brain sphingomyelin commixed with the diacylphospholipid. Data from [79].

ordered structure of dimensions in the μm size range or less are created in fluid lipid bilayers. The formation of liquid-ordered phase was observed with cholesterol, ergosterol, sitosterol and lathesterol but not with lanosterol, stigmasterol or stigmastanol and this was related to the ability of the particular sterol to order the hydrocarbon chain of the phospholipid [68].

Spectroscopic methods have pioneered the definition of the Lo phase which has properties intermediate between a gel, on the one hand, and a liquid-crystal phase on the other. The motion and orientation of spin-label probes embedded in Lo phase show a relatively fast motional rate typical of fluid bilayers whereas the reorientational motion of the axis of rotation of the probe is reminiscent of the order found in gel phases. Complex spectra recorded from probes partitioning between Lo and fluid phases in bilayers comprised of mixtures of sphingomyelin and cholesterol can be simulated to generate parameters reflecting the size of coexisting domains. The evidence is consistent with the creation and dissipation of Lo domains on a timescale approaching 1ns and of dimensions in the order of nm. NMR spectroscopic methods have provided complimentary information on lateral motion of phospholipids in mixed dispersions with cholesterol and other sterols. Order parameters derived from ^{2}H-NMR studies of deuterated lipids confirm that sterols reduce the disorder of the acyl chains of phospholipids. Finally, X-ray diffraction has been used to identify Lo phase in phospholipid–cholesterol bilayers both in small-angle reflections where coexisting bilayers of different d-spacing can be observed and in wide-angle reflections generated by packing arrangements in the hydrocarbon domain of the bilayer.

6. Sphingomyelin and cholesterol: from evolution to cell physiology

Cholesterol interacts with many membrane components and in most cases this leads to demonstrable changes in structure and/or properties of the target component. A pertinent question in the context of the interaction of cholesterol with sphingomyelin is whether such interactions drive lateral phase separations in membranes or are such domains formed when sphingomyelin is phase separated and the structure of this domain is altered by interaction with cholesterol. In the latter case the interaction may not be regarded as a specific interaction as would be assigned to formation of a complex. Some clues to the answer can be gleaned from a consideration of how the two lipids have evolved as membrane components.

The structures of sphingomyelin and cholesterol shown in Fig. 2 have evolved independently. There are examples of organisms (bacteria, yeasts, fungi) with sphingolipids derived of phytosphingosine but no cholesterol (or phytosterol) and *vice versa*. The content of SM and cholesterol varies widely amongst animal species and even for membranes in different organisms with similar functions such as erythrocyte membranes [69]. However, it is well known that SM and cholesterol content range in parallel in subcellular and plasma membranes.

While the two lipids have evolved independently they share the same fate in evolutionary biology [70]. This is evidenced by (a) the relative proportions of the two lipids in biological membranes is highly correlated (b) their metabolism is interdependent as evidenced by concomitant changes in levels when the metabolism of one or other lipid is disturbed (c) they are co-localized in membranes as in membrane rafts and caveolae (d) each when present in membranes enhances the permeability barrier properties of membranes by impeding the flux of solutes and water and (e) they both serve to reduce oxidative damage to membranes. This section will consider whether the association of SM and cholesterol in plasma membranes is adventitious or essential to the properties of the plasma membrane.

6.1. Membrane physiology

Evidence for interactions between sphingomyelin and cholesterol are manifest in physiological properties of cell membranes. A critical point as to whether the chemical structures of cholesterol and SM evolved in concert is the degree to which the stereochemistry of their interaction mimics a macromolecule binding site-ligand interaction. In contrast to the polycyclic planar ring system of the sterol which remained constant over evolutionary time the very long, saturated hydrocarbon chains of SM are able to adopt multiple conformations to

accommodate the sterol ring by thermal *gauche-gauche<>gauche-trans* isomerization. As a result multiple, short-range van der Waals interactions can be created under conditions where *cis* unsaturated chains are hindered. Indeed, the experimental protocols such as temperature scanning and/or the addition of detergent have been criticized as potential artifacts prone to distort the interaction.

A striking observation has been reported recently showing a strong correlation between the resistance of erythrocytes to thermal haemolysis with the life span of erythrocytes for 13 different mammals (human, sheep, cow, goat, dog, horse, rabbit, pig, cat, hamster, guinea pig, rat, and mouse). Resistance against thermal haemolysis is strongly correlated with sphingomyelin content [71]. The ratio of SM/SM + PC was found to vary from 0.15 in guinea pig (red cell life span 30days) to 1 in sheep (life span 160days). These data imply a prominent role of SM in biology with eventual consequences correlated not only for erythrocyte longevity but also for the maximal life span of the organism itself ($r = 0.46$; the correlation is not validated in man).

It is known that cholesterol concentration equilibrates with SM as a result of an affine association, a short term adaptation dependent on cholesterol transfer processes. It is also relevant that concentrations are paralleled in the long term by a concerted cross-regulation of key enzymes. In rat liver nuclear membranes, for example, variations of sphingomyelin content are due to the action of a neutral sphingomyelinase and sphingomyelin synthase. This is evident in the correlation between sphingomyelin and cholesterol contents during cell proliferation in the nuclear membrane examined over the lifetime of the cell [72]. Thus degradation of sphingomyelin by exogenous sphingomyelinase results in a decrease of cholesterol due to either an increase of esterification or to a reduced rate of biosynthesis. The concerted action of sphingomyelin synthase and neutral sphingomyelinase could explain an increase of sphingomyelin content during the first 18h following hepatectomy followed by a decrease during the subsequent 6 h, the variation being paralleled by cholesterol due to a change of the esterification process.

6.2. Specificity of sphingomyelin–cholesterol interactions

Non-specific associations between sphingomyelin and cholesterol have been demonstrated by fluorescence resonance energy transfer between pyrenyl derivatives of PC and SM and NBD-cholesterol and fluorescence quenching between the pyrenyl-phospholipid derivatives and dibrominated cholesterol interpolated into giant unilamellar vesicles of di-C14)-PC and di-C24:1-PC [73]. The distribution of pyrenyl-phospholipid analogues showed temperature-dependent fluorescent properties that indicated their respective co-localization in the bilayer were driven by hydrocarbon chain mismatch between the probe and the matrix phospholipid. In the presence of cholesterol the patterns of fluorescence perturbation were almost the same for the PC and SM probes from which it was concluded that there was no preferential interaction between the PC and SM probes and cholesterol. Thus, co-localization arises not because of any specific interactions between the respective phospholipids and cholesterol but because of a hydrophobic mismatch with the surrounding phospholipids in the bilayer matrix.

A comparison of the interaction between sphingomyelin and natural cholesterol and cholesterol enantiomers has been undertaken using differential scanning calorimetric, X-ray diffraction, and neutral buoyant-density measurements [74]. It was concluded that there was no evidence of any detectible enantioselective cholesterol–sphingolipid interactions. This contrasted with the results of monolayer experiments in which enantiomeric 3-β, Δ5 cholesterol was shown to be about twice as effective in condensing monolayers of egg-SM at sterol concentrations of 30mol% at lateral pressures resembling those in lipid bilayers [75]. One can interpret this difference assuming that simplified lipid mixtures comprised of only few synthetic lipids (2 molecular species and cholesterol) are not accurate models of complex lipid mixtures of biological origin. A reason for this is likely to be that simplistic lipid models do not reflect the consequences of co-miscibility of components of complex lipid mixtures. The heterogeneous assortment of components of complex lipid mixtures result in phase separations of domains that behave cooperatively. Although cooperative characteristics are amenable to biophysical characterisation such partition behaviour may represent an unrealistic arrangement when compared with the phase behaviour of lipid mixtures of biological origin.

An insight into the complexity of lipid–cholesterol interactions has been obtained by studies of egg-SM and C16:0-SM mixtures with cholesterol using attenuated total internal reflection-FTIR spectroscopy to monitor the amide-I and other absorbance bands of the phospholipid [76]. The amide-I band has characteristic band frequencies and widths when the phospholipid is in a gel or fluid phase and the spectral parameters are modified proportionately by the amount of cholesterol co-dispersed with the phospholipid. There is clear evidence that there is a significant change in the hydrogen bonding associated with the interaction of sphingomyelin with cholesterol, however, it was not possible, from a detailed analysis of the spectral changes, to attribute this to intermolecular SM-SM, SM-H_2O or SM-cholesterol hydrogen bonds.

An indication of the tight association between sphingolipids and how cholesterol influences this was inferred from the effect on the rate of glycolipid transfer protein-mediated transfer of fluorescently-labeled galactosylceramide from sphingomyelin bilayers of different composition [77]. Thus transfer rates were 1.0pmol/s from *N*-oleoyl-D-*erythro*-sphingosylphosphorylcholine vesicles, 0.65pmol/s from deoxy *N*-palmitoyl-D-*erythro*-sphingosylphosphorylcholine bilayers, and 0.3pmol/s from *N*-palmitoyl-D-*erythro*-dihydrosphingosylphosphorylcholine vesicles. No transfer is detected between *N*-palmitoyl-D-*erythro*-sphingosylphosphorylcholine matrices. There was a progressive inhibition of transfer on addition of cholesterol to bilayers of *N*-oleoyl-D-*erythro*-sphingosylphosphorylcholine reaching half maximal inhibition with about 20mol% cholesterol. The consequences are noteworthy with respect to the brain where there are abundant hydroxylated, methylated and sphingolipids with varying degrees of unsaturation. The conclusion from these transfer studies was that inter- and intramolecular hydrogen bonding and lipid ordering in the presence of cholesterol modulates the rate of protein-mediated glycolipid transfer.

6.3. Bilayer permeability

The permeability of biological membranes to water and solutes is markedly influenced by the presence of sphingomyelin and cholesterol in the lipid bilayer. Since these lipids are concentrated in the exoplasmic leaflet of the plasma membrane it is believed that this is the dominant barrier to the permeation of water and small solute molecules in cells [78]. The results of many model experiments have been used to support this conclusion. The findings of a detailed study of the effect of cholesterol and sphingomyelin on the permeability of

Table 2
Permeability of water across large unilamellar vesicles comprised of different mixtures of brain-SM, phosphatidylcholines and cholesterol at 35 °C

Vesicle composition	Pw (μm/s)
Brain-SM/cholesterol, 1:1	6.2
Brain-SM/cholesterol/SOPC, 1:1:1	9.0
Brain-SM/cholesterol/DOPC, 1:1:1	11.0
SOPC/cholesterol, 1:1	10.2
SOPC	14.5
DOPC	16.8

Data from [80].

water across bilayers of POPC are summarized in Fig. 4 [79]. This shows that increasing proportions of either sphingomyelin or cholesterol in the POPC bilayers resulted in a proportionate decrease in permeability of the bilayers to water. By contrast the permeability to protons increases on mixing POPC with either sphingomyelin or cholesterol. Addition of both sphingomyelin and cholesterol to a total < 20mol% produced less than additive effect of the individual lipids on POPC permeability to water but halved the proton flux across the membrane. This suggests that hydrogen bonding between sphingomyelin and cholesterol may reduce proton permeability across the bilayer.

More recent studies of giant unilamellar vesicles [80] have shown that the permeability of water across bilayers comprised of an equimolar mixture of brain sphingomyelin and cholesterol was significantly less than that recorded from vesicles formed from equimolar mixtures of stearoyl-oleoyl-phoposphatidylcholine and cholesterol. These results are summarized in Table 2. Bilayers of pure steoroyl-oleoyl- and dioleoyl-phosphatidylcholine were relatively permeable to water. The conclusion from these results together with measurements of bilayer area-stretch moduli and rupture tension was that chain unsaturation dominated these parameters which were modulated, in turn, by ordering the chains by reducing the temperature or by interaction with cholesterol or sphingomyelin.

While sphingomyelin and cholesterol appear to have evolved separately their common occurrence in mammalian cell membranes confers unique properties. Thus, their presence together in cell membranes has a considerable influence on physiological processes such as protein-mediated exchange of sphingolipids between membranes and the permeability of membranes to water and solutes. The molecular mechanisms responsible for these effects have been attributed to the intermolecular hydrogen bonding network particular to sphingolipids and the ordering effect of the sterol on the hydrocarbon substituents of the lipid.

7. Conclusions

The questions addressed in this review are 1. Is the interaction between sphingomyelin and cholesterol specific and, if so, how does it differ from interactions with glycerophospholipids? 2. Under what conditions does the interaction of sphingomyelin with cholesterol form liquid-ordered phase? 3. What is the process for creation of liquid-ordered phase and what are the dynamics of the phase in the midst of disordered fluid bilayer lipids?

Liquid-ordered phase can be distinguished from liquid-disordered phase because the properties of the phase are intermediate between those of a crystal or gel phase on the one hand and a liquid-crystal phase on the other. There are a number of biophysical methods that define the parameters of liquid-ordered phase based on lipid motion and structure and characterise these from other coexisting phases in complex mixtures.

Membrane models employed to represent the Ld/Lo phase separated domains in biological membranes often consist of aqueous dispersions of ternary mixtures of cholesterol and phospholipids that coexist as fluid and solid bilayers at a particular temperature. The phase diagrams of all the three-component mixtures examined so far have been divided by Feigenson into two categories [81]. The temperatures at which all these phase diagrams have been constructed is such that gel–fluid macroscopic phase separation is observed and, the presence of sufficient cholesterol, a coexisting liquid-ordered/fluid phase separation is generated depending on energetically-favourable interactions with one or other of the phospholipids. While these phase diagrams go some way to establish principles underlying phase separations brought about by the interaction between phospholipids and cholesterol it has been pointed out that they are relatively simplistic when considering the complexity of mixtures found in biological membranes [82].

Overall, physical studies of cholesterol–sphingomyelin and cholesterol–phosphatidylcholine interactions suggest that cholesterol exhibits a modestly higher affinity (perhaps of the order of twofold but not higher) for saturated sphingomyelins compared to saturated phosphatidylcholines with comparable hydrocarbon chain lengths. The major difference in cholesterol affinities for phosphatidylcholines vs. sphingomyelin in natural systems is thus attributable to the fact that the phosphatidylcholine molecular species in eukaryotic cell membranes are almost entirely unsaturated while the sphingomyelin fraction includes a majority population of species with long saturated *N*-acyl chains.

The interaction of cholesterol with more complex mixtures of biological origin where there is a range of molecular species differing in polar group and hydrocarbon chains offers a more challenging task. The picture that is emerging is that only particular sterols are able to order the hydrocarbon chain region of phospholipids and cholesterol appears to be the most effective. The hydrocarbon chains of sphingomyelin appear to be more efficiently ordered that those of the glycerophospholipids by sterols. While the interaction energies between particular phospholipids and sterols remains uncertain it is clear that this does not differ to such an extent that strongly-associating complexes are formed between particular molecular species of sphingomyelin and cholesterol. It is more probable that the interaction of cholesterol with sphingomyelin differs from that of phosphatidylcholine. This may involve the hydrogen bonding network of sphingomyelin that would be disturbed by interaction with cholesterol and induce a particular mutual orientation between the interacting molecules. The interaction energy of the association due to the ordering of the hydrocarbon chains of the sphingomyelin could be balanced by disordering of water molecules at the lipid–water interface. In this model the overall interaction energy would be relatively low allowing the liquid-ordered phase to exhibit the dynamic properties of the phase that are observed.

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