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Review

Lipid lateral diffusion and membrane heterogeneity

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ABSTRACT

The pulsed field gradient (pfg)-NMR method for measurements of translational diffusion of molecules in macroscopically aligned lipid bilayers is described. This technique is proposed to have an appreciable potential for investigations in the field of lipid and membrane biology. Transport of molecules in the plane of the bilayer can be successfully studied, as well as lateral phase separation of lipids and their dynamics within the bilayer organizations. Lateral diffusion coefficients depend on lipid packing and acyl chain ordering and investigations of order parameters of perdeuterated acyl chains, using ^{2}H NMR quadrupole splittings, are useful complements. In this review we summarize some of our recent achievements obtained on lipid membranes. In particular, bilayers exhibiting two-phase coexistence of liquid disordered (l_d) and liquid ordered (l_o) phases are considered in detail. Methods for obtaining good oriented lipid bilayers, necessary for the pfg-NMR method to be efficiently used, are also briefly described. Among our major results, besides determinations of l_d and l_o phases, belongs the finding that the lateral diffusion is the same for all components, independent of the molecular structure (including cholesterol (CHOL)), if they reside in the same domain or phase in the membrane. Furthermore, quite unexpectedly CHOL seems to partition into the l_d *and* l_o phases to roughly the same extent, indicating that CHOL has no strong preference for any of these phases, i.e. CHOL seems to have similar interactions with all of the lipids. We propose that the lateral phase separation in bilayers containing one high-T_m and one low-T_m lipid together with CHOL is driven by the increasing difficulty of incorporating an unsaturated or prenyl lipid into the highly ordered bilayer formed by a saturated lipid and CHOL, i.e. the phase transition is entropy driven to keep the disorder of the hydrocarbon chains of the unsaturated lipid.

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Contents

Abbreviations: CHOL, cholesterol; DPPC, dipalmitoylphosphatidylcholine; D_L, lipid lateral diffusion coefficient; θ_{LD}, the angle between the bilayer normal and the main magnetic field, B_0; MA, magic angle=54.7°; S_{CD}, order parameter of the C–^{2}H bond; $\Delta\nu_q$, quadrupole splitting; PSM, palmitoylsphingomyelin, POPC, palmitoyloleoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; SOPC, stearoyloleoylphosphatidylcholine; SLPC, stearoyllinoleoylphosphatidylcholine; SAPC, stearoylarachidonoylphosphatidylcholine; SDPC, stearoyldocosahexaenoylphosphatidylcholine; MGlcDAG, monoglucosyldiacylglycerol; DGlcDAG, diglucosyldiacylglycerol; eSM, egg sphingomyelin; l_d, liquid disordered phase; l_o, liquid ordered phase; T_m, main phase transition temperature between gel and liquid crystalline phases

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1. Introduction

The effect of cholesterol (CHOL) on the phase behaviour and dynamics of simple lipid bilayers has fascinated researchers since at least 80 years ago. Much interest was focussed on the increase in the packing and molecular ordering of the lipid molecules in mono- and bilayers (the so-called "condensing effect" [1]) accomplished by the steroid that could be observed by spectroscopic methods such as ^{2}H NMR on perdeuterated acyl chains of the lipids [2]. The influence of CHOL on a number of measured physico-chemical properties in the lipid bilayer have ever since intrigued scientists, such as the change in elasticity, lateral diffusion, and permeability of water and other small molecules [3]. A careful determination of the phase behaviour of CHOL/diacylphosphatidylcholine/water systems turned out to be more complicated than expected and it was not until 1990 the first phase diagram of a binary[1] system of CHOL/dipalmitoylphosphatidylcholine (DPPC)/water was published [4]. This phase diagram included a two-phase region where two fluid phases, one disordered (l_d) and one ordered (l_o) were in equilibrium, as theoretically described by Ipsen et al. [5]. Around this time an intense study of lateral phase separation of domains, especially the biologically functional domains in living cells, often referred to as rafts, was launched forth [6]. This tickled our curiosity because of our long-standing interest in lipid phase behaviour and it was a natural step for us to start investigations of the proposed lipid domains more closely using NMR spectroscopy. In this review we will summarize our investigations of bilayers containing one or two different lipids, in particular sphingomyelins and phosphatidylcholines with different levels of unsaturation, together with various sterols including CHOL. The lipid dynamics have mainly been investigated by pulsed field gradient (pfg)-NMR spectroscopy to obtain the lipid lateral diffusion coefficients, and the molecular ordering has been measured by ^{2}H NMR quadrupolar splittings on deuterated lipids.

2. Methods

2.1. The making of oriented lipid bilayers

Both the determinations of lipid diffusion coefficients by pfg-NMR and the lipid ordering by ^{2}H NMR quadrupole splittings have been achieved by using macroscopically oriented bilayers. A brief review of the method of preparation of such samples will be given in this section. The advantage of using oriented samples is that it is a convenient way of creating the necessary conditions for spin-echo formation required for diffusion measurements (see Section 2.2). An additional benefit is that the quadrupole splittings can be recorded with good accuracy with relatively small amounts of lipids. Furthermore it is possible to explore orientation-dependent characteristics of the systems that would be difficult to observe otherwise.

To obtain macroscopically oriented lipid bilayers the alignment is assisted by glass plates. A solution of the appropriate lipids is placed on glass plates followed by solvent removal. Subsequent hydration of the thin film of lipids usually results in a high degree of orientation in which approximately 100–1000 bilayers are stacked between each glass plate. The solvent needs to be chosen to obtain sufficient solubility of all lipid components and to give the correct wetting properties for the solution to form a thin film during solvent evaporation [7]. In some cases the glass surface has to be modified in order to get a suitable degree of hydrophilicity for the lipids to adhere to it. The way the dry lipid layers are hydrated is also important, and for example just addition of liquid water will disrupt the bilayers and results in vesicular structures, while hydration in a humid atmosphere generally gives a better result [8]. Especially, this latter hydration technique has to be used when more than a single glass plate is used. If the stack of glass plates is made before hydration, addition of liquid water will disrupt the bilayers when water is sucked in between the plates, and attempts to stack prehydrated plates often results in mechanical disruption of the lipid bilayers. However, if only one supporting surface is sufficient, methods have been reported that give good oriented samples with excess water [9]. A more detailed discussion on orienting techniques can be found elsewhere [10,11].

2.2. NMR measurements of lipid lateral diffusion coefficients

Pfg-NMR spectroscopy provides one of the most attractive techniques for studies of molecular transport, and lipid lateral diffusion coefficients (D_L) can be directly measured on macroscopically aligned bilayers [11,12]. The first diffusion measurements by pfg-NMR spectroscopy on oriented lipid bilayers were performed already in the early 70s [12,13].

For an unoriented sample the static interactions, always present in anisotropic lipid bilayers, will create broad, featureless spectra in NMR spectroscopy. The pfg-NMR method, which is based on the refocussing of a spin-echo, cannot be used straightforwardly for such samples unless special techniques are employed [14]. However, by using oriented bilayers several technical problems are avoided. Fortunately, in fluid lipid bilayers the interactions are averaged due to rapid molecular motion and the NMR spectrum is greatly simplified (compared to a solid). Thus, the fast translational and rotational motions of the lipid molecules result in that all the static interactions are projected along the bilayer normal. The residual interaction includes a scaling term, namely the second Legendre polynomial $P_2(\theta_{LD}) = \frac{1}{2}(3\cos^2\theta_{LD}-1)$ where θ_{LD} is the angle between the bilayer normal and the main magnetic field, B_0 [15]. For $\cos\theta = \frac{1}{\sqrt{3}}$, *i.e.* θ_{LD}=54.7° (the "magic angle" (MA)), $P_2(\theta_{LD})$ becomes zero and the residual static interaction "magically" disappears.

With all the bilayer normals oriented in the same direction the static interactions can thus be conveniently cancelled by rotating the sample so that the bilayer normals makes an angle of 54.7° with respect to B_0, thereby cancelling the static interactions and narrow peaks are observed in the NMR spectrum. Generally, the NMR spectra for such samples show linewidths that are reduced from several kHz (unoriented) to the order of hundreds of Hz or less (oriented) (Fig. 1). In Fig. 1 it is also clear that the sensitivity on the magic angle orientation is directly dependent on the magnitude of the residual interaction. Thus, the signal from the protons of the hydrocarbon chain at 1.1 and 0.7 ppm is more sensitive to the bilayer orientation than the protons of the more

[1] It is common to exclude the water component and regard the ternary system as a pseudobinary lipid system. This should be a reasonable assumption as long as water is present in excess.

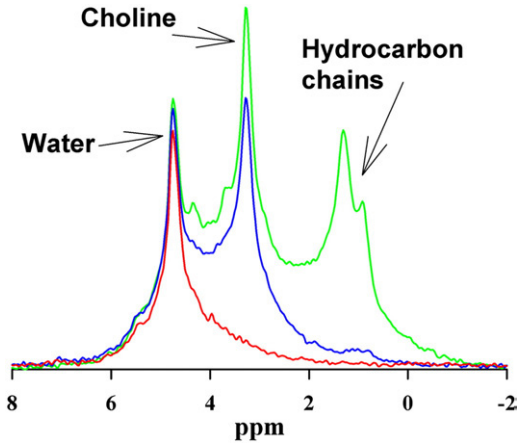

Fig. 1. Pfg-NMR spectra recorded on oriented dimyristoyl-PC (DMPC) bilayers with θ_{LD} at MA and when it is deviating by 0.3° (blue) and 3° (red) from MA.

flexible lipid head group at 3.1 ppm. Finally, the water signal at 4.6 ppm is much less sensitive to a deviation of θ_{LD} from the MA because of its very small residual static interactions.

For the diffusion measurements the stimulated echo pulse sequence is generally used in which the echo amplitude of each spectral component, A_i, is given by [16]:

$$A_i = A_{0i}\exp\left(-\gamma^2\delta^2 g^2 D_i\left(\Delta-\frac{\delta}{3}\right)\right) \quad (1)$$

where A_{0i} is a factor proportional to the proton content in the system, γ is the gyromagnetic ratio, Δ is the time interval between two identical gradient pulses, $(\Delta-\delta/3)$ is the diffusion time, δ and g are the duration and amplitude of the pulsed field gradients, respectively, and D_i is the self-diffusion coefficient of spectral component i. The observed spectrum will be a superposition of all spectral components present in the sample. Varying one or more of the variables δ, Δ or g results in a series of attenuated spectra, from which D_i can be extracted. The so-called CORE method for global analysis of the entire data set is used for the extraction of the diffusion coefficients [17]. This method improves the quality of the evaluated data and provides the spectral features of each of the components in the multi-component analyses. The CORE analysis generally gives one fast component corresponding to the water, and one or two lipid components, together with the corresponding spectral shapes.

In the NMR experiment the translational diffusion is measured in the direction of the magnetic field gradient, which normally is directed parallel with B_0. For a lipid membrane the observed diffusion coefficient, D, depends on the two diffusion coefficients, D_L and $D_\perp$. D_L is the lateral diffusion coefficient for motion parallel with the membrane plane, and $D_\perp$ stands for the diffusion perpendicular to the bilayer. Then, [12,18]

$$D = D_L\sin^2\theta_{LD} + D_\perp\cos^2\theta_{LD}. \quad (2)$$

For a bilayer oriented at the magic angle $\sin^2\theta_{LD} = \frac{2}{3}$ and D_L is given by $D_L = 1.5D$, since it is reasonable to assume that $D_\perp$ is orders of magnitude smaller than D_L and the second term in Eq. (2) can be neglected.

2.3. ^{2}H-NMR quadrupole splittings for determination of molecular ordering

2.3.1. Molecular order

Since the packing of lipids and the molecular ordering are important properties that seem to strongly affect the lateral diffusion coefficients (see e. g. the free area model mentioned in Section 3.1.1), it is sometimes desirable to be able to determine the order parameters of the acyl chains. This is usually done by ^{2}H NMR quadrupole splittings of deuterated acyl chains. For a lamellar arrangement of lipids, the ^{2}H NMR signal of a C–^{2}H group consists of a doublet at resonance frequencies $\pm\nu_q$, where the separation between the two lines in the doublet, defined as the quadrupole splitting of the methylene or methyl groups, is given by [19,20]

$$\Delta\nu_q = \frac{3}{2}\chi\left(\frac{3\cos^2\theta_{LD}-1}{2}\right)S_{CD}. \quad (4)$$

The order parameter of the C–^{2}H bond, S_{CD}, is given by

$$S_{CD} = \frac{1}{2}\langle 3\cos^2\beta-1\rangle \quad (5)$$

where the angular brackets $< >$ denotes a time average and β is the angle between the C–^{2}H bond and the bilayer normal. Thus, the order parameter is determined both by structural terms (i.e. the average orientation of the molecular axes) and by the dynamics and magnitude of the fluctuations of the C–^{2}H bond around the average direction.

For a sample consisting of a random distribution of liquid crystalline microdomains, the spectrum will adopt a so-called "powder pattern", where the intensities at each θ_{LD} is scaled by the probability density, $p(\theta_{LD}) = 1/2\sin\theta_{LD}$. This gives characteristic ^{2}H NMR spectra with "peaks" ($\theta_{LD}=90°$) and "shoulders" ($\theta_{LD}=0°$). For macroscopically oriented samples one orientation will dominate and all intensity will be collected into two lines at frequencies $\pm\nu_q$ with a tremendous increase in the signal to noise ratio. Fig. 2 exemplifies the spectra obtained from macroscopically oriented systems at 60 °C and with θ_{LD} set to 0°. The spectra for palmitoyloleoyl-PC-d_{31} (POPC-d_{31}) consist of one narrow splitting (4 kHz) belonging to the terminal C^2H_3-group (carbon number 16) of the deuterated chain. (The intensity of this doublet is smaller than expected, since the pulse delays in the experiment were short compared to T_1 for this group). Then follows 8 Pake-pairs approximately equally spaced in the splitting range of 30–40 kHz, corresponding to the 8 C^2H_2-groups of carbons 8–15. The peak, corresponding to the largest splitting of these, overlaps with the broad peak at the edge of the spectrum corresponding to the C^2H_2-groups of carbons 3–7. The remaining carbon (number 2) contains two inequivalent deuterons with splittings that usually are found at about 60 and 40 kHz [21–23] for palmitoylsphingomyelin (PSM) at 56 °C [22]. The order parameter calculated from Eq. (4) gives a so-called order profile with a constant S_{CD} for about the first eight carbons in the chains and with a subsequent strong decrease in the molecular ordering down to the ω-carbon. Addition of the saturated PSM results in a small increase in ordering, while an addition of CHOL produces significantly larger order and a prolongation of the plateau region of the order profile.

2.3.2. Chemical exchange

Here, we pay the attention to an extraordinary property of ^{2}H NMR splittings from oriented bilayers, namely the possibility to set the magnitude of the residual static interactions to any desirable value simply by varying θ_{LD}. This can be quite useful for example in studies of chemical exchange.

Fig. 3 shows an example where ^{2}H NMR quadrupole splittings have been used to detect small domains in ternary lipid bilayers.

The figure shows stack plots of deuterium spectra obtained for a sample containing POPC-d_{31}/PSM/CHOL (35:35:30 mol%). The temperature is varied from 0 °C (bottom) to 60 °C (top) in steps of 3°. Fluorescence anisotropy measurements indicate that this composition resides in a two-phase area of l_o and l_d phases (Halling, K. K.,

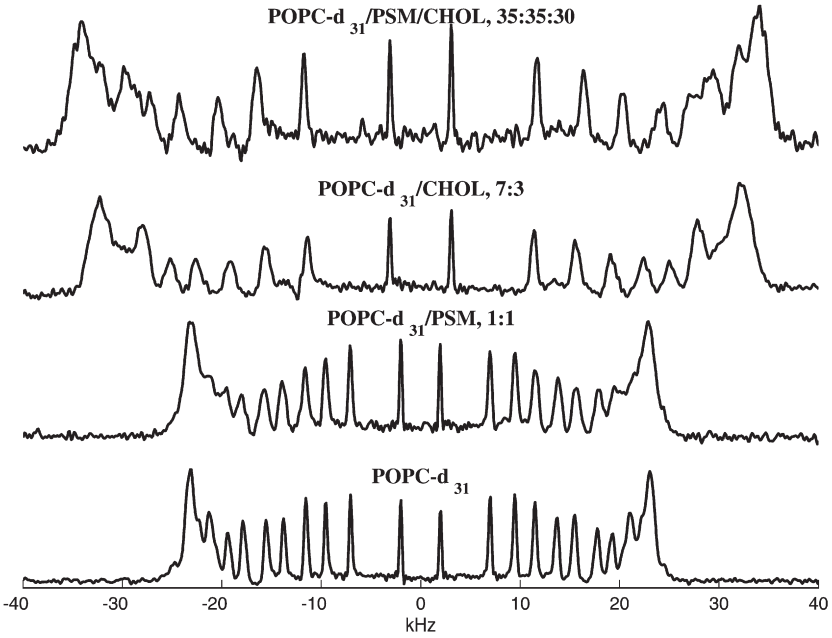

Fig. 2. ^{2}H-NMR spectra of various macroscopically oriented bilayers at 60 °C and with θ_{LD}=0° recorded with a quadrupole echo sequence with composite pulses [55]. Note the slight increase in ordering when a high-T_m lipid is mixed with a low-T_m lipid, as compared to the large ordering imposed by the addition of CHOL.

Ramstedt, B., Nyström, J. H., Slotte, J. P., and Nyholm, T. K. M. to be published). The broadening and disappearance of the signal in the temperature region of 9–36 °C is indicative of a situation, where the molecules experience an exchange between two environments, in which the exchange rate is comparable to the difference in the quadrupole splittings in the two phases [24].

Fig. 4 shows spectra of the same sample taken at 24 °C, where the lineshape is smeared out by the exchange at θ_{LD}=0°. Four orientations of the bilayer normal between 0 and 50° are shown in the figure. The left hand side of the plot shows the frequency axis, in which the lineshape is seen to contract as the $P_2(\theta_{LD})$ term gets smaller. The right hand side of the plot is given in terms of an "order" axis by dividing the frequency axis by $2*P_2(\theta_{LD})$. In this representation all peaks would stay in the same position for all orientations, since the order parameter is not expected to change by a sample rotation. This is indeed the case as can be seen for the innermost splitting that is not affected by the exchange smearing effect. By varying θ_{LD} it is possible to reduce the residual quadrupole coupling to such an extent that the difference in the quadrupole splittings becomes smaller than the exchange rate. This results in a condition of fast exchange, where the observed lineshape narrows around the mean value of the peaks for the two different phases and

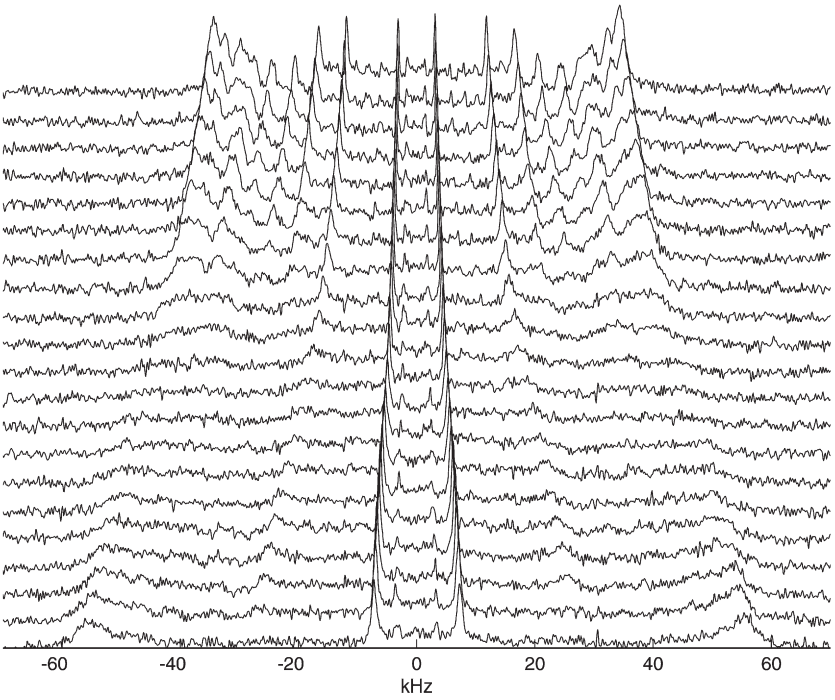

Fig. 3. Stacked ^{2}H-NMR plots of oriented bilayers of POPC-d_{31}/PSM/CHOL (35/35/30 mol%) and θ_{LD}=0°. Temperature is varied from 0 (bottom) to 60 °C (top) in steps of 3°.

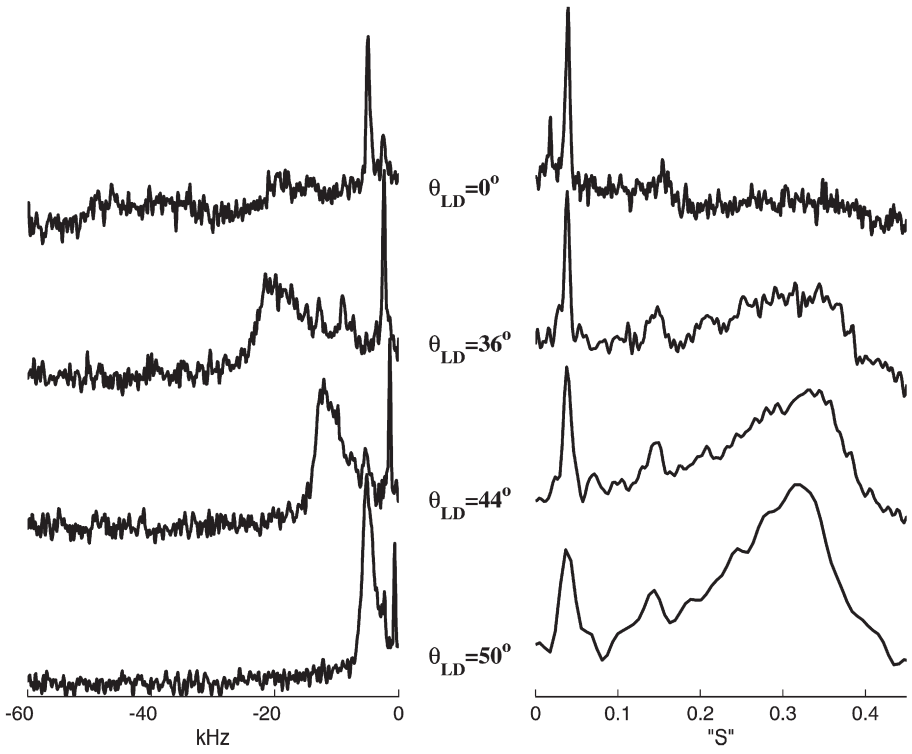

Fig. 4. ^{2}H NMR spectra of the same sample as in Fig. 3 and taken at 24 °C. The left hand side of the figure has an *x*-axis in frequency units, while the right hand side *x*-axis is given in "order" units by dividing the frequency by $2*P_2(\theta_{LD})$. Four different bilayer orientations have been used as indicated in the plot.

the signal amplitude is regained. A detailed analysis, will be presented in a forthcoming publication (Orädd, G., Halling, K., Slotte, P., and Lindblom, G.), and the results indicate that the domain sizes must be in the range of 10–100 nm.

3. Heterogeneity in lateral organization in bilayers studied by pfg-NMR

Lateral phase separation into two fluid phases in a bilayer can be achieved in several ways and it can be relatively easy detected by the pfg-NMR diffusion technique [10,11,25–30]. A common theme in our studies is that in the lipid bilayer a sterol is always solubilized together with one or two other lipids, and all experiments are performed for bilayer systems in equilibrium with excess water. High water content has to be used, since the lipid diffusion coefficients depend on the water content, as illustrated in Fig. 5 for dioleoyl-PC (DOPC) and POPC with three different CHOL contents and water concentrations between 20 and 55 wt%. As can be inferred from this figure D_L increases with the water content between 20 and about 35% and then stays constant [28].

3.1. Effect of lipid packing — influences of lipid headgroup area on D_L

In general, a closer packing of the lipids in the bilayers results in a decrease in D_L. This result is predicted in the free area theory of lipid diffusion [31–33]. This theory considers a particle performing a random walk in two dimensions, and leads to the following relation between the lateral diffusion coefficient and the free area (a_f) [34]

$$D_L = D^* \exp\left(\frac{-\beta a^*}{a_f}\right) \quad (6)$$

where D^* is a constant and a^* is a critical size of the free area that needs to be present next to the diffusing particle for a step to take place, β is a factor to correct for overlapping free volumes, which is typically in the range of 0.5–1. Usually a^* is taken to be the van der Waals area and the free volume is calculated as the difference between this value and the average area measured in bilayers or monolayers.

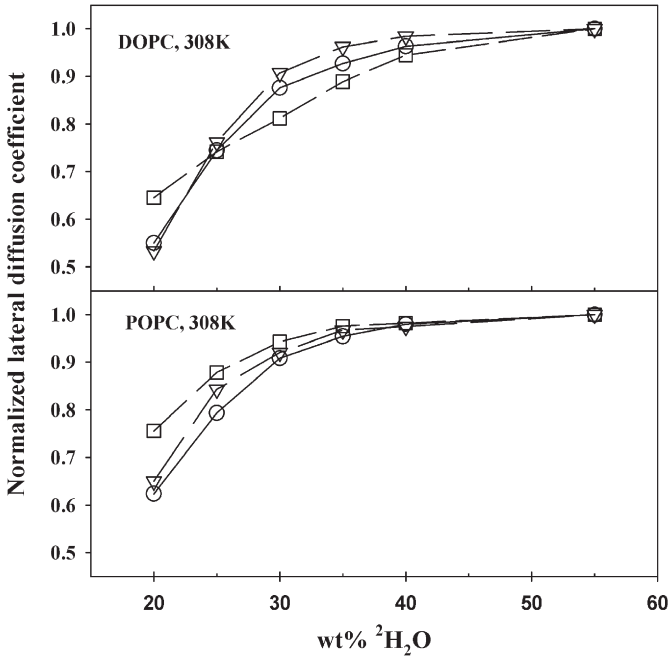

Fig. 5. Normalized lipid lateral diffusion coefficients for two different lipid bilayers at 35 °C and varying water content. The CHOL content in the upper panel is: 0% (circles); 14.5% (triangles), and 42% (squares), and for the lower panel: 0% (circles); 13% (triangles), and 48% (squares).

3.1.1. Bilayers with one lipid species

Table 1, where D_L for a number of lipid bilayer system are collected, gives support for the free area model. It can be inferred from this Table that D_L increases in the order DOPC>POPC>DPPC>DMPC in line with the increase in the headgroup area caused mainly by the acyl chain unsaturation. Moreover, eSM has a lower D_L than DPPC, and dioleoylphosphatidylglycerol (DOPG) with its repulsive charged headgroup exhibits a larger diffusion coefficient than DOPC, again in agreement with what can be expected from the headgroup areas [34]. Also the more tightly packed lipids extracted from the *Acholeplasma laidlawii* membrane have a slower translational diffusion than the phospholipids coming from *Escherichia coli* [35]. In fact there is a striking difference in D_L for the *A. laidlawii* lipids, which consist of 70–80 mol% glucolipids [36], and the *E. coli* lipids, which consist of only phospholipids [37]; the diffusional motion of the latter is much more rapid. Surface balance studies on monolayers [38] and X-ray scattering on Lα phases [39–41] have shown that the area per lipid molecule at the water/lipid interface is smaller for a mixture of glucolipids (monoglucosyldiacylglycerol (MGlcDAG) and diglucosyldiacylglycerol (DGlcDAG)) than for phosphatidylcholines (PCs) with corresponding acyl chains.

Recently, the effect of increased unsaturation in the *sn*-2 fatty acyl chain of PCs on the lipid lateral diffusion has been investigated [30]. Bilayers, where the number of double bonds in the PC was 1, 2, 4 or 6, have been studied. It was found that the lateral diffusion increased with increasing number of double bonds, as a consequence of the increased headgroup area caused by the unsaturation. Fig. 6 nicely illustrate the good fit to Eq. (6) for the D_L dependence on the increasing area per lipid molecule with increasing number of double bonds in the acyl chains [30].

3.1.2. The condensing effect of CHOL

In mixed lipid monolayers, formed by spreading lipids in a film balance, it is frequently observed that the area of the mixture is significantly smaller than that calculated from the weighted sum of the areas of each pure component at a given pressure [1,38]. In a recent study the effect of CHOL on phospholipid bilayers was systematically investigated by X-ray diffraction [42]. The phosphate-to-phosphate distance across the bilayers was measured as a function of CHOL concentration and it was found, as expected for a nonideal fluid, that this distance increased nonlinearly with the CHOL content until it reaches a maximum. The mechanism behind the observed condensing effect of CHOL is still not fully understood and a number of theoretical studies have been published. A crucial question has arisen on how to partition the total area in simulation of membranes consisting of more than one kind of molecule into the average areas for each type of molecule. Recently, a solution to this problem was published by Edholm and Nagle [43], who in analogy with the definition of partial-specific volume, used a partial-specific area that could be obtained from simulations of DPPC/CHOL bilayers. Using this formalism they demonstrated the well-known condensing effect of CHOL and this also lead to a theoretical model that accounted for the area of CHOL and the lipid over the entire range of CHOL concentrations in the bilayer. In our studies the condensing effect is illustrated for the binary system of DMPC:CHOL (Table 1). The flat, rigid ring system of CHOL is effective in increasing the packing of the hydrocarbon chains, resulting in a reduced diffusional motion.

An anomaly is observed for the DOPC/CHOL system at low CHOL contents and low temperature. A deviation from the normally monotonous decrease in D_L with increasing CHOL content is observed at low water contents (Fig. 7) [28]. At high temperatures the dependence is close to linear, but much weaker than at higher water contents. As the temperature decrease a plateau at low CHOL content become prominent and for the two lowest temperatures there is even a small increase in D_L for small additions of CHOL. A possible explanation to this increase in the lipid mobility could be that a decrease in the intermolecular interactions between the phospholipid molecules is accomplished by CHOL. It was proposed that incorporation of the flat steroid molecule resulted in a decrease in the entanglement between the unsaturated acyl chains, slightly increasing the molecular ordering, and at the same time allowing for the lipid molecules to move more independent of each other.

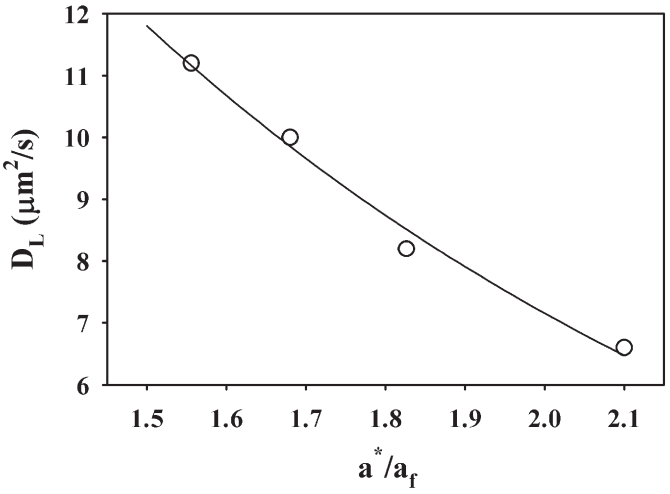

Fig. 6. D_L vs. free area at 30 °C for a series of oriented bilayers of SXPC, where X stands for a 18:1, 18:2, 20:4 and 22:6 acyl chains. The curve represents the best fit to Eq. (6) with β set to 1.

Table 1
Headgroup areas and corresponding D_L for some lipids in bilayers

Lipid	Area, nm^2	D_L,$\mu m^2 s^{-1}$	*t*, °C
DOPC	0.72[a]	8.25[b]	25
POPC	0.68[c]	7.79[b]	25
DPPC	0.64[c]	17.8[d]	45
DMPC	0.61[c]	5.82[e]	25
SOPC[f]	0.63[c]	6.6[g]	25
SLPC[h]	0.66[c]	8.2[g]	25
SAPC[i]	0.68[c]	10.0[g]	25
SDPC[j]	0.70[c]	11.2[g]	25
eSM	0.53[c]	4.5[k]	50
DOPG	0.80[l]	15[m]	30
A. laidlawii lipid extract[n]	Ca 0.6	2.7	30
CHOL/(CHOL+DMPC)			
0.0	0.61[o]	9.0[e]	30
0.1	0.53[o]	4.9[e]	30
0.2	0.48[o]	2.6[e]	30
0.3	0.44[o]	2.0[e]	30

[a] From [57].
[b] From [28].
[c] From LIPIDAT and [30].
[d] From [25].
[e] From [56].
[f] SOPC is stearoyloleoylphosphatidylcholine.
[g] From [30].
[h] SLPC is stearoyllinoleoylphosphatidylcholine.
[i] SAPC is stearoylarachidonoylphosphatidylcholine.
[j] SDPC is stearoyldocosahexaenoylphosphatidylcholine.
[k] From [27].
[l] From [58].
[m] From (Filippov, A. Orädd, G. And Lindblom, G. to be published).
[n] From [35,38].
[o] From [43].

3.2. Domain formation and phase diagrams with lipids and CHOL

As mentioned above the well known and striking effect of CHOL on the lipid bilayer, especially saturated lipids, is the strong ordering of

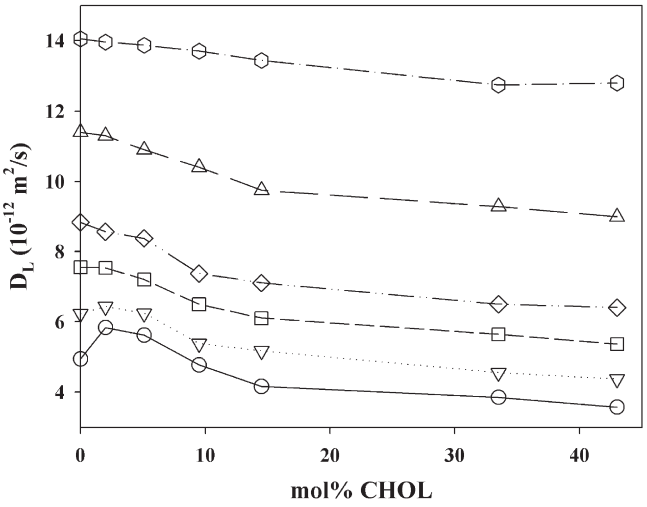

Fig. 7. Lateral diffusion coefficients as a function of CHOL content in oriented DOPC bilayers at a water content of 20wt% and at temperatures 25 (circle), 30 (triangle down), 35 (square), 40 (diamond), 50 (triangle up) and 60 °C (hexagon).

the lipid acyl chains that results in a tighter packing of the molecules in the bilayer and the so-called condensing effect.

When the CHOL level reaches some threshold value a separation into two phases can occur, depending on if the temperature is above T_m — two liquid crystalline phases form or below T_m — one liquid crystalline and one solid phase form (see Fig. 8, left panel). The generic phase diagram suggested in Fig. 8, left panel is based on several studies of binary CHOL/high-T_m lipid systems [4,5,44,45]. CHOL is in many ways an amazing molecule; for example low CHOL concentrations are soluble in the solid (s_o) phase (often called the gel phase) and the effect on T_m is small. This is an unusual property for a solid that is normally not a good solvent. The solubility of CHOL in saturated PCs is almost as high in the s_o as in the l_d phase. Note also that (for thermodynamic reasons) there is a narrow coexistence region ending in a eutectic point separating the s_o and l_d phases. Then, with increasing CHOL content the phase transition between gel and liquid crystalline phase gradually disappears, and CHOL has been called the "crystal breaker" as it disturbs the translational order of the PC molecules in the gel phase. At high CHOL contents the bilayer system strongly favours a fluid l_o phase over the solid phase at a large range of temperatures.

Fig. 8, right panel shows a typical ternary phase diagram built up of CHOL and one low-T_m lipid and one high-T_m lipid [46,47] at a temperature above the highest T_m. As can be inferred from Fig. 8, right panel there is a closed area of coexisting l_d and l_o phases. According to Fig. 8, left panel there is a region of lateral phase separation also along the CHOL/high-T_m lipid edge. In experiments by

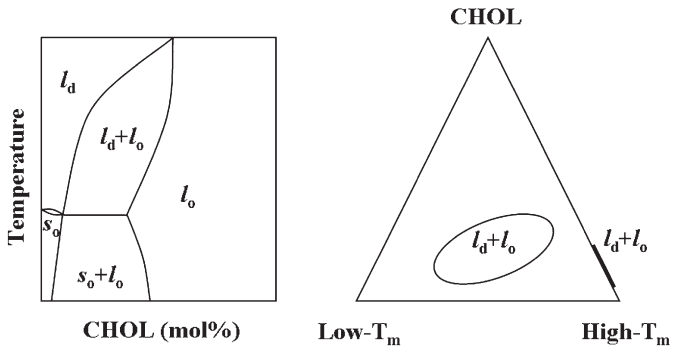

Fig. 8. Generic phase diagrams of binary (left) and ternary (right) bilayer systems. In the ternary phase diagram only the fluid-fluid phase coexistence areas are depicted.

Veatch et al. [48] on a ternary system composed of diphytanoyl-PC (low T_m)/DPPC/CHOL they observed that the tie-lines in the ternary two-phase area were directed nearly perpendicular to the binary DPPC/CHOL edge. Veatch et al. [48] concluded that it was unlikely that a phase separation were present in the binary mixture, and that their data suggest that DPPC/CHOL bilayers do not form coexisting l_d and l_o phases above T_m of DPPC but this system only form a single uniform fluid lamellar phase at these temperatures (i.e. not like in Fig. 8, left panel). This seems to be at variance with studies we have performed on the binary eSM/CHOL and DMPC/CHOL systems, where the lateral diffusion data indicated that there are small domains, between which there is rapid exchange (see also the next section) [27]. Recently, we performed careful studies on the phase behaviour in the DOPC/eSM/CHOL system close to the proposed l_d and l_o coexistence in the binary eSM/CHOL edge of the phase triangle with only 0.5 wt% DOPC and only a single phase was observed (A. Filippova, G. Orädd and G. Lindblom, unpublished results). From these findings we concluded that there are two different l_d/l_o two-phase areas in the DOPC/eSM/CHOL phase triangle.

3.3. Lateral diffusion in heterogenous systems

Influence of domains on the translational motion of lipids enables the pfg-NMR diffusion method to detect lateral phase separation in lipid bilayers. Lipids will either diffuse in and out between the separated phases in the bilayer by an exchange mechanism or, provided that the border between different phase domains presents an obstacle to lipid diffusion, the lipids will encounter restrictions in their diffusional motion. Fig. 9 shows how D_L depends on the CHOL content for three different fluid bilayers at 50 °C that will be discussed below in *i*)–*iii*).

i) No domain formation. The system of DOPC/CHOL (triangles) forms a microscopically homogeneous system in which the addition of CHOL causes a gradual increase of ordering of the bilayer. As the CHOL concentration increases the lipid diffusion varies continuously due to the increase in the packing of the lipids [27].

ii) Domain formation with fast exchange. The system of egg sphingomyelin (eSM)/CHOL (squares) exhibits domain formation in the range of 6–22 mol% CHOL, and this is seen as a sudden break in the curve D_L vs CHOL [27]. Due to fast exchange between the separated phases, l_o and l_d, the observed diffusion coefficient, D_L

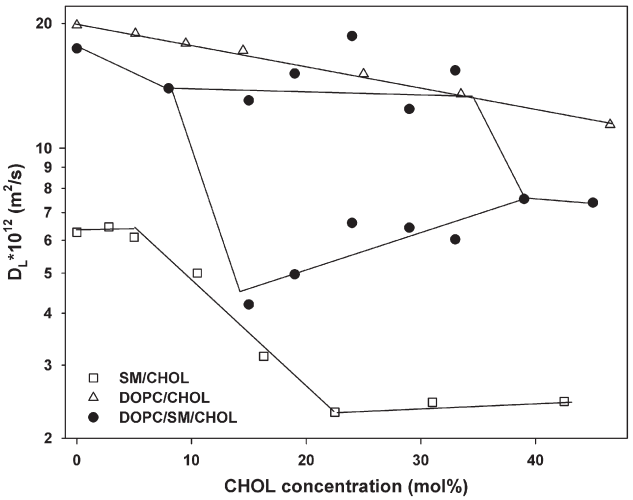

Fig. 9. D_L as a function of CHOL content at 50 °C for three different bilayer systems. Squares: eSM/CHOL; triangles: DOPC/CHOL; circles: DOPC/eSM/CHOL.

(2ϕ), will be a weighted average of the diffusion coefficients in the separate phases

$$D_L(2\phi) = p_o D_L(l_o) + (1-p_o) D_L(l_d) \quad (6)$$

in which p_o is the relative amount of the l_o phase. As p_o increases the diffusion will decrease as $D_L(l_o)$ becomes dominant. A fast exchange is an indication that the domains are smaller than the mean diffusion length (ca 1 μm) calculated from $r^2 = 4D_L\Delta$. In the one-phase regions on each side of the two-phase area the diffusion coefficients are almost constant.

iii) Domain formation with slow exchange. In the ternary system of DOPC/eSM/CHOL (circles) macroscopic phase separation occurs between 10 and 37 mol% CHOL [29]. Because of the large size of the domains the lipids will mainly move within the same domain during the time of measurement (50–200 ms) and, therefore, exchange between the phases will be slow on this timescale. This will occur when the average domain sizes exceed 1 μm and results in separately observed diffusion coefficients corresponding to diffusion in the two phases [29].

A fourth case also is possible (not illustrated in Fig. 9), in which the exchange time between the domains is comparable with the diffusion time. In this case two-component diffusion will also be observed, but both the preexponential factors and the apparent diffusion coefficients will be functions of diffusion time, relaxation times, diffusion coefficients and lifetimes of the lipids in the two phases [49,50]. Such a case can thus be distinguished from the case *iii*) above, since D_L will vary as the diffusion time, Δ, changes.

3.3.1. Summarizing the properties of the l_o and l_d phases

At this point we conclude that valuable information about the domain structure and lipid dynamics can be obtained from pfg-NMR diffusion experiments. By investigating the D_L dependence on the temperature and CHOL concentration we were able to observe lateral phase separation in several phospholipid/CHOL/water systems. The findings can be summarized as follows [26,27,29,30,51]:

- D_L is about 2–10 times smaller in the l_o phase, depending on the lipid system studied, and it is almost independent on CHOL content in this phase.
- The temperature dependence is of the Arrhenius type in the one-phase regions, but deflects from this behaviour in the two-phase areas, in which fast exchange occurs because of the changing relative amounts of the two phases.
- The apparent activation energies for the diffusion process is higher in the l_o than in the l_d phase.
- Only small domains seem to form for binary systems while both small and large domains are observed for ternary systems.

3.3.2. Isotopically labelled lipids give detailed information

The observation of two distinct lipid lateral diffusion coefficients is not by itself unambiguous indication of phase separation in systems containing several lipid species. Instead, for example different lipids could have different D_Ls due to differences in shape and/or interactions in the headgroup. Since there is a severe overlap of the ^{1}H NMR signals from the lipids, it is difficult to assign any specific lineshape to the individual lipids and it is therefore impossible to separate the contributions from the different lipid species by pfg ^{1}H NMR. This problem can be overcome by isotopic labelling. By observing e.g. ^{2}H NMR on a deuterated lipid it is possible to separately study each labelled lipid species. We have employed this strategy for the DOPC/DPPC/CHOL system, where DPPC has been perdeuterated and CHOL has been ^{19}F labelled at the 25-position on the hydrocarbon chain of the sterol (25FCH) [25] (Fig. 10). It could be concluded that the diffusion of the lipids is governed by the properties of the phase in which they reside, rather than the properties of the molecules themselves. This means that all lipid species, including CHOL, have the same diffusion coefficient as long as they are in the same phase. Finally, from the diffusion data the partitioning of the lipids into the two phases could be estimated. While DPPC partitioned exclusively into the l_o phase it was found that both DOPC and CHOL were present in both phases. Thus, CHOL does not seem to favour any of the phases in any large degree.

Further studies with isotopically labelled molecules are in progress and the results show that it is the overall (biophysical) properties of the bilayer rather than the individual lipids that governs the lateral diffusion (Orädd, G., Halling, K. K., Nyholm, T. K. M., Slotte, J. P., and Lindblom, G. to be published).

4. Criteria for lateral phase separation in ternary lipid systems

Systematic pfg-NMR studies have revealed that CHOL together with one low-T_m lipid and one high-T_m lipid are essential components for domain formation and that small changes in the structure of the components can have a large impact on the domain-forming process [26,29,51,52].

4.1. The homogeneity of the high-T_m lipid is important

In order to address the importance of the high-T_m lipid we have investigated the system of DOPC/SM/CHOL for SMs of three different origins: egg, brain and milk [26]. These three natural compounds differ in the composition of both the long-chain base and the fatty acid such that eSM has the most homogeneous composition and milk SM the most heterogeneous one. Although all three SMs have the same diffusional behaviour in single component bilayers the ability to form domains differs in the multi-component systems. The region of large domain formation is more extended for eSM than for brain SM and no such region is found for milk SM. The results indicate that a crucial element in the domain-forming process is highly packed bilayers of SM and cholesterol and that the introduction of different chain lengths and/or unsaturation can disturb this packing to such an extent that large domains no longer form.

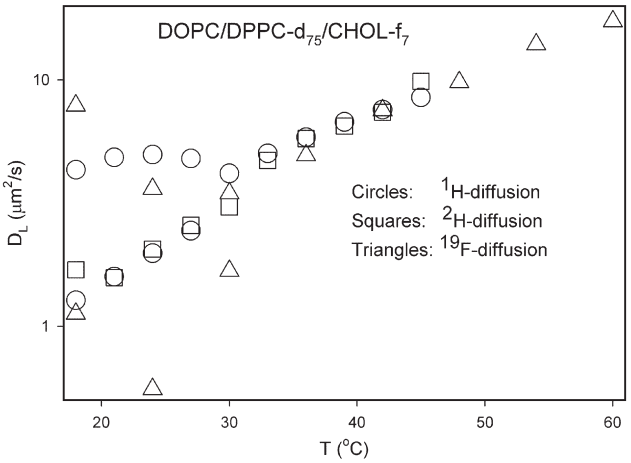

Fig. 10. D_L in oriented bilayers of DOPC/DPPC-d_{75}/CHOL-f_7. By using differently labelled lipids it is possible to study the lateral diffusion of each lipid species individually. As the temperature goes below 30 °C the two-phase area is entered and two separate diffusion coefficients are observed for DOPC and CHOL. DPPC, on the other hand, seems to partition only into the l_o phase [25].

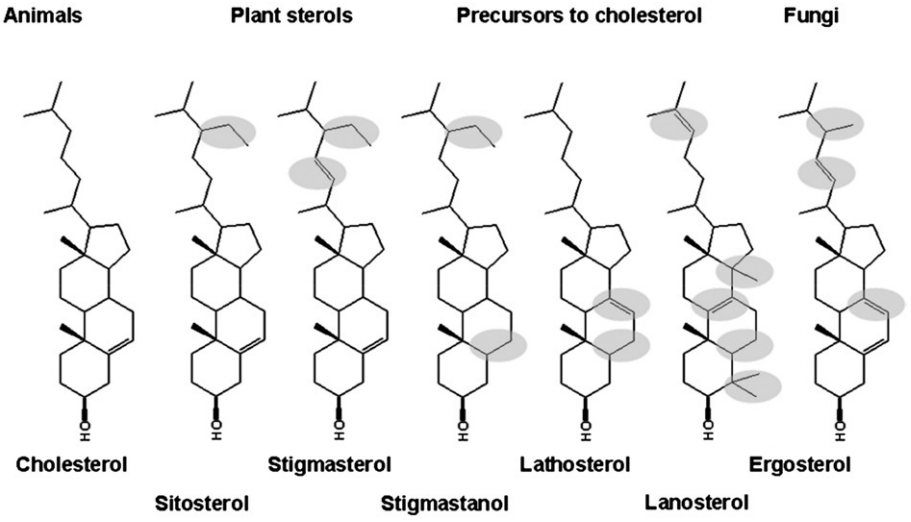

Fig. 11. Chemical structures of seven sterols. The grey areas highlight the differences in the structures compared to CHOL.

4.2. The structure of the sterol is important

A series of seven biologically relevant sterols (Fig. 11), including CHOL were used in mixtures of DOPC/eSM/sterol (37.5:37.5:25 mol %) [51]. The occurrence of two lipid diffusion coefficients was used as evidence of lateral phase separation into large domains of l_o and l_d phases. It was found that additions of cholesterol, ergosterol, sitosterol and lathosterol gave rise to two lipid diffusion coefficients, while lanosterol, stigmasterol and stigmastanol resulted in only one observed D_L in the temperature interval of 24–70 °C. For the sterols that were able to induce large domains the stability of these were sensitive to the sterol structure, indicating that lathosterol forms the most stable domains, followed by ergosterol, CHOL and sitosterol.

It is obvious that even very small changes in sterol structure can have a large impact on the domain formation. A bulkier hydrocarbon chain, created by the introduction of either double bonds or side chains, reduces the domain-forming capability of the sterol, as does the presence of bulky sidechains on the ring skeleton. Such modifications will obviously interfere with the tight packing of lipids and sterol that is needed for the domain formation. The position of the double bonds in the ring skeleton also seems to have a large impact on the domain-forming potential of the sterol. This behaviour is harder to rationalize, but could possibly be related to a change in the planarity of the ring skeleton. An NMR-relaxation study also indicates that the domain-forming sterols increase the bending rigidity of the bilayers to a higher degree than other sterols (Orädd, G., Shahedi, V., and Lindblom, G.

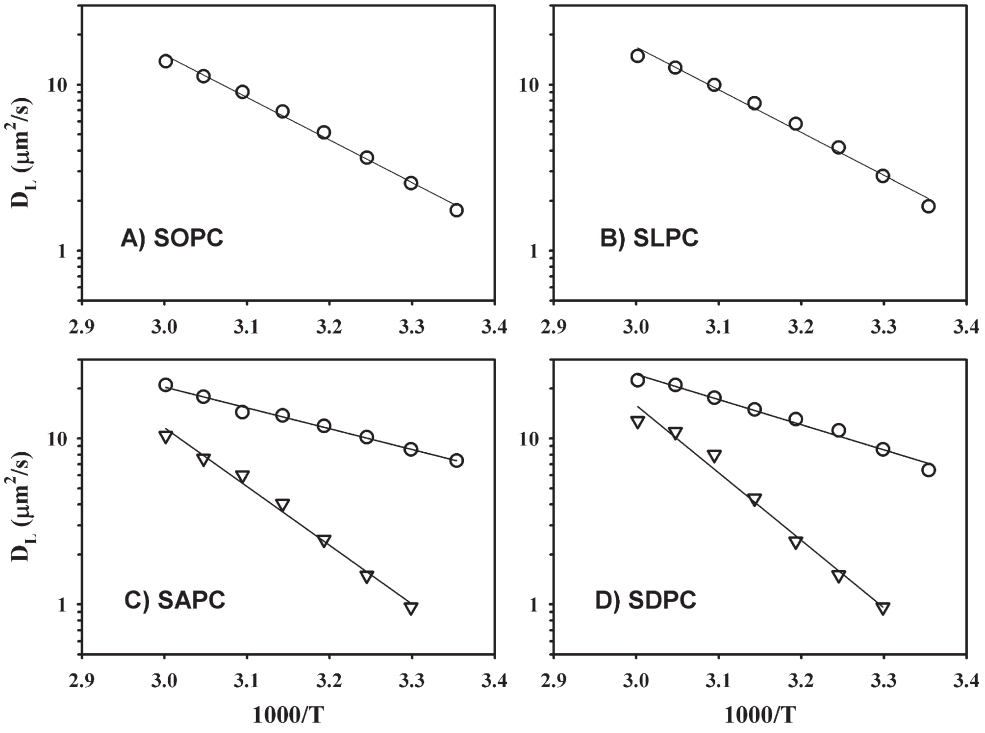

Fig. 12. Temperature dependence of D_L for oriented bilayers of SXPC/eSM/CHOL, where X stands for a 18:1, 18:2, 20:4 and 22:6 acyl chains.

to be published). This is to be expected if the packing of the bilayers is increased by the sterol.

4.3. The degree of unsaturation of the low-T_m lipid is important

Large domains have been observed for the diunsaturated DOPC with eSM and CHOL [26,53], while for the monounsaturated POPC it is less clear whether domains form or not. There are large differences in the two phase diagrams that have been reported [46,53] and with pfg-NMR methods we have been unable to detect large domains in this system. This discrepancy probably originates from differences in spatial resolution of the methods. Fluorescence microscopy and pfg-NMR rely on large (>μm) domains, while fluorescent anisotropy measurements can detect smaller domains[46]. However, our recent ^{2}H-NMR studies have revealed evidence for small domains in the POPC/PSM/CHOL system (see Section 2.3.2). The corresponding system with DPPC as the high-T_m lipid shows no signs of exchange effects. Instead a smooth transition in quadrupole splittings is observed. This is indicative of a fast exchange between domains smaller than 10 nm or a homogeneous membrane.

In an attempt to address the role of the degree of unsaturation of the low-T_m lipid we have investigated systems of SXPC/eSM/CHOL, in which X stands for hydrocarbon chains with differing degree of unsaturation (Fig. 12). For the systems stearoyloleoylphosphatidylcholine (SOPC)/eSM/CHOL and stearoyllinoleoylphosphatidylcholine (SLPC)/eSM/CHOL we found no evidence for large domains, although the existence of small domains could not be excluded. However, increasing the degree of unsaturation in the lipid chains results in phase separation into the l_d and l_o phases for both the stearoylarachidonoylphosphatidylcholine (SAPC)/eSM/CHOL and the stearoyldocosahexaenoylphosphatidylcholine (SDPC)/eSM/CHOL systems. Therefore, systems that are on the verge of forming larger domains can be triggered into this behaviour by an increase in the number of double bonds in the PCs. The possible existence of small domains in the SOPC and SLPC systems is currently investigated, using deuterated SM (Orädd, G., Halling, K., Slotte, P., and Lindblom, G. to be published).

5. The driving force(s) for lipid lateral phase separation

The lateral phase separation in the systems studied can be rationalized in terms of lipid order and miscibility of unsaturated lipids in ordered phases. First, we emphasize that the interpretation of our experimental findings is based on the assumption that the lipid lateral diffusion in bilayers is strongly dependent on the lipid headgroup interfacial area, i.e. the lipid packing. Furthermore, for an understanding of the changes in the lipid lateral diffusion upon an alteration of the composition in the bilayers there is no need to include specific interactions between molecules in the bilayer, like lipid–cholesterol complexes, as have been suggested by McConnell and coworkers [54]. Thus, it is seen that high-T_m lipids, such as DPPC and eSM, form more ordered phases than low-T_m analogs, such as DOPC and SDPC, and that addition of CHOL greatly enhances the ordering, especially for the high-T_m lipids. We propose that *the driving force behind the phase separation into l_d and l_o phases is the increasing difficulty for low-T_m lipids to be incorporated into a highly ordered phase*. Our results suggest that the low-T_m lipids have a preference to be located in a disordered phase, while high-T_m lipids prefer the ordered phase. Interestingly, and quite unexpectedly, CHOL seems to partition into both phases to roughly the same extent, indicating that CHOL has no particular preference for any of these phases, and there are no specific interactions between CHOL and saturated lipids. This property probably stems from the rigid nature of the sterol structure, making it rather insensitive to the molecular order of the environment. The role of CHOL in the phase separation process is to increase the ordering of the (homogeneous) lipid membrane to such an extent that the system finally favours phase separation, where a portion of the low-T_m lipid is squeezed out from the l_o phase. This will drastically increase the entropy of the low-T_m lipid, while leaving the entropy of the high-T_m/CHOL phase, i.e. the l_o phase, relatively unchanged. Thus, a phase separation will result in a decrease in the free energy of the system and we propose that a change in entropy is the main driving force for the formation of the l_o phase.

Acknowledgements

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