Sphingomyelin is much more effective than saturated phosphatidylcholine in excluding unsaturated phosphatidylcholine from domains formed with cholesterol

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Abstract In this study, we compared domain formation in raft-like mixtures of cholesterol and dioleoylphosphatidylcholine (DOPC) with either sphingomyelin (SM) or dipalmitoylphosphatidylcholine (DPPC). Using ^{2}H nuclear magnetic resonance, we studied the properties of the lipid enriched in the fluid phase, DOPC. We found that acyl chain ^{2}H-labeled DOPC is much less ordered in SM-containing mixtures than in those containing DPPC, suggesting that DOPC in the SM-containing mixture senses a lower concentration of cholesterol in its direct environment. Atomic force microscopy experiments demonstrated large differences in the size and shape of domains in the different mixtures. We propose that these various differences are a consequence of the preferential interaction of cholesterol for sphingolipids over glycerophospholipids.

Key words: ^{2}H nuclear magnetic resonance; Atomic force microscopy; Raft; Model membrane; Sphingolipid; Glycerophospholipid

1. Introduction

The isolation of detergent-insoluble membrane fractions, enriched in cholesterol, sphingolipids and certain proteins, from eukaryotic cells [1,2] has led to the postulation of the existence of detergent-resistant domains in the plasma membrane, called rafts [3]. The physical principles underlying raft formation have been extensively studied using model membranes and these studies have contributed greatly to the understanding of domain formation. For example, it was shown that domain formation can be driven by the properties of the lipids themselves [4,5]. A major contribution was the definition of the liquid-ordered phase (L_o) [6], which is characterized by a high ordering of the lipid acyl chains combined with a high lateral diffusion. The L_o phase arises from the interaction of cholesterol with lipids with long saturated acyl chains, resulting in a tight packing of these acyl chains. This tight packing would explain the detergent insolubility of lipids in the L_o phase [7,8]. In the presence of cholesterol, domain formation can occur in a mixture of lipids due to phase separation of a cholesterol-rich L_o phase and a cholesterol-poor liquid-disordered (L_d) phase.

A mixture that is often used in model membrane studies on domain formation is that of dioleoylphosphatidylcholine (DOPC), sphingomyelin (SM) and cholesterol. In equimolar ratios, this mixture will display phase separation at ambient temperatures, forming a DOPC-enriched L_d phase and a SM-and-cholesterol enriched L_o phase [9], which is detergent resistant [5]. Domain formation in these mixtures has been visualized by techniques such as atomic force microscopy (AFM) [10] and fluorescence microscopy [11,12].

Instead of SM, dipalmitoylphosphatidylcholine (DPPC) can also be used in model membrane studies on domain formation. DPPC, like SM, has saturated hydrocarbon chains and a phosphocholine headgroup. When mixed with sufficient amounts of cholesterol, both SM and DPPC form a detergent-resistant L_o phase [5,13]. In fact, the existence of the L_o phase was first derived from mixtures of DPPC and cholesterol [6]. The differences in behavior of the cholesterol itself in binary mixtures with either SM or DPPC are only subtle [14].

However, from a biological point of view, the behavior in more complex ternary mixtures is more relevant. Rafts in natural membranes are enriched in sphingolipids [1,2] and cholesterol shows a preferential interaction for sphingolipids over glycerophospholipids [15–17], possibly due to differences in the hydrogen bonding capabilities [18,19] of these phospholipids. This may have consequences for the behavior of ternary mixtures containing DPPC as compared to those containing SM. In this study, we compare the behavior of ternary mixtures of cholesterol, DOPC and either SM or DPPC. Although the behavior of lipids in the L_o phase in such mixtures has been extensively studied [20–23], little is known about the effects of domain formation on the lipids in the L_d phase. As the more fluid phase may be relatively more sensitive to subtle differences in the behavior of ternary mixtures than the highly ordered phase, we direct our main attention to the lipid that is enriched in the L_d phase, the DOPC. In order to disturb the phase behavior as little as

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Abbreviations: DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; SM, sphingomyelin; NMR, nuclear magnetic resonance; AFM, atomic force microscopy; L_o, liquid-ordered; L_d, liquid-disordered; T_m, transition temperature

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possible, we use DOPC, labeled with ^2H on both acyl chains at the C_{11} position ($^2H_4$-DOPC) and we study its properties in different lipid mixtures using ^2H nuclear magnetic resonance (NMR). Additionally, the lipid organization on a molecular level is coupled to the macroscopic organization of domains in ternary mixtures with SM or DPPC by directly visualizing the domains using AFM. The results show large differences between these mixtures in the size and shape of the domains and in the composition of the two phases.

2. Materials and methods

DOPC, DPPC and egg SM were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Egg SM consists of a mixture of SMs with exclusively saturated, mainly $C_{16:0}$, acyl chains. Cholesterol was obtained from Avanti Polar Lipids or Merck (Darmstadt, Germany). DOPC, labeled with two deuterium atoms on both acyl chains at the C_{11} position ($^2H_4$-DOPC) was synthesized as described previously [24]. Deuterium-depleted water was purchased from Isotec Inc (Miamisburg, USA).

2.1. NMR sample preparation

Lipids were dissolved in $CHCl_3$/MeOH (1/1, v/v) as 10–20 mM stock solutions. The exact concentrations of the phospholipid stocks were determined by a phosphorous assay according to Rouser [25]. The appropriate amounts of indicated lipids were mixed in solution, after which the organic solvents were evaporated under a flow of nitrogen. The resulting films were further dried by overnight storage under high vacuum. Multilamellar vesicles were made by hydrating the mixed dry lipid films with deuterium-depleted water (typically 100 μl) at 60°C under repeated vortexing until the film was dispersed. Vesicles were pelleted in glass tubes (3 cm long, ∅∼0.5 cm) and freeze–thawed 20 times.

2.2. ^2H-NMR measurements

^2H-NMR spectra were recorded on a Bruker Avance 500 WB at the indicated temperatures. ^2H-NMR measurements were performed at 76.8 MHz, using a quadrupolar echo sequence [26] with a 6 μs 90° pulse and a recycling delay of 100 ms. Typically, 100 000 scans were acquired for samples containing 3 μmol $^2H_4$-DOPC. A line broadening of up to 100 Hz was used. At the end of a series of measurements at different temperatures the measurement at the initial temperature (30°C) was repeated to check the reversibility of the temperature effects on the sample.

2.3. AFM sample preparation

AFM samples were prepared as described previously [10]. Briefly, lipids were mixed in organic solution and dried in a rotary evaporator, followed by overnight storage under high vacuum. The dry mixed lipid films were hydrated with 20 mM NaCl solution, resulting in a lipid concentration of 1 mM. After freeze–thawing, the suspension was sonicated at 45°C. Possible remaining large vesicles were pelleted by centrifugation (20 800×g for 1 h at 4°C) and the supernatant containing small unilamellar vesicles (SUV) was used within 5 days. The AFM results were not dependent on the time of storage within this time period. Supported lipid bilayers were prepared by depositing 75 μl SUV suspension onto freshly cleaved mica. After 1 h at room temperature, the sample was rinsed with the NaCl solution and heated for 1 h at 65°C. After cooling down to room temperature, the sample was rinsed again.

2.4. AFM imaging

The supported bilayers were covered by the NaCl solution during the measurements. The samples were mounted on an E-scanner, which was calibrated on a standard grid, of a Nanoscope III AFM (Digital Instruments, Santa Barbara, CA, USA). A fluid cell without O-ring was fitted and the sample was scanned in contact mode, using oxide sharpened Si_3N_4 tips attached to a triangular cantilever with a spring constant of 0.06 N/m (NanoProbe, DI, Santa Barbara, CA, USA). All images were recorded at temperatures between 23 and 28°C and at a minimal force (100–200 pN) to ensure stable scans and well-resolved images.

3. Results

3.1. $^2H_4$-DOPC in binary mixtures

First the effects of SM, DPPC and cholesterol were determined on the ordering of $^2H_4$-DOPC in equimolar binary lipid mixtures. Fig. 1 shows the ^2H-NMR spectra at 37°C of $^2H_4$-DOPC in these mixtures, compared with that of pure $^2H_4$-DOPC. The quadrupolar splittings (ν_q), which are the distances between the doublet peaks in the ^2H-NMR spectra, are a direct measure for the ordering of the acyl chains of $^2H_4$-DOPC at the C_{11} position in the membrane [26]. For simplicity, this will be referred to as the ordering of $^2H_4$-DOPC. Clearly, the effect of cholesterol on the ordering of $^2H_4$-DOPC is much larger than that of either SM or DPPC. The values of $\Delta\nu_q$ of $^2H_4$-DOPC in the temperature range from 30°C to 60°C are shown in Fig. 2. In all mixtures, $\Delta\nu_q$ decreases with increasing temperature, due to the increase of thermal mobility with temperature. In the presence of SM or DPPC, $\Delta\nu_q$ is slightly higher than in pure $^2H_4$-DOPC, caused by the mixing of the saturated lipids with the unsaturated $^2H_4$-DOPC. At all measured temperatures, the mixing of $^2H_4$-DOPC with cholesterol has a much larger effect on the ordering of $^2H_4$-DOPC than mixing with either SM or DPPC.

3.2. $^2H_4$-DOPC in ternary mixtures

The behavior of $^2H_4$-DOPC was then studied in ternary lipid mixtures, in which domain formation is proposed to occur [4,5,7]. $\Delta\nu_q$ of $^2H_4$-DOPC was first determined in an equimolar mixture with cholesterol and SM. Fig. 3A shows the ^2H-NMR spectra of $^2H_4$-DOPC in this mixture at 37°C (top) and at 60°C (bottom). Strikingly, $\Delta\nu_q$ is larger at 60°C than at 37°C, contrary to the effect of the increase of thermal mobility with temperature.

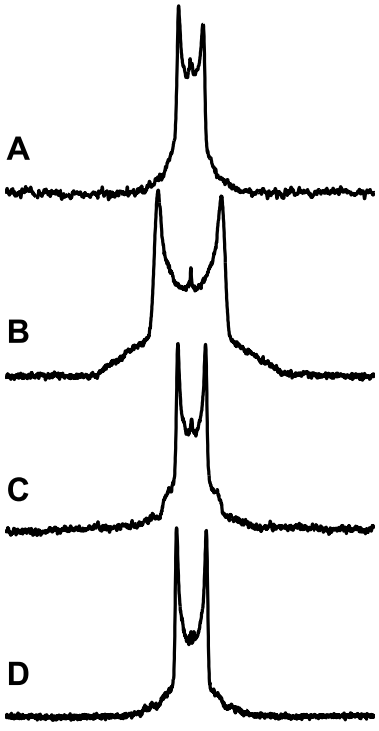

Fig. 1. 2H-NMR spectra at 37°C of pure $^2H_4$-DOPC (A) and of $^2H_4$-DOPC in an equimolar mixture with cholesterol (B), SM (C) or DPPC (D).

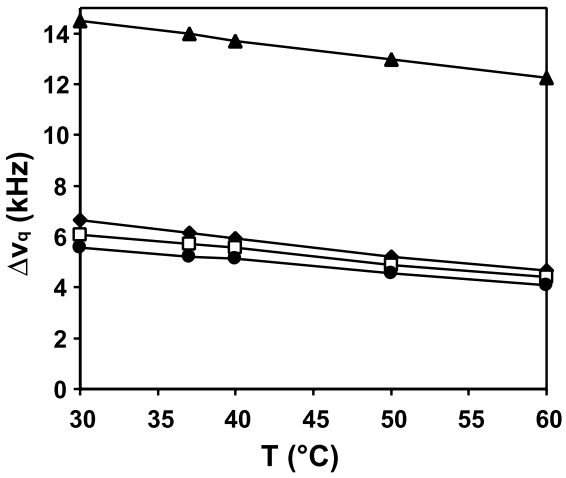

Fig. 2. Effect of the temperature on $\Delta\nu_q$ of pure $^2H_4$-DOPC (●) and $^2H_4$-DOPC in an equimolar mixture with cholesterol (▲), SM (□) or DPPC (♦). The error in $\Delta\nu_q$ is estimated to be ~0.2 kHz.

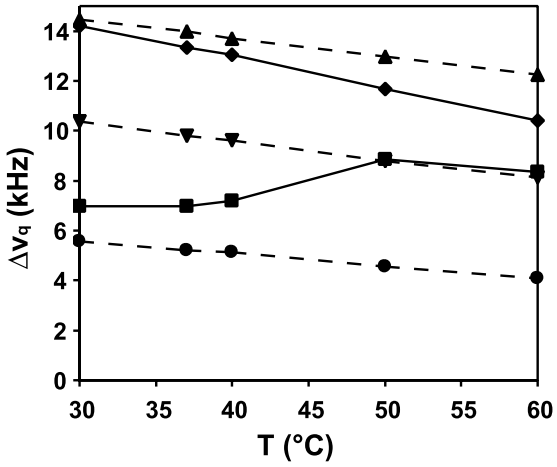

Fig. 4. Effect of the temperature on $\Delta\nu_q$ of $^2H_4$-DOPC in ternary mixtures with cholesterol and either SM (■) or DPPC (♦), compared to that in pure $^2H_4$-DOPC (●) and of $^2H_4$-DOPC in binary mixtures with cholesterol in equimolar (▲) or 2/1 ratio (▼). The error in $\Delta\nu_q$ is estimated to be ~0.2 kHz.

A different behavior is observed for $^2H_4$-DOPC in an equimolar ternary mixture with DPPC and cholesterol. $\Delta\nu_q$ in the ^{2}H-NMR spectra of $^2H_4$-DOPC in this mixture (Fig. 3B) is much higher than in the SM-containing mixture (Fig. 3A), both at 37°C (top) and at 60°C (bottom). Moreover, $\Delta\nu_q$ is larger at 37°C than at 60°C, contrary to the temperature-dependent behavior in the SM-containing mixture.

The temperature-dependent behavior of $^2H_4$-DOPC in the ternary mixtures is shown in more detail in Fig. 4, which displays the values of $\Delta\nu_q$ in the temperature range from 30°C to 60°C. In all lipid mixtures, $\Delta\nu_q$ decreases with increasing temperature, except in the equimolar ternary mixture with SM and cholesterol. The most noticeable increase in $\Delta\nu_q$ of $^2H_4$-DOPC in this mixture occurs between 40°C and 50°C. The transition temperature (T_m) of the SM used was 40°C, as determined by DSC (data not shown). Therefore, the increase in $\Delta\nu_q$ between 40°C and 50°C suggests a phase transition in the mixture, related to the T_m of SM. Below 40°C, $\Delta\nu_q$ is only a little above the level of pure $^2H_4$-DOPC. This would be consistent with $^2H_4$-DOPC being present in a L_d phase and containing only small amounts of cholesterol, as the ordering of $^2H_4$-DOPC will be most sensitive to the presence of cholesterol (Fig. 2). At 50°C and higher temperatures, $\Delta\nu_q$ in the ternary mixture is comparable to that of $^2H_4$-DOPC in a mixture with cholesterol at a 2/1 ratio. The latter contains the same fraction of cholesterol as the ternary mixture, which suggests that the ternary mixture is homogeneously mixed above 50°C.

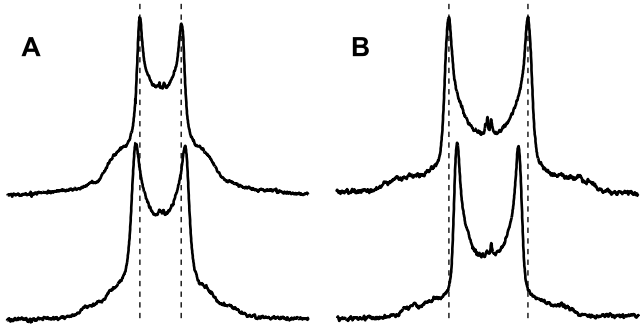

Fig. 3. ^{2}H-NMR spectra of $^2H_4$-DOPC in ternary mixtures with cholesterol and either SM (A) or DPPC (B) at physiological (37°C, top) or high temperatures (60°C, bottom).

In contrast, $\Delta\nu_q$ of $^2H_4$-DOPC in the ternary mixture with DPPC gradually decreases with increasing temperature and does not display behavior reminiscent of a phase transition, although T_m of DPPC (41°C) is comparable to that of SM (40°C). At all temperatures, $\Delta\nu_q$ of $^2H_4$-DOPC in the ternary mixture with DPPC clearly remains larger than that of $^2H_4$-DOPC in a mixture with cholesterol at a 2/1 ratio. This would suggest that the presence of both cholesterol and DPPC in the ternary mixture has a 'cooperative' effect on the ordering of $^2H_4$-DOPC.

In short, DPPC- and SM-containing ternary mixtures have very different properties, as illustrated both by the differences in the ordering of the $^2H_4$-DOPC acyl chains and by the differences in temperature-dependent behavior.

3.3. Visualization by AFM of domains in ternary mixtures

To gain a better understanding of the differences in behavior of the ternary mixtures with either SM or DPPC, the domains in these mixtures were visualized using AFM. A ternary mixture of DOPC, SM and cholesterol displays elevated domains in a surrounding bilayer (Fig. 5A), in agreement with results from a previous study [10]. The elevated phase covers 45–47% of the total area. The height difference between the two phases is 0.7 ± 0.1 nm. The height difference between the lower phase and the supporting mica is ~4.7 nm, which was measured at a defect in the bilayer. Consequently, the height difference between the elevated phase and the mica would amount to 5–6 nm. This corresponds to the thickness of a detergent-resistant, SM- and cholesterol-enriched bilayer combined with a layer of water between the mica and this bilayer [10]. Therefore, the elevated domains are assigned to L_o phase domains, enriched in SM and cholesterol. The surrounding bilayer would then be a DOPC-enriched L_d phase.

Domains are also visible in the corresponding DPPC-containing ternary mixture (Fig. 5B). However, these domains are much smaller, more branched and more irregular in shape as

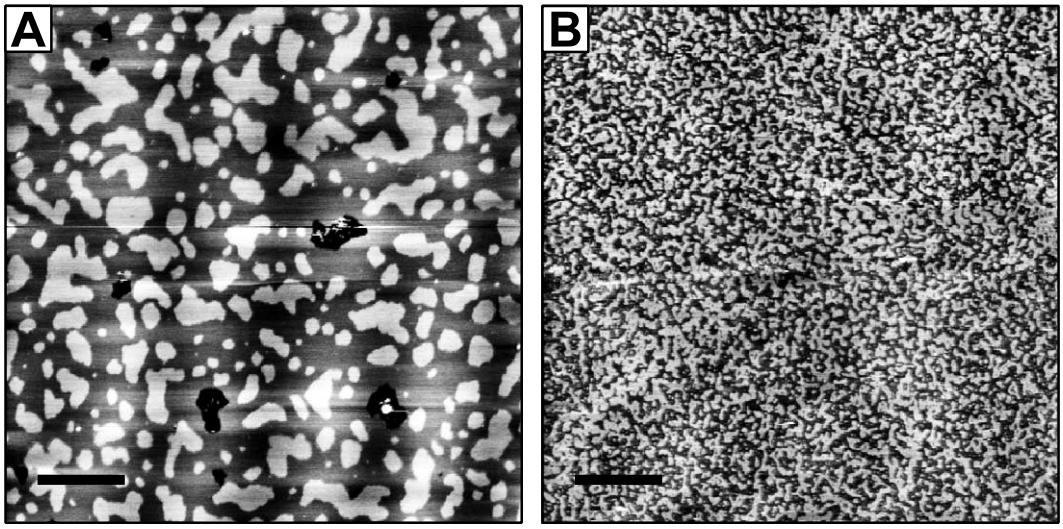

Fig. 5. AFM images (6×6 μm, scale bar 1 μm, *z*-scale of 5 nm) showing domains in bilayers of SM/DOPC 1/1 with 30% cholesterol and of DPPC/DOPC 1/1 with 30% cholesterol. Lighter areas are higher than darker areas.

compared to the domains in the SM-containing mixture. The DPPC-containing mixture appears to be on the verge of percolation, as the connectivity of the domains is difficult to trace. Due to the shape and small size of the domains, the area that they cover is difficult to determine accurately, but is estimated to be 45–50% of the total area. The height difference between the two phases is 0.9 ± 0.2 nm, which is slightly larger than that in the SM-containing mixture. Based on the height difference between the phases, the elevated phase is assumed to be a DPPC- and cholesterol-enriched phase and the lower phase to be a DOPC-enriched phase. This was confirmed by varying the relative amounts of DOPC and DPPC in the mixture and monitoring the resulting changes in the area distribution of the phases (data not shown).

4. Discussion

This study clearly shows that the behavior of ternary mixtures of cholesterol, DOPC and SM is different from that of corresponding mixtures containing DPPC instead of SM. Not only do the domains in these mixtures differ in shape and size, but also the extent of ordering of $^2H_4$-DOPC and the temperature dependence of this ordering are quite different. First, the behavior of the SM-containing mixture will be discussed, and compared with that of the DPPC-containing mixture. Next, the implications for the lipids enriched in the L_d phase will be discussed, both in model and natural membranes.

4.1. SM-containing ternary mixture

SM- and cholesterol-enriched domains are visualized by AFM in a ternary mixture of DOPC, SM and cholesterol (Fig. 5A). The bilayer thicknesses of these domains and of the DOPC-enriched bilayer surrounding these domains are in agreement with X-ray data [27] and with results from a previous AFM study [10].

The DOPC-enriched bilayer in the ternary mixture is thicker than a pure DOPC bilayer, suggesting the presence of some cholesterol in the DOPC-enriched bilayer [10], which is in agreement with the ^{2}H-NMR results. At temperatures below 40°C, the $^2H_4$-DOPC-enriched phase in an equimolar ternary mixture with SM and cholesterol is estimated to contain 10–15% cholesterol. This is based on comparison of $\Delta\nu_q$ of $^2H_4$-DOPC in the ternary mixture with those of pure $^2H_4$-DOPC and $^2H_4$-DOPC in binary mixtures with cholesterol in equimolar or 2/1 ratios (Fig. 4), and assuming that the effect of SM in the L_d phase is negligible compared to that of cholesterol (Fig. 2).

This estimation would imply that most of the cholesterol is not in the vicinity of the bulk $^2H_4$-DOPC. Instead, the bulk cholesterol most likely forms L_o phase domains together with SM, consistent with the preferential interaction of cholesterol with sphingolipids over glycerophospholipids [15–17,20,28]. Due to the strong interaction between SM and cholesterol, such L_o phase domains may not be readily accessible to $^2H_4$-DOPC. It is possible that very small amounts of $^2H_4$-DOPC are present in the domains, but they are not detectable under the experimental conditions used.

Between 40°C and 50°C, the ordering of $^2H_4$-DOPC in the ternary mixture increases to the level that would be expected when this mixture is homogeneous. The onset of this transition correlates with T_m of the SM (40°C). This suggests that the transition that is apparent at these temperatures is the melting of the SM- and cholesterol-enriched domains into the $^2H_4$-DOPC-enriched L_d phase. Such melting of domains has been demonstrated using fluorescence microscopy [11,29] and was correlated to T_m of the saturated lipid species [11].

4.2. Comparison of ternary mixtures containing DPPC vs. SM

The behavior of $^2H_4$-DOPC in the equimolar ternary mixture with DPPC and cholesterol is very different from that in the SM-containing ternary mixture. The ordering of $^2H_4$-DOPC in the DPPC mixture is much higher and there is no transition visible in the measured range of temperatures (30–60°C, Fig. 4). Moreover, the AFM images show that domains in the DPPC-containing mixture are much smaller and more irregular in shape than those in the SM-containing mixture.

These observations can be explained by the fact that cholesterol shows a preferential interaction for sphingolipids over glycerophospholipids [15–17]. As the interaction between DPPC and cholesterol is not as strong as that between SM

and cholesterol, the domains in the DPPC-containing mixture might be more easily disrupted. This would result in small, irregularly shaped domains, as is seen using AFM (Fig. 5B). It would also cause these domains to be more accessible to DOPC. The high ordering of $^2H_4$-DOPC in the DPPC mixture (Fig. 4) may then be due to the presence of a relatively large amount of $^2H_4$-DOPC in the highly ordered L_o phase of DPPC and cholesterol. In principle, this would result in two signals with different values of $\Delta\nu_q$ in the ^{2}H-NMR spectra (Fig. 3B), corresponding to the $^2H_4$-DOPC in the L_o phase and to that in the L_d phase. That only one signal is detected suggests that the diffusion is sufficiently fast on the NMR timescale to lead to the averaging of the signals of both pools of $^2H_4$-DOPC. $\Delta\nu_q$ of such an average signal would be large, as especially the ordering in the L_o phase of DPPC and cholesterol can be very high. This high ordering could be directly demonstrated by measuring $\Delta\nu_q$ of only 5 mol% $^2H_4$-DOPC in an equimolar mixture of DPPC and cholesterol (data not shown). At all temperatures, $\Delta\nu_q$ in this mixture was $\sim$14 kHz higher than in pure $^2H_4$-DOPC. Therefore, it is likely that the large $\Delta\nu_q$ that is observed in the equimolar ternary mixture is indeed caused by the averaging of the signals of $^2H_4$-DOPC present in both phases[1]. If the direct detection of each separate phase is required, techniques with a shorter timescale should be used, such as fluorescence quenching, which has been shown to detect domains in DPPC-containing ternary mixtures [4,23,30]. The presence of $^2H_4$-DOPC in the L_o phase domains would not only explain the high ordering of $^2H_4$-DOPC in the DPPC mixture, but also why no transition is detected in this mixture. In the SM-containing mixture, the $^2H_4$-DOPC does not appear to sense the environment in the L_o phase domains, but instead directly reflects the situation in the L_d phase. Therefore, the compositional changes in this phase upon melting of the SM- and cholesterol-enriched domains are easily detected through the increased ordering of $^2H_4$-DOPC. It is likely that the DPPC-containing mixture undergoes a similar transition [31]. However, $^2H_4$-DOPC in this mixture already senses the environments in both phases in the presence of domains below T_m. Therefore, the melting of these domains might not have such a large effect on the average signal of $^2H_4$-DOPC.

4.3. Concluding remarks

In this study, it is shown that the behavior of an equimolar ternary mixture of SM, DOPC and cholesterol is not comparable to that of the corresponding mixture containing DPPC instead of SM. It is known that cholesterol interacts more strongly with SM than with DPPC [15–17]. Our results show that this difference has dramatic consequences for the lipids enriched in the L_d phase, like DOPC. In SM-containing mixtures, the behavior of DOPC directly reflects the behavior of the L_d phase, whereas in DPPC-containing mixtures, the DOPC senses the L_o phase as well.

Since natural rafts are enriched in sphingolipids [1,2], the behavior of sphingolipid-containing ternary mixtures will be more relevant to the behavior of natural raft-containing membranes. Natural rafts may be smaller and more dynamic than domains in model membranes. However, this study shows that the strong interaction between cholesterol and sphingolipids leads to a well-defined phase separation and that the components in the L_d phase would hardly be influenced by those in the L_o phase. This seems plausible to occur in natural membranes as well, as rafts are proposed to function by concentrating certain proteins and/or keep certain proteins apart in the plasma membrane [34]. By raft formation, the plasma membrane can offer two distinctly different environments for proteins; the highly ordered environment of rafts and that of the surrounding bilayer, which may be more fluid than is thought typical for the plasma membrane.

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[1] Based on the difference in $\Delta\nu_q$ between pure $^2H_4$-DOPC and 5 mol% $^2H_4$-DOPC in an equimolar mixture of DPPC and cholesterol, the timescale on which diffusion between the phases in the equimolar ternary mixture begins to lead to the averaging of the signal can be estimated to be about 140 μs. The average diffusion constant is estimated to be 3×10^{-8} cm^2/s [32,33]. Assuming a random walk diffusion caused by Brownian motion, this amounts to a root mean square distance of approximately 40 nm. On close inspection of the AFM image of the DPPC-containing ternary mixture, we estimate that $>$95% of the lipids would cross a phase boundary when traveling this distance.

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