Effects of Cholesterol and Saturated Sphingolipids on Acyl Chain Order in 1-Palmitoyl-2-oleoyl- sn -glycero-3-phosphocholine Bilayers—A Comparative Study with Phase-Selective Fluor...

ARTICLE *in* LANGMUIR · MARCH 2015

Impact Factor: 4.46 · DOI: 10.1021/acs.langmuir.5b00403 · Source: PubMed

4 AUTHORS, INCLUDING:

Oskar Engberg

Åbo Akademi University

5 PUBLICATIONS **4** CITATIONS

SEE PROFILE

Thomas K M Nyholm

Åbo Akademi University

54 PUBLICATIONS **1,073** CITATIONS

SEE PROFILE

Langmuir

Article

pubs.acs.org/Langmuir

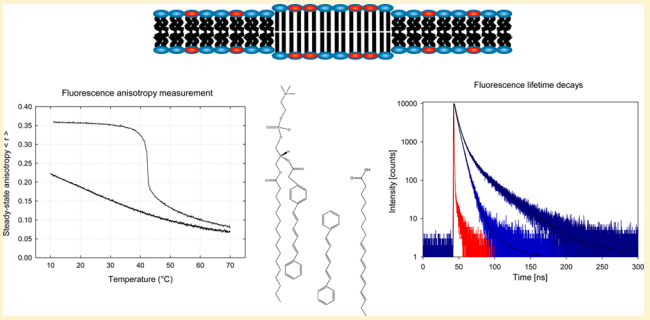
Effects of Cholesterol and Saturated Sphingolipids on Acyl Chain Order in 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine Bilayers—A Comparative Study with Phase-Selective Fluorophores

Oskar Engberg, Henrik Nurmi, Thomas K.M. Nyholm, and J.Peter Slotte*

Biochemistry − Department of Biosciences, Åbo Akademi University, Turku, Finland

Supporting Information

ABSTRACT: Saturated sphingolipids have high acyl chain order. Our aim was to study how palmitoylated sphingomyelin (PSM), ceramide (PCer), glucosyl (GlcPCer)-, and galactosylceramide (GalPCer) were able to order the bulk acyl chains of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), in comparison with cholesterol. For this reason, we used lipid probes which had preferred phases that were either the disordered phase (1-oleoyl-2-propionyl[DPH-*sn*-glycero-3-phosphcholine (18:1-DPH-PC) or the ordered phase (*trans* parinaric acid (tPA). DPH was also used, although it has no clear phase preference. We measured steady-state anisotropy (all probes) and performed fluorescence lifetime analysis (tPA) as a function of composition and temperature. At concentrations where the saturated sphingolipids were not aggregated into ordered domains (and 23 °C), they did not increase POPC acyl chain order as determined from 18:1-DPH-PC anisotropy. As expected, cholesterol increased the POPC acyl chain order linearly as a function of concentration (0−28 mol %). Since PCer already forms ordered domains below 5 mol % (at 23 °C), we measured the acyl chain ordering effect of PCer at 50 °C (0−13 mol %) and observed that PCer ordered POPC acyl chains as efficiently as cholesterol. We conclude that the bulk acyl chain order of POPC was not markedly affected in bilayers where disordered and ordered domains coexist.



1. INTRODUCTION

The lateral structure of complex model phospholipid bilayer membranes is always heterogeneous.[1,2] Even a single component phospholipid bilayer can show lateral heterogeneity with regard to packing densities, if the observations are made close to a phase transition.[3] Variations in packing densities may also imply that acyl chain order is influenced. In complex bilayer systems, heterogeneity can result from many different phenomena, such as component immiscibility[4,5] and preferential/selective interaction between molecules.[6−8]

Addition of increasing amounts of cholesterol to a one-component saturated phospholipid mono- or bilayer membrane eventually results in the formation of cholesterol-poor and cholesterol-enriched domains.[9] In monolayers, such domains can easily be visualized using fluorescent reporter molecules whose lateral distribution into cholesterol-enriched domains is hindered.[10,11] In bilayer systems containing saturated phosphatidylcholines (PC), the cholesterol-induced formation of a liquid-ordered phase can be determined with e.g., NMR,[12] calorimetry,[13] and FCS.[14] The association of cholesterol with phospholipids having disordered acyl-chains results in a well-documented acyl-chain ordering by cholesterol.[15,16] The acyl-chain ordering induced by cholesterol also results in an increased lateral packing density (condensing effect[17,18]) as well as increased bilayer thickness[19] (resulting from more extended acyl chains). Acyl chain ordering in bilayer membranes by cholesterol (or another bilayer component) has been directly determined using ^{2}D-NMR (with site-specifically deuterated,[20] or perdeuterated phospholipid acyl chains[21]), and indirectly from the steady-state anisotropy of fluorescent reporter molecules such as 1,6-diphenyl-1,-3,5-hexatriene (DPH).[22]

Ceramide is another membrane-competent lipid whose polar function (C1-hydroxyl) is similar to that of cholesterol's (3-beta-hydroxyl). *N*-Palmitoyl ceramide (PCer) is a well-studied ceramide species,[23] and like cholesterol, it cannot by itself form bilayers, but needs a colipid with a water-shielding headgroup to interact with.[24] One could therefore assume that PCer would be able to order the acyl chains of disordered phospholipids in a way similar to that of cholesterol. In a complex bilayer with unsaturated PCs and saturated sphingomyelin (SM), PCer (again like cholesterol) appears to prefer interacting with the saturated phospholipids over the unsaturated phospholipid species, and together with SM readily forms highly ordered domains.[7,8,25−29] In 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) bilayers, PCer has very low monomer solubility[8] and readily clusters to ceramide-rich ordered domains. While POPC included in the PCer-enriched cluster must be highly ordered, it remains largely unknown to what extent bulk

Received: January 31, 2015
Revised: March 9, 2015
Published: March 25, 2015

DOI: 10.1021/acs.langmuir.5b00403
Langmuir 2015, 31, 4255−4263

POPC is ordered by the inclusion of PCer. In a ^{2}H NMR study with perdeuterated POPC and PCer conducted at 57 °C (no ordered phase present), PCer (at 10 and 15 mol %) was shown to increase acyl chain order (S_{CD}) of POPC, suggesting a cholesterol-like ordering effect.[30] However, a comparison of the extent of POPC acyl chain ordering by cholesterol and PCer was not performed in the study.

In this study, we have used fluorescent probes with varying degrees of phase selectivity to determine acyl chain order in POPC bilayers to which increasing amounts of either cholesterol or saturated sphingolipids were added. POPC was selected to produce the fluid matrix bilayer, since it is a prevalent monounsaturated phosphocholine species in cell membranes. The probes were as follows: DPH, which is not strictly phase-selective; 1-oleoyl-2-propionyl[DPH]-*sn*-glycero-3-phosphocholine, which is selective for the disordered phase; and *trans*-parinaric acid which is highly selective for ordered phases (because of the all trans configuration of the tetraene double bond system). (See Scheme S1 in Supporting Information, SI, for molecular structures.) We show that the saturated sphingolipids used are weakly ordering the bulk acyl chains of POPC bilayers, even though they sphingolipids form highly ordered domains.

2. MATERIALS AND METHODS

2.1. Materials. POPC, PCer, *N*-palmitoyl-galactosyl ceramide (GalPCer), *N*-palmitoyl-glucosyl ceramide (GlcPCer), and egg SM of the highest available purity were purchased from Avanti Polar Lipids (Alabaster, AL, U.S.A.). *N*-Palmitoyl SM (PSM) was isolated from egg SM with reverse-phase HPLC as previously described.[31] All acyl chain-defined sphingolipids contained (2*S*,3*R*)-2-aminooctadec-4-ene-1,3-diol as the long-chain base. Cholesterol was purchased from Sigma Chemicals (St, Louis, MO, U.S.A.). The lipids were dissolved in either methanol (phospho- and sphingolipids) or hexane/isopropanol (3:2 by vol; cholesterol) and stored at −20 °C. The concentrations of phospholipids was determined by an inorganic phosphate assay as described previously.[32] A surface barostat was used to determine cholesterol concentration as described in.[33] PCer, GalPCer and GlcPCer concentrations were determined by weight. *trans*-Parinaric acid (tPA) was synthesized in house from the methyl ester of α-linolenic acid according to the method described in ref 34. HPLC with a Discovery C18 column (Supelco) was used to purify the tPA. The identity and purity of tPA was ascertained by ESI-MS (Bruker Daltronics), analytical HPLC (coelution with authentic tPA), absorbance, and emission spectra (identical to published spectra). DPH was purchased from Molecular Probes (Leiden, Netherlands). Fluorophores were stored at −87 °C under argon in brown vials until dissolved in argon-purged methanol. Dilute stock solutions of fluorophores were stored at −20 °C and used within a week. Tris buffer (50 mM Tris pH 7.4, 140 mM NaCl) was used in all experiments. Water used in buffer was purified by reverse osmosis and passage through a Millipore UF Plus water purification system to a final resistivity of 18.2 MΩcm. The solvents used were of spectroscopic grade and all other inorganic and organic chemicals used were of highest available purity.

18:1-DPH-PC was prepared from 1-oleoyl-2-OH-*sn*-glycero-3-phosphocholine and DPH-propionic acid (SETAREH BIOTECH, Eugene, OR, U.S.A.), according to published methods.[35,36] The product was purified using preparative HPLC (Supelco Discovery C18 column eluted with methanol). The identity and purity of 18:1-DPH-PC was ascertained by ESI-MS, analytical HPLC, and absorbance and emission spectra (identical to published spectra).

2.2. Preparation of Vesicles. Multilamellar vesicles were prepared from POPC to a concentration of 50 μM (100 nmol POPC in 2 mL buffer). POPC vesicles also contained the indicated amounts of colipids (cholesterol or sphingolipids at 0−28 mol %). Lipids and fluorophores (1 mol %) in desired amounts were mixed in glass tubes, and the solvent was evaporated under a flow of nitrogen at 40 °C. Dry lipid samples were redissolved in chloroform to give homogeneous mixing of the lipids and finally dried extensively under a nitrogen flow. The fully dried lipid films were hydrated in prewarmed argon-purged Tris buffer at 65 °C for 1 h, vortexed and sonicated at 80 °C for 5 min using a FinnSonic M3 Bath Sonicator (40 kHz, ultrasonic power 80 W, FinnSonic Oy, Lahti, Finland). Samples were let to cool down slowly to room temperature in the dark before any measurements were performed. Since no extrusion or vesicles was performed (where one can have selective loss of material[37]), the final multilamellar vesicle lipid composition should reflect very accurately the amounts of added lipids for each sample preparation.

2.3. Lifetime Analysis of *trans*-Parinaric Acid. To detect the possible formation of an ordered phase, lifetime analysis of tPA was performed for all compositions indicated. tPA prefers to partition into ordered domains (because of its rod-like structure dues to the all trans double bonds). Its excited state lifetime is greatly stabilized in the gel/ordered phase, probably in part because of less diffusion-induced quenching by water and oxygen). The samples contained 1 mol % tPA and measurements were performed at constant temperature (23 °C) with a FluoTime 100 instrument (PicoQuant Gmb, Berlin, Germany) using a PLS LED laser source for excitation (298 nm). Emission was recorded above 350 nm. Ten 000 counts were collected for each measurement. FluoFit Pro software obtained from PicoQuant was used to analyze lifetimes.

2.4. Steady-State Anisotropy Measurements. Steady-state anisotropy was measured with an ISS Photon Counting spectrofluorimeter in L-format (Innovations in fluorescence, IL, Champaign, U.S.A.). The excitation polarizer was in the vertical position (0°), while the emission polarizers were switched between the vertical (0°) and horizontal (90°) positions for each measurement point. The G-factor (ratio of sensitivities of the detection system for vertically and horizontally polarized light) was determined with the excitation polarizer in the horizontal position (90°). The anisotropy was calculated as follows:[38]

$$r = \frac{I_W - GI_{VH}}{I_W + GI_{VH}}$$

The anisotropy of samples containing POPC and the indicated amounts of either cholesterol, PCer, PSM, GalPCer, or GlcPCer, and 1 mol % fluorophore (either DPH, 18:1-DPH-PC, or tPA) was recorded at constant temperature (23 °C). For some experiments, 18:1-DPH-PC anisotropy was measured at 50 °C. tPA and 18:1-DPH-PC steady state anisotropy was also measured as a temperature function for selected compositions. Experimental conditions were as indicated above, except that temperature ramping was performed at 2 °C/min between 15 and 65 °C. Excitation/emission wavelengths were 360 nm/430 nm for both DPH and 18:1 DPH-PC, and 305 nm/405 nm for tPA.

2.5. Quenching of Fluorescence As a Temperature-Function. To detect probe partitioning into ordered domains, and to demonstrate ordered domain melting, 7SLPC-induced quenching of 18:1-DPH-PC, DPH, or tPA was measured in the following membrane composition: POPC:PCer (100 nmol +40 nmol; F_0 sample). When 7SLPC was present (F-samples) 30 nmol of 7SLPC replaced 30 nmol of POPC (i.e., 70 nmol POPC + 30 nmol 7SLPC + 40 nmol sphingolipid). Since probes in the ordered phase are not quenched by 7SLPC (which prefers the disordered phase because of the bulky doxyl group[39]), the ratio of quenched to unquenched fluorescence (as a temperature function) can reveal probe partitioning into the ordered phase.[40,41] The temperature gradient was 2 °C/min.

3. RESULTS

3.1. Fluorescence Lifetime Analysis of tPA in POPC Vesicles Containing Increasing Amounts of Cholesterol or Sphingolipids. Fluorescence lifetime analysis of *trans*-parinaric acid is a well-suited method to detect increasing acyl chain order in a mixed bilayer, since the lifetime of tPA is sensitive to packing properties in bilayers. The lifetime of tPA is very short in fluid bilayers (<5 ns), intermediate in a liquid-

DOI: 10.1021/acs.langmuir.5b00403
Langmuir 2015, 31, 4255−4263

ordered phase (20−30 ns), and very long (40−50 ns) in highly ordered gel or solid phases.[42,43] In addition, tPA prefers to partition into ordered/gel-phase domains.[44] We used lifetime analysis of tPA to detect the possible formation of ordered phases in POPC when increasing amounts of cholesterol or saturated sphingolipids were added (Figure 1). In pure POPC (see Scheme 1 for structures of the lipids used in this study), the intensity-weighted average lifetime of tPA was low (about 4 ns). It increased modestly when cholesterol was added to the POPC bilayer (10 ns at 28 mol % cholesterol), whereas addition of PSM to the POPC bilayer did not significantly increase the average lifetime of tPA (in the range 0−28 mol %). However, when PCer was included in the POPC bilayer (between 5 and 28 mol %), the tPA average lifetime increase curvi-linearly with increasing PCer, as shown in Figure 1. Addition of GalPCer or GlcPCer to POPC bilayers also increased the average lifetime of tPA, but their effect was smaller compared to PCer (in the concentration regimen examined).

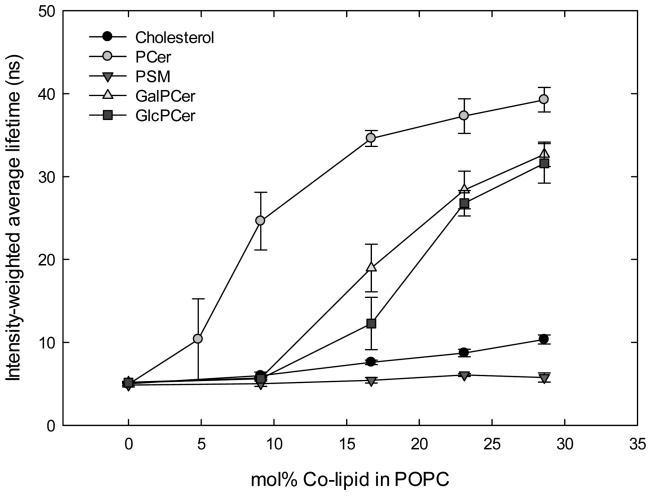
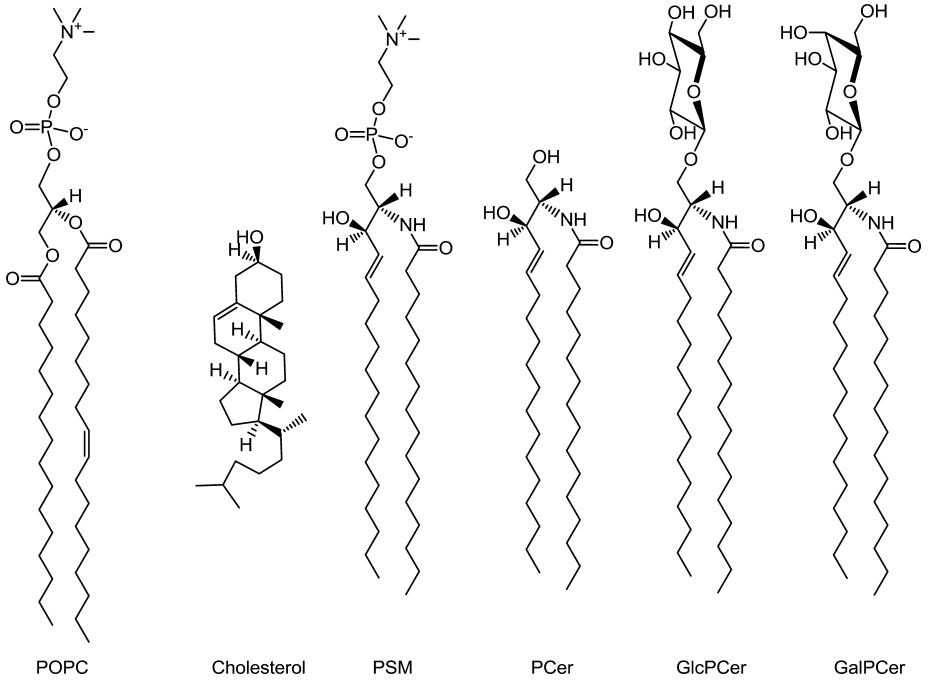


Figure 1. Lifetime analysis of tPA in POPC vesicles containing increasing amounts of cholesterol or saturated sphingolipids. The vesicles (50 μM POPC) contained 1 mol % tPA and were prepared as described in the Materials and Methods. Intensity-weighted average lifetimes are presented for each bilayer composition (average ± SD, n = 3−5).

On the basis of the tPA lifetime profile presented in Figure 1, we conclude that inclusion of PCer or the glycosphingolipids into the POPC bilayer led to a concentration-dependent formation of highly ordered domains in which these lipids (and tPA) were enriched. Similar curves for PCer in POPC bilayers have been reported.[8] Neither cholesterol nor PSM appeared to enhance the formation of similarly ordered domains in the POPC bilayer in the concentration range used. The reason for the aggregation of ceramide and glycosphingolipids into ordered domains at fairly low concentration (compared to PSM) is most likely due to the small headgroups of these sphingolipids compared to PSM.

3.2. Steady-State Anisotropy Measurements Using Three Different Reporter Fluorophores. Next, we measured acyl chain order from the steady-state anisotropy function of three different reporter fluorophores: DPH, 18:1-DPH-PC, and tPA. While DPH is known to partition equally between liquid disordered and liquid-ordered phases ($K_p^{Lo/Ld} \approx 1$;[25]), and to be excluded from some gel phases,[25] 18:1-DPH-PC prefers the disordered phase over the solid ordered one ($K_p^{So/Ld} \approx 0.31$ (S_o = PSM), SI Figure S1, see also ref 45), and tPA favors the solid ordered phase ($K_p^{gel/fluid}$ between 2 and 6.3, depending of the gel and fluid phase composition.[8,44,46] Thus, we have two probes reporting acyl chain order more or less selectively from the fluid (18:1-DPH-PC) or the ordered phase (tPA). DPH reports from the disordered phase and from some ordered phases

Scheme 1. Chemical Structures of the Lipids Used in the Study[a]



[a]1-Palmitoyl-2-Oleoyl-*sn*-glycero-3-phosphocholine (POPC), cholesterol, *N*-palmitoyl sphingomyelin (PSM), *N*-palmitoyl ceramide (PCer), *N*-palmitoyl-glucosylceramide (GlcPCer), and *N*-palmitoyl-galactosylceramide (GalPCer).

DOI: 10.1021/acs.langmuir.5b00403
Langmuir 2015, 31, 4255−4263

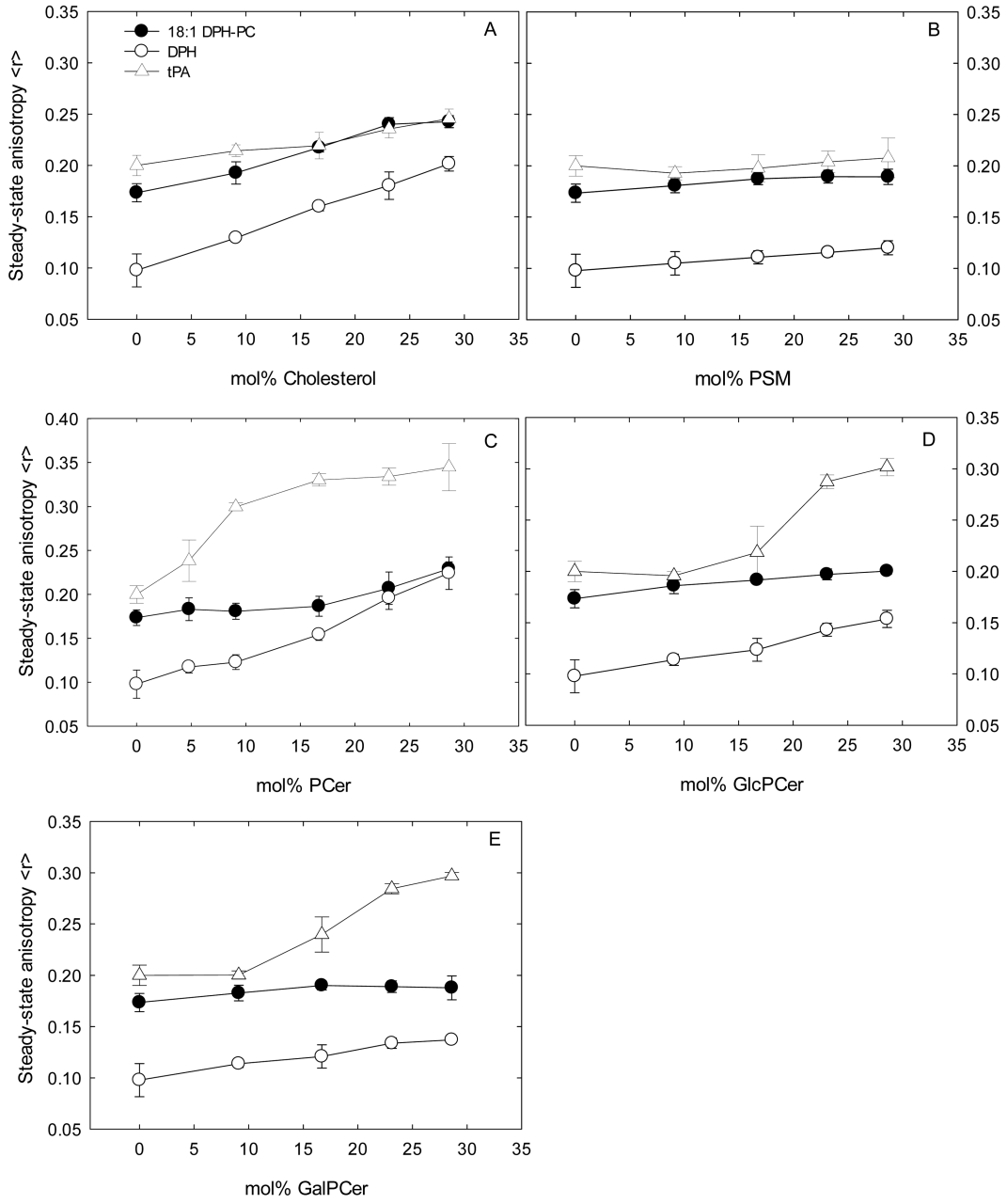


Figure 2. Steady-state anisotropy measurements with three different reporter probes. Vesicles with indicated compositions were prepared (50 μM POPC), and contained 1 mol % of either 18:1-DPH-PC, DPH, or tPA as reporter probe. Measurements were performed at 23 °C and values are averages ± SD with n = 3−5. Panel A shows data for POPC and cholesterol, panel B for POPC and PSM, panel C for POPC and PCer, panel D for POPC and GalPCer, and panel E for POPC and GlcPCer, respectively.

(e.g., DPPC[47]) but not all (reported to be excluded from PCer[25]).

The acyl-chain ordering effect of cholesterol (panel A in Figure 2) is evident with all three probes, their anisotropy increasing with an increase in cholesterol concentration (up to 28 mol %). The increase in anisotropy (from 0 to 28 mol % cholesterol) was larger for DPH compared to either 18:1-DPH-PC or tPA. This is probably due to it being less restricted compared to the PC-conjugated DPH and even tPA, and thus being more sensitive to changes induced by cholesterol.

The addition of increasing amounts of PSM (up to 28 mol %) to the POPC bilayer (panel B in Figure 2) increased the anisotropy only slightly (for all three probes), compared to the PSM-free situation, suggesting that POPC acyl chain ordering by PSM was very modest.

PCer-addition to POPC led to the formation of an ordered phase that increasingly became gel-like at higher PCer

DOI: 10.1021/acs.langmuir.5b00403
Langmuir 2015, 31, 4255−4263

concentrations (Figure 1). The acyl chain ordering in the fluid POPC phase, as reported by 18:1-DPH-PC, was almost unaffected by PCer in the concentration range 0−16 mol %) but the anisotropy (and acyl chain ordering) started to increase slightly between 16 and 28 mol % PCer (panel C in Figure 2). With free DPH, the anisotropy increased almost linearly with PCer concentration (from 0.1 (0 mol % PCer) to 0.23 (28 mol % PCer—panel C in Figure 2). The tPA anisotropy increased curvilinearly from 0.2 (0 mol % PCer) to 0.34 at 28 mol % PCer. The difference in DPH and 18:1-DPH-PC anisotropy suggest that whereas the 18:1-DPH-PC did not significantly enter the PCer phase, DPH apparently did so (explaining the high anisotropy at increased PCer concentrations).

For the two glycosphingolipids (GalPCer and GlcPCer), all three probes reported very similar anisotropy values, irrespective of the headgroup function. Increasing the glycosphingolipid concentration did not markedly increase acyl chain order in the fluid phase, as reported by 18:1-DPH-PC (panels D and E, Figure 2). With free DPH, a small increase in anisotropy was seen as the glycosphingolipid concentration increased, suggesting some partitioning of DPH also into the glycosphingolipid rich phase that was formed. With tPA, the anisotropy started to increase at or above 16 mol % of glycosphingolipid, in good agreement with the tPA lifetime data presented in Figure 1.

Another comparison of anisotropy values for the different bilayer compositions is given in Figure 3, with data for each reporter molecule shown in separate panels. In the top panel (Figure 3) it can be seen that 18:1-DPH-PC responded most clearly to increasing cholesterol concentrations, and to the highest PCer concentrations, but was more or less unresponsive to lower concentrations of PCer or the addition of PSM or the glycosphingolipids. Free DPH (mid panel Figure 3) reported increased anisotropies in all systems as a function of added colipid. This is expected of a probe that reports the average anisotropy from most phases in a bilayer system. The tPA data (lower panel, Figure 3) reveal the formation of ordered phases by PCer and the two glycosphingolipids, and the concentration response is very similar to the tPA lifetime data in Figure 1. tPA anisotropy sensed the increased presence of cholesterol, but not that of PSM (lower panel, Figure 3).

To further determine whether 18:1-DPH-PC and DPH reported ordered domain melting to some extent, both steady-state anisotropy and quenching experiments were performed as a function of temperature (domain melting). As shown in Figure 4, 18:1-DPH-PC did not clearly detect ordered domain melting (50% PSM in POPC; 28 mol % PCer, GlcPCer or GalPCer in POPC), whereas tPA did (Figure 4). Since cholesterol does not form gel-phase ordered domains in POPC, it was not included in Figure 4. When DPH, 18:1-DPH-PC, and tPA quenching was performed as a temperature function in a POPC/PCer system (28 mol % PCer), tPA revealed a clear PCer domain melting (end of domain melting at around 45 °C; Figure 5). The reason for the discontinuity in the tPA quenching curve is the gradually increased availability of tPA for quenching by 7SLPC, as the ordered domain (where tPA enjoys protection against quenching by 7SLPC) melts as the temperature increases. Free DPH also indicated the end of domain melting of the PCer ordered phase, although $\Delta F/F_0$ for the melting was very small compared to tPA. 18:1-DPH-PC did not clearly indicate an end of domain melting, although the F/F_0 line was not completely linear with temperature. Nevertheless, both DPH and 18:1-DPH-PC differed markedly from tPA in their capacity to report domain melting for PCer in POPC, which is consistent with their

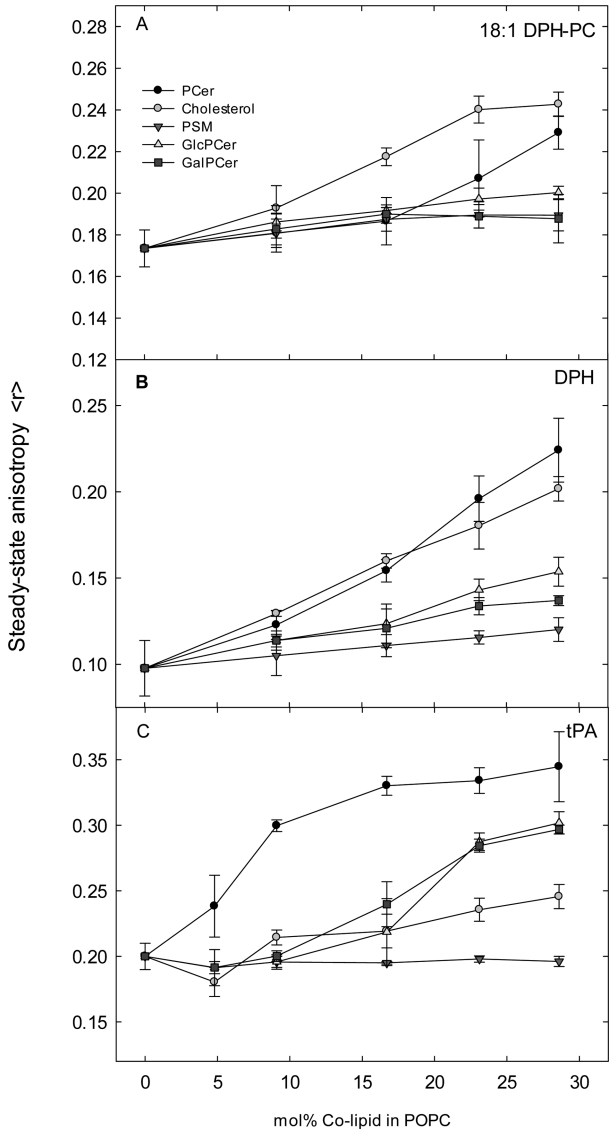


Figure 3. Steady-state anisotropy measurements with three different reporter probes. The experimental values presented in Figure 2 have been redrawn as follows: panel A shows 18:1-DPH-PC data for all compositions, panel B DPH-data for all compositions, and panel C tPA-data for all compositions. This redrawing was motivated to allow better comparison of data for each probe type.

markedly reduced preference for the ordered over the disordered phase.

The response of 18:1-DPH-PC to inclusion of order-forming lipids (PCer and the glycosphingolipids) was very modest, in good agreement with the low partitioning of 18:1-DPH-PC into ordered phases (SI Figure S1.[45] These results also suggest that the acyl chain ordering of these sphingolipids on the bulk disordered phase was modest, apparently because they segregated efficiently to an ordered phase. However, to further examine whether PCer or a glycosphingolipid could increase POPC acyl chain order (and affect the anisotropy of 18:1-DPH-PC) in a situation where an ordered phase is not present (and the monomer solubility of the sphingolipids is increased), we measured 18:1-DPH-PC anisotropy at 50 °C. At this temperature (below 14 mol % sphingolipid), an ordered phase was not present.[24] At 50 °C, both cholesterol and PCer appeared to order the acyl chains of the fluid phase in a similar fashion (Figure 6).

DOI: 10.1021/acs.langmuir.5b00403
Langmuir 2015, 31, 4255−4263

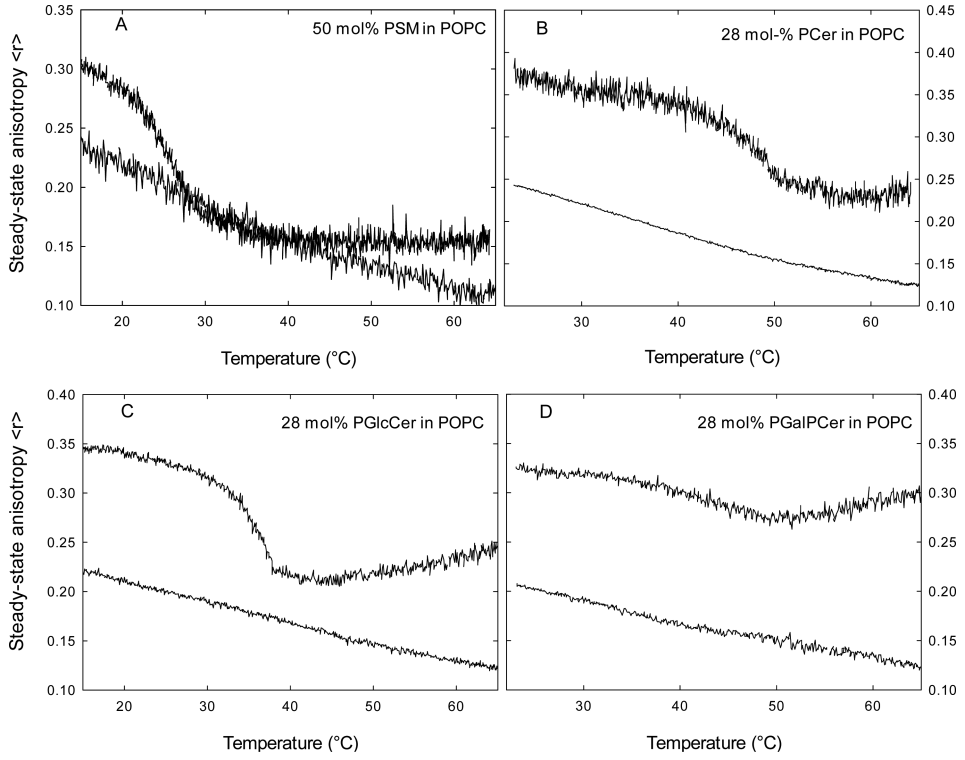


Figure 4. Steady-state anisotropy of tPA or 18:1-DPH-PC as a function of temperature. Bilayer compositions which formed an ordered phase at ambient temperature were analyzed with the two probes, inducing domain melting by temperature ramping (2 °C/min). Panel A shows data for 50 mol % PSM in POPC (50 + 50 nmol), panel B shows the composition 28 mol % PCer in POPC (40 + 100 nmol), panel C 28 mol % GlcPCer in POPC (40 + 100 nmol), and panel D 28 mol % GalPCer in POPC (40 + 100 nmol). The upper curve in each panel is for tPA, the lower curve for 18:1-DPH-PC. The probe was present at 1 mol %. The curves are representative scans from at least three separate experiments.

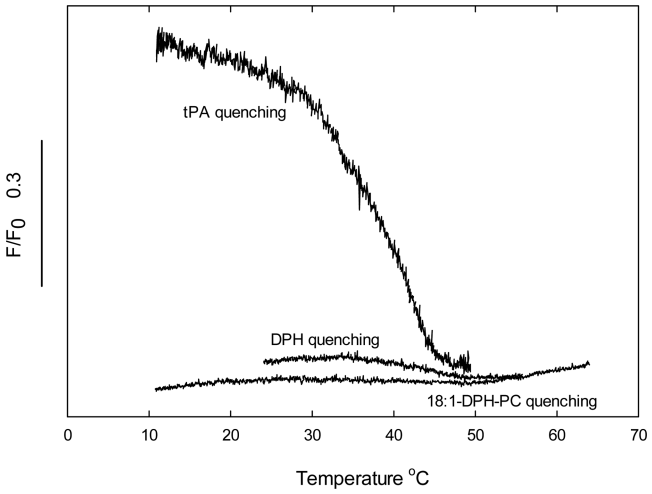


Figure 5. Quenching of tPA or 18:1-DPH-PC fluorescence during temperature ramping. The bilayer composition was 28 mol % PCer in POPC. To study how different probes reported the melting, 7SLPC was used to quench emission as a function of temperature (ramping at 2 °C/min). The F_0 composition was 100 nmol POPC, 40 nmol PCer and 1 nmol probe. The F-composition was 70 nmol POPC, 30 nmol 7SLPC, 40 nmol PCer, and 1 nmol probe. Each curve is a representative scan from three separate experiments. The *y*-axis bar (0.3 F/F_0 unit) indicates the scale proportion, which is identical for all three compositions.

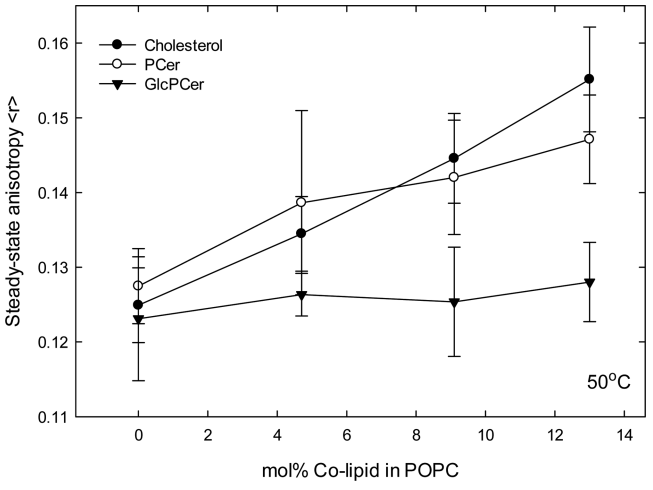


Figure 6. Steady-state anisotropy measurements of 18:1-DPH-PC. Vesicles with indicated compositions were prepared (50 μM phospholipid) and contained 1 mol % 18:1-DPH-PC. Measurements were performed at 50 °C, and values are averages ± SD with n = 3–5.

However, GlcPCer failed to increase acyl chain order as reported by 18:1-DPH-PC, suggesting that the sugar headgroup prevented close interaction necessary for acyl chain ordering.

4. DISCUSSION

The main aim of this study was to examine to what extent saturated sphingolipids are able to order acyl chains in the bulk POPC phase, and compare the possible ordering to that induced by cholesterol. This possible ordering effect is, of course, most likely to happen when the saturated sphingolipids exist nonaggregated in the POPC phase. To study this, we first characterized a fluorescent probe (18:1-DPH-PC) that hopefully

DOI: 10.1021/acs.langmuir.5b00403
Langmuir 2015, 31, 4255–4263

would report exclusively from the disordered phase. Although DPH has frequently been used to assess acyl chain ordering in bilayer membranes (based on anisotropy measurements), it does not have an exclusive preference for the disordered phase. The $K^{Lo/Ld}$ for DPH has been reported to be close to unity,[25] and while it may be excluded from dense ceramide-rich phases,[25] it is soluble in ordered domains formed by saturated phospholipids (e.g., PSM and DPPC) in unsaturated phospholipid bilayers (e.g., POPC).[48] 18:1-DPH-PC has a $K_p^{So/Ld} \approx 0.31$ (SI Figure S1), indicating high preference for the disordered phase. In our experiments, 18:1-DPH-PC did not produce the ordered phase melting (saturated sphingolipids in POPC, Figures 4 and 5), but instead produced cholesterol-induced acyl chain ordering in POPC (Figure 2A). The presence of a sphingolipid-induced ordered phase was determined from tPA fluorescence lifetime analysis (Figure 1), tPA anisotropy (Figure 4), and tPA quenching (Figure 5). tPA is known to prefer ordered phases over disordered ones.[8,44,46]

The inclusion of saturated sphingolipids in the POPC bilayer led to formation of an ordered phase for PCer and the saturated glycosphingolipids, but not for PSM (Figure 1). PCer is known to form a ceramide-rich ordered phase already at very low concentrations[8] at 24 °C. The saturated glycosphingolipids formed an ordered phase at concentrations above 10 mol % (at 23 °C; Figure 1). PSM did not form an ordered phase in POPC at the highest concentration tested (28 mol %, Figure 1), but is known to form a PSM-rich ordered phase in POPC above 30 mol %.[49]

PSM in the POPC bilayer (up to 28 mol % at 23 °C; Figure 2B) failed to significantly increase acyl chain order, as determined by tPA, DPH or 18:1-DPH-PC. In the same concentration regimen, cholesterol markedly increased acyl chain order, as reported by all three probes (Figure 2A). The large headgroup of PSM prevents close interaction between PSM and POPC, and is one likely contributor to the lack of acyl chain ordering by PSM. Cholesterol, however, can interact more closely with POPC and thus can also order up the acyl chains.[21] The lack of acyl chain ordering by PSM may also suggest that the disordered acyl chains of POPC caused a disordering of PSM acyl chains at these low PSM concentrations, and therefore the overall acyl chain order did not markedly increase.

The addition of PCer to POPC (0−17 mol %) failed to order the (bulk) POPC acyl chains, as reported by 18:1-DPH-PC (Figure 2C). However, POPC sequestered by PCer in the PCer-rich ordered phase must have ordered acyl chains, although tPA will not report separately POPC and PCer acyl chain order in the PCer-rich phase. Free DPH reported increased anisotropy with increasing PCer concentration in the POPC bilayer, suggesting that some of the DPH actually entered the PCer-rich phase. Indeed, quenching of DPH by 7SLPC indicated that a small portion of the DPH was protected against quenching as long as the PCer ordered phase remained (Figure 5). PCer has been shown to order the acyl chains of POPC during conditions where no ordered phase is present (10 and 15 mol % PCer in POPC at 57 °C.[30] Using 18:1-DPH-PC as a probe, we also detected PCer-induced ordering of POPC acyl chains when no PCer ordered phase was present (0−13 mol % PCer in POPC at 50 °C; Figure 6). The ordering induced by PCer was similar to that induced by cholesterol under similar conditions.

The ordered phase formation induced by glycosphingolipids required significantly higher concentrations of the respective lipids (above 10 mol %; Figure 2D,E), as compared with the PCer (Figure 2C). Whereas free DPH reported a modestly increased anisotropy with increasing presence of glycosphingolipids (Figure 2D,E), 18:1-DPH-PC reported much smaller changes in anisotropy. This suggests that (i) free DPH entered the glycosphingolipid phase to some extent, and (ii) the disordered phase was not significantly ordered by the glycosphingolipids. This latter conclusion is supported by the lack of acyl chain ordering by GlcPCer (0−13 mol %) at 50 °C, where there is no ordered phase present (Figure 6). The carbohydrate headgroup of the glycosphingolipids most likely imposes the different behavior of the glycosphingolipids compared to PCer. Even though the carbohydrate headgroups are much smaller than the phospholipid headgroups, they reduce T_m of the glycosphingolipids compared to matched ceramides,[50] suggesting that the carbohydrate headgroups prevent such a close interaction, which is possible among saturated ceramides. The glycosphingolipid headgroup is also likely to hinder close interaction with POPC having a large headgroup.

To conclude, we have used 18:1-DPH-PC anisotropy measurements to determine how saturated sphingolipids and cholesterol can affect order of acyl chains in the bulk POPC disordered phase. The cholesterol-induced acyl chain ordering was clearly reported by the 18:1-DPH-PC probe. The saturated sphingolipids failed to markedly increase acyl chain order in the disordered phase, even when they formed highly ordered phases at increasing concentration (at 23 °C) in the POPC bilayer.

■ ASSOCIATED CONTENT

Supporting Information

Method description for determining probe partitioning between gel and fluid phase. One figure showing K_p^{So} for DPH, 16:0-DPH-PC, and 18:1-DPH-PC. One scheme showing structures of fluorophores used. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*jpslotte@abo.fi.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Dr. Pentti Somerharju for help with 18:1-DPH-PC synthesis, Dr. Terhi Maula for valuable comments on the manuscript, and Victor Hautala for help with some measurements. This study was funded by generous grants from the Sigrid Juselius Foundation and the Åbo Akademi Foundation.

■ ABBREVIATIONS:

18:1-DPH-PC-1-oleoyl-2-propionyl[DPH]-*sn*-glycero-3-phosphocholine; DPH-diphenylhexatriene; 7SLPC-1-palmitoyl-2-stearoyl-(7-doxyl)-*sn*-glycero-3-phosphocholine; PC − phosphatidylcholine; PCer-*N*-palmitoyl ceramide; GalPCer-*N*-palmitoyl galactosylceramide; GlcPCer-*N*-palmitoyl glucosylceramide; POPC−1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; PSM-*N*-palmitoyl sphingomyelin; tPA-*trans* parinaric acid

■ REFERENCES

(1) Mouritsen, O. G.; Jorgensen, K. Dynamic lipid-bilayer heterogeneity: a mesoscopic vehicle for membrane function? *Bioessays* **1992**, *14*, 129−136.

(2) Mouritsen, O. G.; Jorgensen, K. Dynamical order and disorder in lipid bilayers. *Chem. Phys. Lipids* **1994**, *73*, 3−25.

DOI: 10.1021/acs.langmuir.5b00403
Langmuir 2015, 31, 4255−4263

(3) Dewa, T.; Vigmond, S. J.; Regen, S. L. Lateral heterogeneity in fluid bilayers composed of saturated and unsaturated phospholipids. *J. Am. Chem. Soc.* **1996**, *118*, 3435−3440.
(4) Kucerka, N.; Marquardt, D.; Harroun, T. A.; Nieh, M. P.; Wassall, S. R.; de Jong, D. H.; Schafer, L. V.; Marrink, S. J.; Katsaras, J. Cholesterol in bilayers with PUFA chains: doping with DMPC or POPC results in sterol reorientation and membrane-domain formation. *Biochemistry* **2010**, *49*, 7485−7493.
(5) Shaikh, S. R.; Locascio, D. S.; Soni, S. P.; Wassall, S. R.; Stillwell, W. Oleic- and docosahexaenoic acid-containing phosphatidylethanolamines differentially phase separate from sphingomyelin. *Biochim. Biophys. Acta* **2009**, *1788*, 2421−2426.
(6) Lonnfors, M.; Doux, J. P.; Killian, J. A.; Nyholm, T. K.; Slotte, J. P. Sterols Have Higher Affinity for Sphingomyelin than for Phosphatidylcholine Bilayers even at Equal Acyl-Chain Order. *Biophys. J.* **2011**, *100*, 2633−2641.
(7) Castro, B. M.; de Almeida, R. F.; Silva, L. C.; Fedorov, A.; Prieto, M. Formation of ceramide/sphingomyelin gel domains in the presence of an unsaturated phospholipid. A quantitative multiprobe approach. *Biophys. J.* **2007**, *93*, 1639−1650.
(8) Silva, L.; de Almeida, R. F.; Fedorov, A.; Matos, A. P.; Prieto, M. Ceramide-platform formation and induced biophysical changes in a fluid phospholipid membrane. *Mol. Membr. Biol.* **2006**, *23*, 137−148.
(9) Darke, A.; Flook, A. G.; Finer, E. G.; Phillips, M. C. Nuclear Magnetic-Resonance Study of Lecithin−Cholesterol Interactions. *J. Mol. Biol.* **1972**, *63*, 265−279.
(10) Slotte, J. P. Lateral domain heterogeneity in cholesterol/phosphatidylcholine monolayers as a function of cholesterol concentration and phosphatidylcholine acyl chain length. *Biochim. Biophys. Acta* **1995**, *1238*, 118−26.
(11) Miller, A.; Knoll, W.; Möhwald, H. Fractal growth of crystalline phospholipid domains in monomolecular layers. *Phys. Rev. Lett.* **1986**, *56*, 2633−2636.
(12) Bartels, T.; Lankalapalli, R. S.; Bittman, R.; Beyer, K.; Brown, M. F. Raftlike mixtures of sphingomyelin and cholesterol investigated by solid-state 2H NMR spectroscopy. *J. Am. Chem. Soc.* **2008**, *130*, 14521−14532.
(13) Vist, M. R.; Davis, J. H. Phase Equilibria of Cholesterol/Dipalmitoylphosphatidylcholine Mixtures: 2H Nuclear Magnetic Resonance and Differential Scanning Calorimetry. *Biochemistry* **1990**, *29*, 451−464.
(14) Honigmann, A.; Mueller, V.; Hell, S. W.; Eggeling, C. STED microscopy detects and quantifies liquid phase separation in lipid membranes using a new far-red emitting fluorescent phosphoglycerolipid analogue. *Faraday Discuss.* **2013**, *161*, 77−89.
(15) Yeagle, P. L.; Albert, A. D.; Boesze-Battaglia, K.; Young, J.; Frye, J. Cholesterol dynamics in membranes. *Biophys. J.* **1990**, *57*, 413−24.
(16) Rog, T.; Pasenkiewicz-Gierula, M.; Vattulainen, I.; Karttunen, M. Ordering effects of cholesterol and its analogues. *Biochim. Biophys. Acta* **2009**, *1788*, 97−121.
(17) Chapman, D.; Owens, N. F.; Phillips, M. C.; Walker, D. A. Mixed monolayers of phospholipids and cholesterol. *Biochim. Biophys. Acta* **1969**, *183*, 458−465.
(18) Demel, R. A.; Deenen, L.L.M.v.; Pethica, B. A. Monolayer interactions of phospholipids and cholesterol. *Biochim. Biophys. Acta* **1967**, *135*, 11−19.
(19) Ipsen, J. H.; Mouritsen, O. G.; Bloom, M. Relationships between lipid membrane area, hydrophobic thickness, and acyl-chain orientational order. The effects of cholesterol. *Biophys. J.* **1990**, *57*, 405−12.
(20) MAtsumori, N.; Yasuda, T.; Okazaki, H.; Suzuki, T.; Yamaguchi, T.; Tsuchikawa, H.; Doi, M.; Oishi, T.; Murata, M. Comprehensive molecular motion capture for sphingomyelin by site-specific deuterium labeling. *Biochemistry* **2012**, *51*, 8363−8370.
(21) Sankaram, M. B.; Thompson, T. E. Modulation of phospholipid acyl chain order by cholesterol A solid state 2H MNR study. *Biochemistry* **1990**, *29*, 10676−10684.
(22) Lentz, B. R.; Hoechli, M.; Barenholz, Y. Acyl chain order and lateral domain formation in mixed phosphatidylcholine−sphingomyelin multilamellar and unilamellar vesicles. *Biochemistry* **1981**, *20*, 6803−6809.
(23) Castro, B. M.; Prieto, M.; Silva, L. C. Ceramide: a simple sphingolipid with unique biophysical properties. *Prog. Lipid Res.* **2014**, *54*, 53−67.
(24) Artetxe, I.; Sergelius, C.; Kurita, M.; Yamaguchi, S.; Katsumura, S.; Slotte, J. P.; Maula, T. Effects of sphingomyelin headgroup size on interactions with ceramide. *Biophys. J.* **2013**, *104*, 604−612.
(25) Silva, L. C.; de Almeida, R. F.; Castro, B. M.; Fedorov, A.; Prieto, M. J. Ceramide-domain formation and collapse in lipid rafts: membrane reorganization by an apoptotic lipid. *Biophys. J.* **2006**, *92*, 502−516.
(26) Goni, F. M.; Alonso, A. Effects of ceramide and other simple sphingolipids on membrane lateral structure. *Biochim. Biophys. Acta* **2009**, *1788*, 169−177.
(27) Montes, L. R.; Ruiz-Arguello, M. B.; Goni, F. M.; Alonso, A. Membrane restructuring via ceramide results in enhanced solute efflux. *J. Biol. Chem.* **2002**, *277*, 11788−11794.
(28) Sot, J.; Ibarguren, M.; Busto, J. V.; Montes, L. R.; Goni, F. M.; Alonso, A. Cholesterol displacement by ceramide in sphingomyelin-containing liquid-ordered domains, and generation of gel regions in giant lipidic vesicles. *FEBS Lett.* **2008**, *582*, 3230−3236.
(29) Veiga, M. P.; Arrondo, J. L.; Goni, F. M.; Alonso, A. Ceramides in phospholipid membranes: effects on bilayer stability and transition to nonlamellar phases. *Biophys. J.* **1999**, *76*, 342−350.
(30) Hsueh, Y. W.; Giles, R.; Kitson, N.; Thewalt, J. The effect of ceramide on phosphatidylcholine membranes: a deuterium NMR study. *Biophys. J.* **2002**, *82*, 3089−3095.
(31) Terova, B.; Slotte, J. P.; Nyholm, T. K. Miscibility of acyl-chain defined phosphatidylcholines with N-palmitoyl sphingomyelin in bilayer membranes. *Biochim. Biophys. Acta* **2004**, *1667*, 182−189.
(32) Rouser, G.; Fkeischer, S.; Yamamoto, A. Two dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. *Lipids* **1970**, *5*, 494−496.
(33) Jungner, M.; Ohvo, H.; Slotte, J. P. Interfacial regulation of bacterial sphingomyelinase activity. *Biochim. Biophys. Acta* **1997**, *1344*, 230−40.
(34) Kuklev, D. V.; Smith, W. L. Synthesis of four isomers of parinaric acid. *Chem. Phys. Lipids* **2004**, *131*, 215−222.
(35) Mason, J. T.; Broccoli, A. V.; Huang, C. A method for the synthesis of isomerically pure saturated mixed-chain phosphatidylcholines. *Anal. Biochem.* **1981**, *113*, 96−101.
(36) Ramstedt, B.; Slotte, J. P. Interaction of cholesterol with sphingomyelins and acyl-chain-matched phosphatidylcholines: a comparative study of the effect of the chain length. *Biophys. J.* **1999**, *76*, 908−15.
(37) Ibarguren, M.; Alonso, A.; Tenchov, B. G.; Goni, F. M. Quantitation of cholesterol incorporation into extruded lipid bilayers. *Biochim. Biophys. Acta* **2010**, *1798*, 1735−1738.
(38) Lakowicz, J.R. *Principles of Fluorescence Spectroscopy*; Kluwer Academic/Plenum Publishers: New York, 1999.
(39) Ahmed, S. N.; Brown, D. A.; London, E. On the origin of sphingolipid/cholesterol-rich detergent-insoluble cell membranes: physiological concentrations of cholesterol and sphingolipid induce formation of a detergent-insoluble, liquid-ordered lipid phase in model membranes. *Biochemistry* **1997**, *36*, 10944−53.
(40) London, E.; Brown, D. A.; Xu, X. Fluorescence quenching assay of sphingolipid/phospholipid phase separation in model membranes. *Methods Enzymol.* **2000**, *312*, 272−90.
(41) London, E.; Feigenson, G. W. Fluorescence quenching in model membranes. 1. Characterization of quenching caused by a spin-labeled phospholipid. *Biochemistry* **1981**, *20*, 1932−1938.
(42) Castro, B. M.; Silva, L. C.; Fedorov, A.; de Almeida, R. F.; Prieto, M. Cholesterol-rich fluid membranes solubilize ceramide domains: implications for the structure and dynamics of mammalian intracellular and plasma membranes. *J. Biol. Chem.* **2009**, *284*, 22978−22987.
(43) Nyholm, T. K.; Lindroos, D.; Westerlund, B.; Slotte, J. P. Construction of a DOPC/PSM/Cholesterol Phase Diagram Based on the Fluorescence Properties of *trans*-Parinaric Acid. *Langmuir* **2011**, *27*, 8339−8350.

DOI: 10.1021/acs.langmuir.5b00403
Langmuir 2015, 31, 4255−4263

(44) Welti, R.; Silbert, D. F. Partition of parinaroyl phospholipid probes between solid and fluid phosphatidylcholine phases. *Biochemistry* **1982**, *21*, 5685−5689.
(45) Wang, T. Y.; Silvius, J. R. Different sphingolipids show differential partitioning into sphingolipid/cholesterol-rich domains in lipid bilayers. *Biophys. J.* **2000**, *79*, 1478−89.
(46) Bjorkqvist, Y. J.; Nybond, S.; Nyholm, T. K.; Slotte, J. P.; Ramstedt, B. *N*-palmitoyl-sulfatide participates in lateral domain formation in complex lipid bilayers. *Biochim. Biophys. Acta* **2008**, *1778*, 954−962.
(47) Megha; Bakht, O.; London, E. Cholesterol precursors stabilize ordinary and ceramide-rich ordered lipid domains (lipid rafts) to different degrees. Implications for the Bloch hypothesis and sterol biosynthesis disorders. *J. Biol. Chem.* **2006**, *281*, 21903−21913.
(48) Bakht, O.; Pathak, P.; London, E. Effect of the structure of lipids favoring disordered domain formation on the stability of cholesterol-containing ordered domains (lipid rafts): identification of multiple raft-stabilization mechanisms. *Biophys. J.* **2007**, *93*, 4307−4318.
(49) de Almeida, R. F.; Fedorov, A.; Prieto, M. Sphingomyelin/phosphatidylcholine/cholesterol phase diagram: boundaries and composition of lipid rafts. *Biophys. J.* **2003**, *85*, 2406−2416.
(50) Saxena, K.; Duclos, R. I.; Zimmermann, P.; Schmidt, R. R.; Shipley, G. G. Structure and properties of totally synthetic galacto- and gluco-cerebrosides. *J. Lipid Res.* **1999**, *40*, 839−849.

DOI: 10.1021/acs.langmuir.5b00403
Langmuir 2015, 31, 4255−4263