

Glycosylation induces shifts in the lateral distribution of cholesterol from ordered towards less ordered domains

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Abstract

Several studies have indicated the involvement of sterol glycosides in the cellular stress response. In this work, we have compared the effect of 1-*O*-cholesteryl- β -D-glucoside, 1-*O*-cholesteryl- β -D-galactoside and cholesterol on the properties of glycerophospholipid and sphingolipid bilayers. The studies were performed in order to gain insight into the change in membrane properties that would follow upon the glycosylation of cholesterol in cells subjected to stress. DPH anisotropy measurements indicated that the cholesteryl glycosides (10–40 mol%) increased the order of the hydrophobic region of a POPC bilayer almost as efficiently as cholesterol. In a PSM bilayer, the cholesteryl glycosides were however shown to be much less effective compared to cholesterol in ordering the hydrocarbon chain region at temperatures above the gel to liquid-crystalline phase transition. Fluorescence quenching analysis of multicomponent lipid bilayers demonstrated that the cholesteryl glycosides, in contrast to cholesterol, were unable to stabilize ordered domains rich in PSM against temperature-induced dissociation. When the sterols were incorporated into bilayers composed of both POPC and PSM, the cholesteryl glycosides showed a higher propensity, compared to cholesterol, to influence the endothermal component representing the melting of POPC-rich domains, as determined by differential scanning calorimetry. Taken together, the results indicate that the glycosylation of cholesterol diminishes the ability of the sterol to reside in lateral domains constituted by membrane lipids having highly ordered hydrocarbon chains.

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Keywords: Model membrane; Cholesteryl glucoside; Cholesteryl galactoside; Cell stress

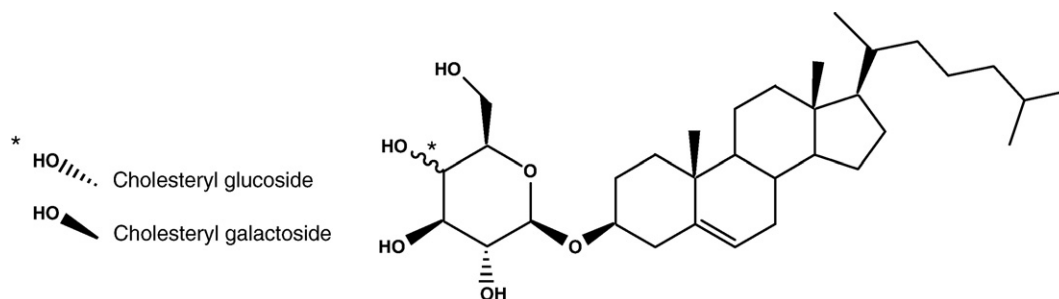
1. Introduction

Steryl glycosides are sterol derivatives in which one or more carbohydrate residues are linked to the C-3 oxygen of the sterol. This class of molecules, as well as the acylated sterol glycosides, in which a fatty acyl chain is linked to one of the carbohydrate units, is commonly found in the membranes of higher plants [1] and fungi [2], while reports on the occurrence of sterol glycosides in animals are more rare. The cellular functions of the sterol glycosides are far from fully characterized. Plant sterol glycosides have been suggested to be involved in e.g. the biosynthesis of cellulose [3] and glucosylceramide [4], and in regulation of the properties of the cellular membranes [1,5]. Steryl glycosides derived from plants have furthermore

been suggested to function as neurotoxins in humans [6]. A yeast sterol glucoside has been found to be required for the degradation of peroxisomes through specific autophagy mechanisms [7]. Additionally, several reports have linked the occurrence of sterol glycosides to the cellular stress response. Sakaki et al. [2] reported that heat shock, as well as increased ethanol concentrations, resulted in the production of sterol glycosides in the yeast *Pichia pastoris*. One of the sterol glycosides was identified as ergosterol- β -D-glucoside. Similarly, heat stress has been reported to induce the production of a poriferasterol- β -glucoside in the slime mold *Physarum polycephalum* [8]. In a study by Kunitomo et al., the concentrations of 1-*O*-cholesteryl- β -D-glucoside was observed to increase in human TIG-3 fibroblasts subjected to heat shock [9]. In subsequent experiments, the cholesteryl glucoside was added to TIG-3 fibroblasts without subjecting the cells to any other form of stress. The researchers observed increased binding of heat

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Scheme 1. Chemical structure of 1-*O*-cholesteryl-β-D-glucoside and 1-*O*-cholesteryl-β-D-galactoside.

shock transcription factor 1 to a heat shock element, as well as an increase in the amount of heat shock protein 70 (HSP70) [10]. Cholesteryl glucoside was furthermore reported to form in the stomach, liver and kidney of rats subjected to cold [10]. Based on their results, Kunimoto et al. proposed a hypothesis according to which the change in cell membrane fluidity upon thermal stress would activate a transferase, which would catalyze the production of steryl glucoside. Steryl glucoside might in turn activate heat shock factors directly or via protein kinases [9,10]. Ly et al. discussed the possibility of steryl glucoside participating in a stress signaling pathway based on their report on increased expression of HSP70 upon the addition of 1-*O*-β-sitosteryl-β-D-glucoside to the mouse motoneuron-derived cell line NSC-34 [6]. They concluded that the upregulation of the production of HSP70 could be due to the steryl glucoside functioning as a signaling molecule but noted that the upregulation could also be a result of damages caused by the steryl glucoside.

The observations that cholesteryl glucoside may play a role in the stress response of mammalian cells motivated our study on the effects of cholesteryl glycosides on glycerophospholipid and sphingolipid model membranes. While there are few reports concerning the effects of steryl glycosides (cholesteryl glycosides in particular) on the properties of lipid membranes, the effects of cholesterol have been studied extensively. The incorporation of cholesterol into a membrane has been observed to modulate e.g. the fluidity and permeability of the bilayer, as well as the lateral organization of membrane components [11,12]. The ability of cholesterol to affect the lateral organization of the membrane, and to induce the formation of liquid-ordered, cholesterol-rich domains, is expected to be a result of its flat and fairly rigid ring structure packing tightly with membrane lipids containing hydrocarbon chains devoid of *cis*-double bonds. Domains rich in cholesterol and sphingolipids, with properties similar to the liquid-ordered domains observed in model membranes, are assumed to form in the membranes of living cells [13,14].

Changes in bilayer fluidity of cellular membranes, occurring as a consequence of a change in temperature or of the incorporation of molecules affecting the packing density of the membrane, have been recognized to be one of several mechanisms through which the cellular heat shock response is induced [15,16]. Recent results by Nagy et al. however indicated that not only a change in fluidity, but also the reorganization of cholesterol-rich domains might be required for

the membrane to mediate cellular stress signals [17,18]. The membrane fluidizer benzyl alcohol was observed to cause a reorganization of domains enriched with fluorescein labeled polyethylene glycol-derivatized cholesterol in the plasma membrane of melanoma cells and to induce the cellular heat shock response [17]. The structurally related phenethyl alcohol, which also has been shown to fluidize membranes, did however neither cause a reorganization of the domains nor induce the cellular heat shock response.

In the present study, we compared the effects of 1-*O*-cholesteryl-β-D-glucoside and 1-*O*-cholesteryl-β-D-galactoside (Scheme 1) to the effects of cholesterol on the fluidity and domain thermostability in glycerophospholipid and sphingolipid model membranes. The observations are expected to be of value for further investigations concerning the part played by cholesterol glycosylation in the cellular stress response, as well as for the general understanding of the importance of steryl glycosides as membrane components.

2. Experimental procedures

2.1. Materials

1-*O*-Cholesteryl-β-D-glucoside and 1-*O*-cholesteryl-β-D-galactoside were synthesized from cholesterol using 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide and 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide (Fluka, Buchs, Switzerland), respectively, as the glycosyl donors. The synthesis was based on previously published procedures by Nagarajan et al. [19], and by Iga et al. [20]. A mixture of 260 μmol of zinc oxide and 130 μmol of cholesterol dissolved in toluene was refluxed for 24 h to produce the zinc salt of cholesterol. 130 μmol of 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide or 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide was added and the mixture was refluxed for 24 h in order to allow the acetylated cholesteryl glycoside derivatives to form. The final products were obtained through alkaline hydrolysis using sodium methylate as the agent. The suspension was mixed with Celite and the undissolved material was removed by filtration. The products were purified by reverse-phase HPLC (Supelco Discovery C18 column, dimensions 250 × 21, 2 mm, 5 μm particle size) with methanol as the mobile phase. Cholesterol, which was included in the study as a reference sterol, was purchased from Sigma Chemicals (St. Louis, MO).

N-Palmitoyl-D-erythro-sphingosylphosphorylcholine (PSM) was purified from egg yolk sphingomyelin (Avanti Polar Lipids, Alabaster, AL) by reverse-phase HPLC using methanol as the eluent. 1-Palmitoyl-2-oleyl-*sn*-glycero-3-phosphocholine (POPC) was obtained from Avanti Polar Lipids. *N*-Palmitoyl-galactosylceramide (PGalCer) was synthesized from lyso-galactosylceramide (D-galactosyl-β1-1'-D-erythro-sphingosine, psychosine) and palmitic anhydride according to a procedure based on a previously published method by Cohen et al. [21]. The product was purified by reverse-phase HPLC using methanol as the eluent. 1-Palmitoyl-2-stearoyl-(7-doxyl)-*sn*-glycero-3-phosphocholine (7SLPC) employed for fluorescence quenching was synthesized from (7-doxyl)-stearic acid (TCI Europe NV, Belgium)

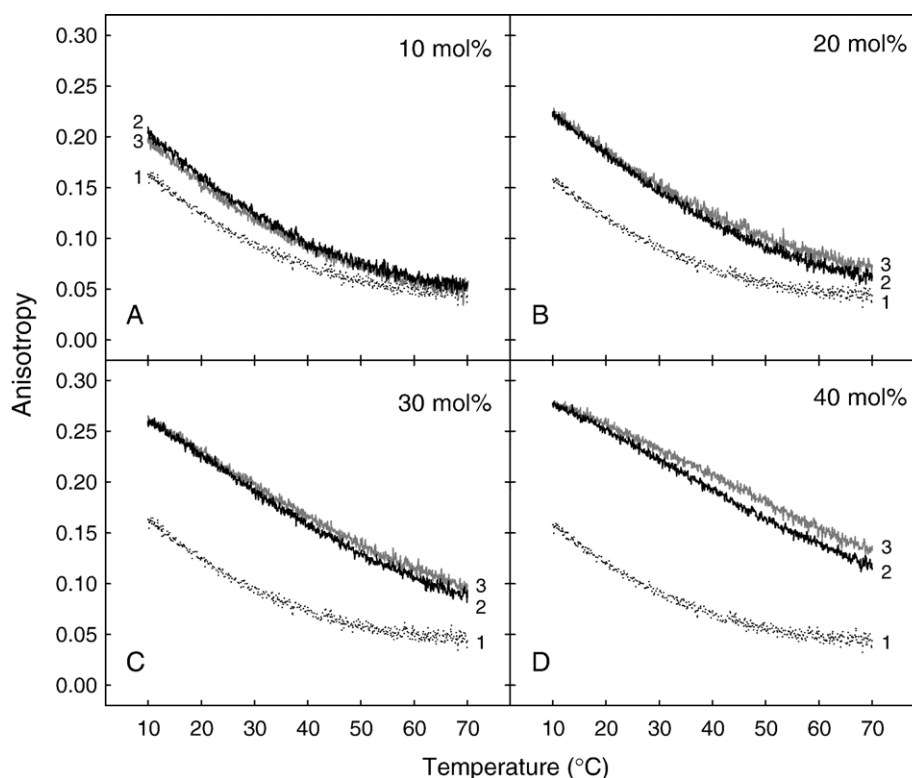


Fig. 1. Anisotropy of DPH in sterol-containing POPC bilayers. The anisotropy of DPH in POPC bilayers containing 10 mol% (A), 20 mol% (B), 30 mol% (C) or 40 mol% (D) of cholesterol glycoside or cholesterol was measured as a function of temperature. Key: dotted black line (1): POPC (shown as a reference in all panels), black line (2): POPC+cholesterol glycoside, grey line (3): POPC+cholesterol. The DPH anisotropy of the POPC+cholesterol galactoside samples (not shown) coincided with that of POPC+cholesterol glycoside samples at the corresponding sterol concentrations.

and 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (Avanti Polar Lipids) according to a procedure based on a previously published method by Szolderits et al. [22]. The product was purified using reverse-phase HPLC with methanol as the eluent. The phospholipid stock solution concentrations were determined using the method described by Rouser et al. [23], while the PGalCer stock solution concentration was determined through mass spectrometric determination of the fatty acid content after acid hydrolysis.

1,6-diphenyl-1,3,5-hexatriene (DPH) was obtained from Molecular Probes (Eugene, OR). *trans*-Parinaric acid (tPA) was produced through an iodine-catalyzed isomerization reaction of *cis*-parinaric acid (Cayman Chemical Company, Ann Arbor, MI). The procedure was based on a previously published method by Kuklev and Smith [24]. The product was purified by reverse-phase HPLC using methanol:water:acetic acid (900:50:3, volume ratio) as the eluent. The concentrations of the fluorophore stock solutions were determined according to previously reported extinction coefficients (DPH: $88,000 \text{ cm}^{-1}\text{M}^{-1}$ at 350 nm in methanol, reported by the vendor; tPA: $92,000 \text{ cm}^{-1}\text{M}^{-1}$ at 299 nm in methanol [25]).

Stock solutions of glycerophospholipids and sphingolipids were prepared in hexane/2-propanol (3:2, volume ratio), and stock solutions of the sterols in dichloromethane:methanol (3:1, volume ratio). The solutions were stored at -20°C and warmed to ambient temperature before use. The water used in all experiments was purified by reverse osmosis followed by passage through a Millipore UF Plus water purification system (Millipore Corporation, Billerica, MA), to yield a product with a resistivity of $18.2 \text{ M}\Omega\text{cm}$.

2.2. Sample preparation

The appropriate amounts of lipids dissolved in organic solvent were mixed and the solvent was evaporated. The lipids were hydrated at a temperature above the gel to liquid-crystalline phase transition temperature of the lipid component having the highest transition temperature. When tPA was included, argon-bubbled water was used to minimize oxidation of the probe. The vesicle suspensions analyzed using anisotropy and fluorescence quenching measurements were prepared through

probe sonication (Branson W-450, Branson Ultrasonics, Danbury, CT) for 2 min (25% duty cycle, power output 10 W). The DSC samples were bath sonicated (Branson 2510, Branson Ultrasonics) at 55°C for 4 min.

2.3. DPH anisotropy

DPH anisotropy measurements were performed on a PTI QuantaMaster-2 spectrofluorimeter (Photon Technology International, Lawrenceville, NJ) operating in the T format. The samples were scanned from 10 to 80°C at a temperature rate of $5^\circ\text{C}/\text{min}$. The excitation and emission wavelengths were 360 and 430 nm, respectively, and all slits were set to a width of 5 nm. The G value was determined prior to each measurement. The DPH to lipid ratio was 1:200 and the total lipid concentration $50 \mu\text{mol}/\text{l}$.

2.4. Fluorescence quenching

Fluorescence quenching of tPA by 7SLPC was applied for the study of the thermostability of lateral ordered domains. The method has been discussed in detail in previous publications [26,27]. Briefly, fluorescence intensity of quencher-containing F samples is compared to the fluorescence intensity of quencher-free F_0 samples giving the fraction of unquenched fluorescence (F/F_0). In lipid bilayers in which ordered and disordered domains coexist, the amount of tPA exposed to quenching by 7SLPC (which is expected to predominantly reside in the disordered domains [28]) gives a measure of the extent of ordered domains formed in the system. The composition of the F samples was POPC:7SLPC:sphingolipid:sterol (30:30:30:10, molar ratio), while POPC replaced the 7SLPC in the quencher-free F_0 samples. We also analyzed control samples containing bilayers devoid of sterol. tPA was included in all samples at a fluorophore:membrane lipid ratio of 1:100. The lipid concentration was $50 \mu\text{mol}/\text{l}$. The fluorescence quenching measurements were performed on a PTI QuantaMaster-2 spectrofluorimeter (Photon Technology International) operating in the L format. The samples were scanned from 10 to 70°C at a temperature rate of $5^\circ\text{C}/\text{min}$. The wavelengths for excitation and

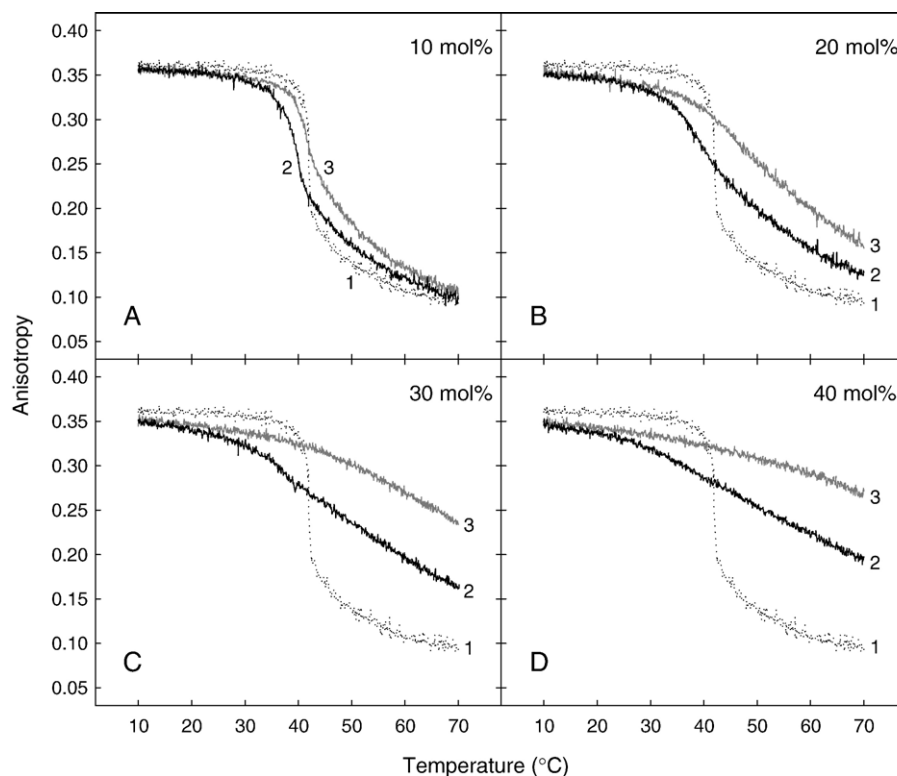


Fig. 2. Anisotropy of DPH in sterol-containing PSM bilayers. The anisotropy of DPH in PSM bilayers containing 10 mol% (A), 20 mol% (B), 30 mol% (C) or 40 mol% (D) of cholesteryl glycoside or cholesterol was measured as a function of temperature. Key: dotted black line (1): PSM (shown as a reference in all panels), black line (2): PSM+cholesteryl glucoside, grey line (3): PSM+cholesterol. The DPH anisotropy of all PSM+cholesteryl galactoside samples (not shown) coincided with that of PSM+cholesteryl glucoside samples at the corresponding sterol concentrations.

emission were obtained using a 308 nm dichroic excitation filter and a 425 nm long bandpass filter, respectively.

2.5. Differential scanning calorimetry

The samples analyzed using differential scanning calorimetry (DSC) were scanned between 0 and 100 °C for a minimum of two rounds at a temperature rate of 0.5 °C/min using a Nano II high performance differential scanning calorimeter (Calorimetry Sciences Corporation, Provo, UT). The phospholipid (PSM+POPC) concentration (according to which the specific heat capacity was calculated) was 4.5 mmol/l in all samples.

2.6. Monolayer collapse pressure determination

The monolayer collapse pressure at room temperature was determined using a KSV surface barostat (KSV, Helsinki, Finland). Sterol dissolved in dichloromethane:methanol (3:1, volume ratio) was applied onto the water surface in a rectangular trough. The solvent was allowed to evaporate for 2 min and the monolayer was then compressed at a speed not exceeding 10 Å²/molecule per minute.

3. Results

3.1. Bilayer fluidity

The incorporation of cholesterol into a liquid-crystalline lipid bilayer decreases the degree of *trans*–*gauche* isomerizations of the lipid hydrocarbon chains [29]. The resulting ordering effect can be observed as an increase in anisotropy of the fluorescent probe DPH, which predominantly partitions into

the hydrophobic core of the bilayer [30]. At temperatures below the gel to liquid-crystalline phase transition temperature, a small decrease in DPH anisotropy can usually be detected upon the incorporation of cholesterol in a phospholipid bilayer [31–33]. In the present study, we compared the effect of cholesteryl glucoside and cholesteryl galactoside to the effect of cholesterol on the anisotropy of DPH as function of temperature in three compositionally different glycerophospholipid and sphingolipid bilayers. The sterol concentrations tested were 10, 20, 30 and 40 mol%. Since cholesteryl glucoside and cholesteryl galactoside always gave indistinguishable results, we do not show data with cholesteryl galactoside-containing bilayers.

When the sterols were added to POPC bilayers, the cholesteryl glycosides were observed to cause an increase in DPH anisotropy, which was almost as large as the increase seen with cholesterol (Fig. 1). A small difference in the effect of the cholesteryl glycosides and cholesterol on DPH anisotropy could be observed at higher temperatures. Due to instrument limitations we could not examine the effect of the sterols on the gel to liquid-crystalline phase transition of POPC, which occurs around –2.5 °C in sterol-free bilayers [34]. When the sterols were incorporated into PSM bilayers, we found that the cholesteryl glycosides increased the order of the hydrophobic region at temperatures above the gel to liquid-crystalline phase transition, but did so much less efficiently compared to cholesterol (Fig. 2). In contrast to cholesterol, the cholesteryl glycosides were seen to cause a considerable decrease in the overall main transition temperature of PSM. The results

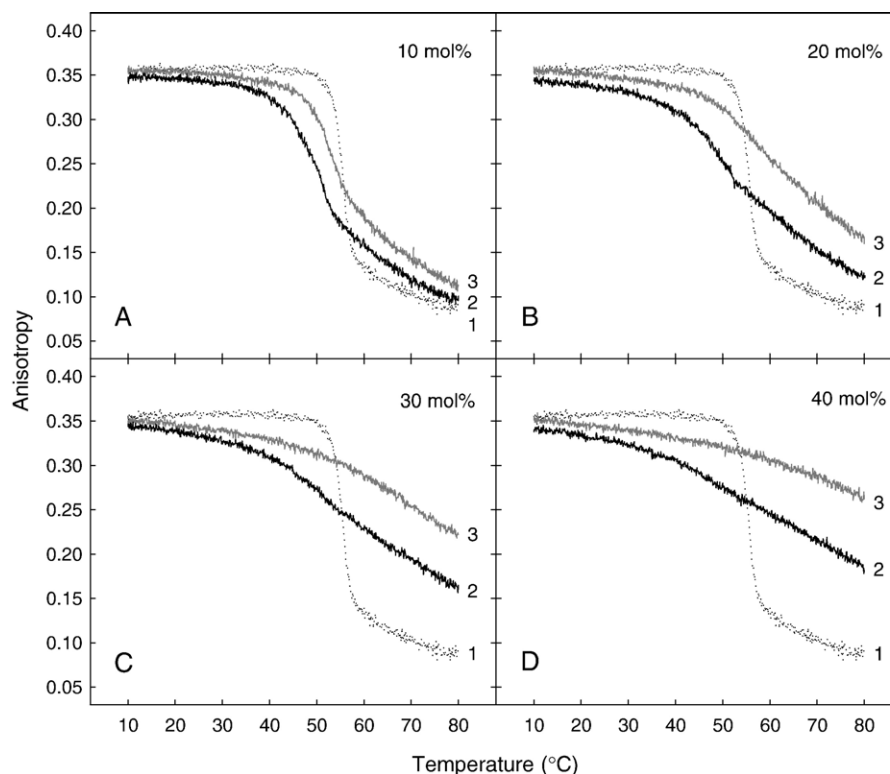


Fig. 3. Anisotropy of DPH in sterol-containing PSM:PGalCer bilayers. The anisotropy of DPH in PSM:PGalCer (1:1) bilayers containing 10 mol% (A), 20 mol% (B), 30 mol% (C) or 40 mol% (D) of cholesteryl glycoside or cholesterol was measured as a function of temperature. Key: dotted black line (1): PSM:PGalCer (shown as a reference in all panels), black line (2): PSM:PGalCer+cholesteryl glucoside, grey line (3): PSM:PGalCer+cholesterol. The DPH anisotropy of all PSM:PGalCer+cholesteryl galactoside samples (not shown) coincided with that of PSM:PGalCer+cholesteryl glucoside samples at the corresponding sterol concentrations.

thus suggest that the cholesteryl glycosides differ more from cholesterol in their effect on the hydrocarbon chain fluidity in bilayers composed of PSM than in bilayers composed of POPC. We performed control experiments measuring the anisotropy of 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH), which due to its amphiphilic character preferentially resides in or close to the hydrophilic/hydrophobic interfacial region of a lipid bilayer [35]. The effects of 30 mol% of cholesterol, cholesteryl glucoside or cholesterol galactoside on the anisotropy of TMA-DPH were practically identical to the effects seen with DPH. The cholesteryl glycosides increased the anisotropy of TMA-DPH in a POPC bilayer as efficiently as cholesterol, while their impact on a liquid-crystalline PSM bilayer was considerably less pronounced than the impact of cholesterol (results not shown).

The third bilayer composition chosen for anisotropy measurements included the glycosphingolipid PGalCer. Analyzing the effect of the cholesteryl glycosides on bilayers composed of equimolar amounts of PSM and PGalCer, we aimed at detecting indications of possible sugar–sugar interactions between the head groups of adjacent cholesteryl glycoside and PGalCer molecules (Fig. 3). The gel to liquid-crystalline phase transition of sterol-free PSM:PGalCer (1:1) bilayers was seen to occur at 56 °C, which is in accordance with previously reported DSC data [26]. The effect of the cholesteryl glycosides on the PSM:PGalCer bilayers were roughly identical to their effect on bilayers composed of only PSM. The cholesteryl glycosides destabilized

the gel phase, and were considerably less effective, compared to cholesterol, to increase the hydrocarbon chain order at temperatures above the gel to liquid-crystalline phase transition. The incorporation of PGalCer in the PSM membrane did thus not seem to influence the effect of the cholesteryl glycosides on the bilayer.

3.2. Lateral phase separation

Fluorescence quenching was used to examine the effect of the sterols on multicomponent bilayers containing both saturated sphingolipids (PSM or PSM and PGalCer) and an unsaturated glycerophospholipid (POPC), in addition to the doxyl-labeled phosphatidylcholine quencher 7SLPC and the fluorescent probe tPA. In the quenching experiment, the susceptibility of tPA to quenching by 7SLPC (shown as the fraction of unquenched fluorescence F/F_0) was monitored as a function of temperature. The coexistence of ordered and disordered domains is detected as a high F/F_0 value, and the increased desegregation of membrane components upon an increase in temperature can be observed as a decrease in F/F_0 . Using fluorescence quenching, the melting of ordered domains (expected to be rich in PSM) in bilayers composed of POPC:7SLPC:PSM (30:30:30, molar ratio) was observed to occur at approximately 20 °C (Fig. 4, panel A). The incorporation of 10 mol% of cholesterol (POPC:7SLPC:PSM:chol, 30:30:30:10) into the bilayer caused a marked increase in thermostability of the ordered domains

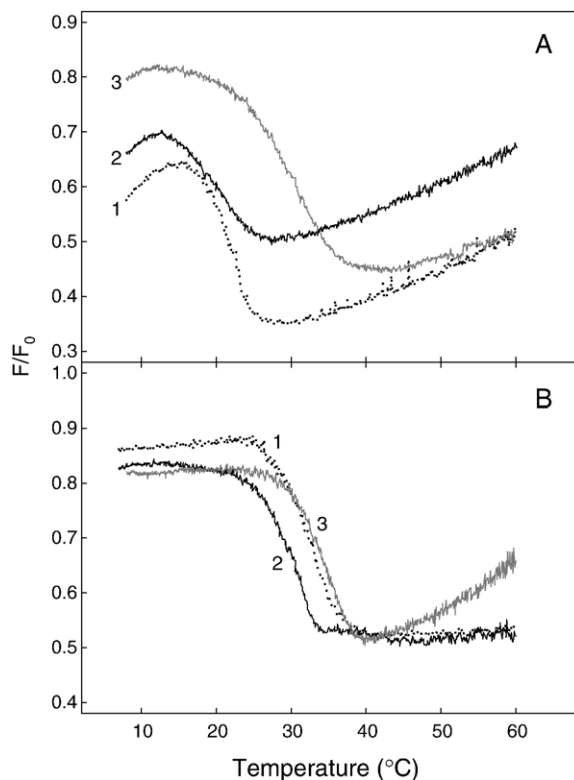


Fig. 4. Effect of cholesterol glycosides on the stability of ordered domains. The relative intensity (F/F_0) of tPA in quencher-containing (F) samples and quencher-free (F_0) samples was measured for multicomponent bilayers as a function of temperature. A. POPC:7SLPC:PSM:sterol (30:30:30:10; F sample composition), B. POPC:7SLPC:PSM:PGalCer:sterol (30:30:15:15:10; F sample composition). POPC replaced 7SLPC in the F_0 samples. tPA was included in all samples at a fluorophore:membrane lipid ratio of 1:100. Key: dotted black line (1): no sterol, black line (2): cholesterol glycoside, grey line (3): cholesterol. The results obtained for bilayers containing cholesterol galactoside (not shown) were equivalent to the results for bilayers containing cholesterol glycoside.

[26,36]. Ordered domains formed in the presence of cholesterol were observed to melt at temperatures around 30 °C. The quenching of the fluorescent probes cholestatrienol and *trans*-parinaroyl sphingomyelin has been shown to report a miscibility transition in approximately the same temperature interval as the quenching of tPA in the cholesterol-containing bilayers [26,36]. We can thus expect the ordered domains formed in POPC:7SLPC:PSM:chol (30:30:30:10) bilayers to contain both cholesterol and PSM. In contrast to cholesterol, the cholesterol glycosides were not able to increase the thermostability of the ordered PSM-rich domains. The cholesterol glycosides rather seemed to decrease the miscibility transition temperature with a few degrees (Fig. 4, panel A). These results are in line with the DPH anisotropy data, supporting the idea of the cholesterol glycosides having a lower ability, compared to cholesterol, to interact tightly with the saturated PSM.

When half of the PSM in the sterol-free bilayers was replaced by the glycosphingolipid PGalCer (POPC:7SLPC:PSM:PGalCer, 30:30:15:15, molar ratio), ordered domains (expected to be rich in both PSM and GalCer [26]) were seen to melt at temperatures around 33 °C. The addition of 10 mol% of cho-

lesterol to these bilayers (POPC:7SLPC:PSM:PGalCer:chol, 30:30:15:15:10) caused a small increase in the thermostability of the ordered domains [26,36]. In accordance to the results obtained for bilayers containing only PSM as the saturated lipid, the incorporation of 10 mol% of the cholesterol glycosides in the PGalCer-containing bilayers resulted in a small destabilization of the ordered domains (Fig. 4, panel B). We could thus conclude that neither the DPH experiments nor the quenching experiments gave any indications for the presence of stabilizing sugar–sugar interactions between the cholesterol glycosides and PGalCer.

Differential scanning calorimetry (DSC) was applied for studies of the effect of the cholesterol glycosides on the thermotropic transitions of POPC- and PSM-containing bilayers. The DSC analysis of bilayers composed of POPC and PSM (60:30, molar ratio) revealed two endothermic transitions (see Fig. 5). The endothermal components observed at temperatures around 3 and 18 °C can be assumed to represent the melting of POPC- and PSM-rich domains, respectively. Due to technical limitations, we were not able to observe thermotropic transitions below approximately 1 °C. The addition of 10 mol% of cholesterol appeared to result in a decrease in the transition enthalpy of the endothermal component representing the melting of PSM-rich domains [27]. Cholesterol seemed to influence the endothermal component representing the melting of POPC-rich domains to a lower extent. A small downward shift in transition temperature of this component could however be discerned [27]. Compared to cholesterol, the cholesterol glycosides seemed to have a much stronger influence on the low-temperature part of the endotherm. In addition to an apparent decrease in enthalpy of the component representing the transition of a PSM-rich phase, 10 mol% of cholesterol glycoside caused a considerable broadening of the endothermal component representing the melting of a POPC-rich phase. We did not observe any significant differences between the endotherms recorded upon sample heating and cooling.

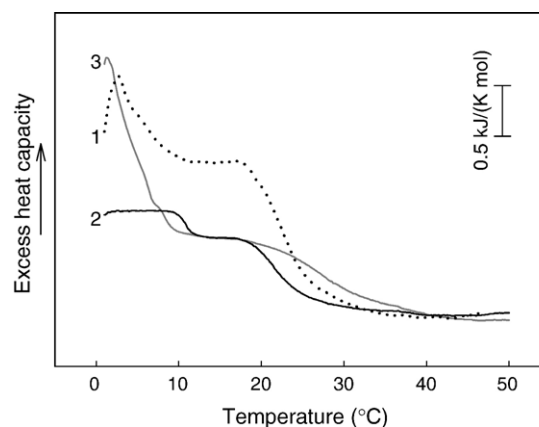


Fig. 5. Differential scanning calorimetry endotherms of bilayers containing cholesterol glycoside. The second heating scans are shown. Key: dotted black line (1): POPC:PSM (60:30), black line (2): POPC:PSM:cholesterol glycoside (60:30:10), grey line (3): POPC:PSM:cholesterol (60:30:10). The results obtained for bilayers containing cholesterol galactoside (not shown) coincided with that of bilayers containing cholesterol glycoside.

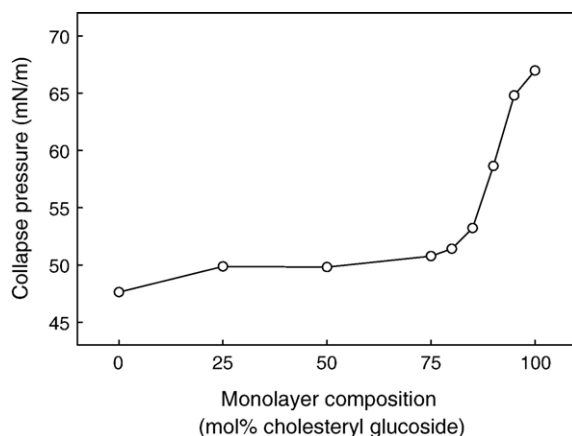


Fig. 6. Sterol monolayer collapse pressure. The collapse surface pressure of binary cholesterol/cholesteryl glucoside monolayers is shown as a function of their cholesteryl glucoside content.

3.3. Sterol monolayer collapse pressure

Pressure versus area isotherms of sterol monolayers revealed that the cholesteryl glucoside monolayers reached a much higher surface pressure prior to monolayer collapse compared to monolayers composed of cholesterol. The cholesteryl glucoside and the cholesteryl galactoside monolayers collapsed at surface pressures of 67 mN/m and 63 mN/m, respectively, while the collapse pressure of a cholesterol monolayer was observed to be 47 mN/m (in agreement with previously reported values [37,38]). The change in molecular features of the sterol (e.g. shape and number of polar functions) upon glycosylation thus seemed to increase the stability of the pure sterol monolayer. Analyses of binary cholesteryl glucoside/cholesterol monolayers revealed that the stabilizing effect of the carbohydrate residues in a cholesteryl glucoside monolayer was efficiently counteracted by the incorporation of only a few mol% of cholesterol (Fig. 6). This observation suggests a good miscibility of cholesterol in the cholesteryl glucoside monolayer, and the ability to interfere with the interactions between neighboring cholesteryl glucoside molecules or between cholesteryl glucosides and water molecules surrounding the polar head groups.

4. Discussion

In the present study, we have investigated the effect of two cholesteryl glycosides on the membrane fluidity and lateral phase organization of model membranes composed of PSM, POPC and PGalCer. Glycosylation of the hydroxyl group of cholesterol has been reported to occur in cells subjected to stress [9,10]. In addition to cholesteryl glucoside, which was the sterol observed to be synthesized in cells upon thermal stress, we chose to include its isomer cholesteryl galactoside in the study. Glucose and galactose moieties in the head groups of glycosphingolipids have previously been shown to have dissimilar effects on the properties of lipid model membranes [36,39,40]. We however found that cholesteryl glucoside and cholesteryl galactoside had practically identical influence on the aspects of lipid model membranes that were analyzed in this study.

4.1. The sterol carbohydrate head group prevents tight interactions with PSM

4.1.1. Binary bilayers

We examined the effect of the cholesteryl glycosides on the steady-state anisotropy of DPH in lipid bilayers. DPH partitions into the hydrophobic core of the bilayer and its fluorescence anisotropy consequently reflects the fluidity of this part of the membrane [30]. The cholesteryl glycosides were observed to be almost as effective as cholesterol in ordering the hydrocarbon chain region of a POPC bilayer. When incorporated into a liquid-crystalline PSM bilayer, however, the cholesteryl glycosides were significantly less efficient, compared to cholesterol, in ordering the hydrocarbon chain region. The relatively low ability of the cholesteryl glycosides to order the hydrocarbon chains of the liquid-crystalline PSM bilayer is most likely a result of both the size and hydrophilicity of the carbohydrate head groups. As a cholesterol molecule is incorporated into a membrane, it is oriented with the plane of its ring structure at a small angle against the bilayer normal. According to ^2H -NMR measurements, the most likely tilt angle, i.e. the angle between the vector connecting carbon atoms 3 and 17 in the sterol and the bilayer normal, is $16\text{--}19^\circ$ for $[3\alpha\text{-}^2\text{H}]$ cholesterol in bilayers composed of phosphatidylcholines having a saturated (palmitoyl or stearoyl) chain in the *sn*-1 position (with a small dependence on sterol concentration and temperature) [41,42]. The cholesterol hydroxyl is situated in the vicinity of the carbonyl residues of the fatty acyl chains, while the ring structure and the isooctyl side chain protrudes into the core of the bilayer [43,44]. The head groups of cholesteryl glucoside and cholesteryl galactoside are considerably larger than the hydroxyl group of cholesterol. This is likely to result in steric constraints in the polar and interfacial regions of the cholesteryl glycoside-containing bilayer preventing tight interactions between the sterol ring structure and the hydrocarbon chains of neighboring phospholipids. Hydrogen bonds between the polar functions of the cholesteryl glycoside head groups and surrounding water molecules can be expected to increase the apparent bulkiness of the head group even further [45,46]. The steric constraints caused by the increase in size of the polar portion of the sterol might moreover reduce the depth at which the sterol is embedded in the bilayer. This could in turn increase the hydrophobic mismatch [47,48] and reduce the hydrophobic contact area between the sterol and surrounding membrane lipids. The attachment of a glucose or galactose moiety to the cholesterol head group can be expected to affect the sterol tilt angle, which has been suggested to be tightly linked to the ability of the sterol to increase the order in a lipid bilayer [49]. Because of the *cis*-unsaturated oleoyl chain in POPC, the ratio of the cross sectional area of the head group to that of the hydrophobic portion is smaller for POPC than for PSM. The glycosyl residues in the cholesteryl glycosides might therefore be expected to cause less steric constraints in a POPC than in a PSM bilayer, and thus show less interference with the interactions between the sterol ring system and the hydrocarbon chains.

As the structural differences between cholesterol and the cholesteryl glycosides lie in the part of the sterol which is

positioned in the polar/interfacial region of a lipid bilayer, the differences in effect might be accentuated in this part of the membrane. We therefore performed control experiments in which we monitored the effect of the sterols (30 mol%) on the fluorescence anisotropy of TMA-DPH in POPC and PSM bilayers (results not shown). The polar trimethylammonium group of TMA-DPH is expected to anchor the DPH residue to the upper hydrophobic portion of the bilayer and to report on the fluidity in this region [35]. The difference in effect of the sterols were observed to be practically identical to what was observed using DPH. This suggests that the constraints inferred by cholesterol glycosylation would have an effect on the fluidity of the hydrocarbon chain segments closest to the polar residues that would not be very different from the effect on the average fluidity of the hydrophobic region of the bilayer.

The steric constraints imposed by the large head group of the cholesteryl glycosides are most probably the cause of the decrease in gel to liquid-crystalline transition temperature seen with the cholesteryl glycosides but not with cholesterol. The packing difficulties are expected to be accentuated in the highly ordered gel state, thus destabilizing the bilayer and allowing it to undergo a chain-melting transition at lower temperatures. In a previous electron spin resonance study, a soy bean-derived steryl glucoside mixture was shown to cause a considerable decrease in the gel to liquid-crystalline transition temperature of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), while no such evident shift was seen with cholesterol or non-glycosylated soy bean-derived sterol [50]. In contrast to cholesterol, the glucosylated soy bean-derived sterol was moreover shown to have only a very small ordering effect on the hydrophobic portion of the DPPC bilayer at temperatures above the main transition of DPPC [50]. Our results regarding the effect of the cholesteryl glycosides on PSM bilayers are in excellent agreement with these findings.

Our DPH anisotropy data, which demonstrated that the effect of the cholesteryl glycosides on model membrane bilayers resembled the effect of cholesterol more in bilayers composed of POPC compared to bilayers composed of PSM, suggest a preference of the cholesteryl glycosides to interact with relatively fluid membrane lipids. This is in line with previously published observations on the hydrocarbon chain-ordering effect of the cholesterol head group analog cholesteryl sulfate [51], which is found in e.g. the plasma membrane of the spermatozoon head region [52] and in skin epidermis [53,54]. Based on results from DSC, monolayer condensation, fluorescence and erythritol permeability measurements, Schofield et al. concluded that the sulfate head group renders cholesteryl sulfate more prone to interact favorably with the polyunsaturated 1-stearoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine than with the mono-unsaturated 1-stearoyl-2-oleyl-*sn*-glycero-3-phosphocholine (SOPC), while cholesterol shows a reversed preference [51]. Schofield et al. hypothesized that the anionic sulfate head group of cholesteryl sulfate positions the sterol less deep in the bilayer where it might fit into a pocket (not accessible to cholesterol) formed due to the $\Delta 4$ *cis*-double bond in the docosahexaenoyl chain in 1-stearoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine. No considerable difference in vertical position

could be detected when Faure et al. determined the order profile of 1-myristoyl-2- $^2\text{H}_{27}$ -myristoyl-*sn*-glycero-3-phosphocholine in binary bilayers containing either cholesterol or cholesteryl sulfate [55]. The order profile plateau region was demonstrated to include the same number of carbon atoms regardless of which sterol (cholesterol or cholesteryl sulfate) was included in the membrane, which would indicate that the depths at which cholesterol and cholesteryl sulfate are embedded in a bilayer composed of a saturated phosphatidylcholine are roughly equal. The findings regarding cholesteryl sulfate might reflect properties shared with cholesteryl glucoside and cholesteryl galactoside. If this is the case, the relatively low ability of the cholesteryl glycosides to increase the hydrocarbon chain order in a PSM bilayer can be expected to be a consequence of the effect of the head group on the spacing between phospholipid molecules and the sterol tilt angle rather than of the head group preventing the sterol ring structure and side chain from protruding deep into the bilayer [55].

In a previous study regarding the effect of plant sterols on model membranes, Mudd and McManus examined the effect of soy bean-derived steryl glycoside, which to a large part consists of β -sitosteryl glycosides, on DPPC bilayers [56]. Mudd and McManus observed that the soy bean-derived steryl glycoside broadened the gel to liquid-crystalline phase transition of DPPC. They noted, however, that the steryl glycoside was never able to eliminate all traces of the phase change. Our DPH anisotropy results seem to point in the same direction. At 40 mol% of sterol in PSM bilayers, cholesterol has eliminated all traces of the sudden fluidity change occurring at the phase transition temperature. In bilayers containing 40 mol% of cholesteryl glycoside, a kink in the curve describing the DPH anisotropy as a function of temperature can, however, still be detected. This might indicate that a higher concentration of cholesteryl glycosides, compared to cholesterol, would be needed in order to eliminate the transition. It is however possible that the solubility of the cholesteryl glycosides in the gel phase of PSM is limiting and too low for complete elimination of the transition to be reached [57].

4.1.2. Multicomponent bilayers

The heterogeneous lateral organization of membrane components has been recognized as a crucial feature of cell membranes in processes such as cell signaling, protein and lipid sorting, and in the interactions between a cell and various pathogens (see [58] and [59] for recent reviews). Also the membrane-mediated stress signal transduction has been suggested to require the reorganization of the components of certain types of lateral domains [17,18]. The composition of ordered domains in live cells has frequently been expected to be reflected by the composition of detergent resistant membrane fractions derived from the cells. Triton X-100 resistant membrane fractions from plant cells have been shown to be enriched in steryl glycosides, as well as acylated steryl glycosides, free sterol and sphingolipids [60,61], which might be considered an indication of these molecules partitioning into ordered domains in plant cell membranes. As the connection between detergent resistant membrane fractions and ordered domains in live cells is probably

very weak, or non-existent, such results should however be interpreted with caution [62–64].

Due to preferential interactions between cholesterol and membrane lipids having hydrocarbon chains devoid of *cis*-unsaturations, cholesterol is capable of inducing phase separation in multicomponent bilayers [11,12]. The composition and size of the domains that form in a multicomponent bilayer are dependent on the specific lipid species that are included, as well as on the temperature of the system. In a bilayer containing equimolar amounts of cholesterol, POPC and PSM, two phases, a POPC-rich liquid-disordered phase and a PSM- and cholesterol-rich liquid-ordered phase coexist at 23 °C [65]. The cholesterol concentrations of the liquid-ordered and the liquid-disordered phase have been estimated to be around 40 and 5 mol%, respectively [65]. If however, the cholesterol fraction of the bilayer is increased at the expense of POPC and/or PSM, the lipid components mix and form a uniform liquid-ordered phase. Based merely on the experiments performed in this study, the exact lateral distribution of the cholesteryl glycosides between various bilayer domains cannot be determined. Our DSC results, (together with the DPH anisotropy and fluorescence quenching data) however, give us some indications. DSC experiments provide us with information regarding the effects of the different sterols on enthalpies and temperatures of phospholipid phase transitions. Incorporation of an increasing amount of cholesterol into a bilayer composed of one glycerophospholipid or sphingolipid species gradually induces the formation of a liquid-ordered phase and thereby decreases the enthalpy and cooperativity of the gel to liquid-crystalline phase transition. In phosphatidylcholine bilayers, the shift in transition temperature upon the incorporation of cholesterol is dependent on the length of the phospholipid acyl chains. Due to the hydrophobic mismatch effect, cholesterol stabilizes the gel phase of phosphatidylcholines having saturated acyl chains shorter than 17 carbon atoms and destabilizes it when the acyl chains contain less than 17 carbon atoms [48]. When 10 mol% of cholesterol is added to a POPC:PSM (60:30, molar ratio) bilayer, a decrease in the transition enthalpy and cooperativity of the endothermal component representing the melting of PSM-rich domains can be observed [27]. In contrast to cholesterol, the cholesteryl glycosides seemed to have a significant effect also on the endothermal component representing the melting of POPC-rich domains. This can be considered an indication of the cholesteryl glycosides showing a higher propensity, compared to cholesterol, to reside in the more disordered POPC-rich domains [51,66]. The assumption is supported by our steady-state fluorescence anisotropy results, which strongly indicated that the glycoside residue of the cholesteryl glycosides rendered the sterol much less prone to create favorable, order-promoting interactions with PSM. In fluorescence quenching experiments, cholesterol was seen to increase the thermostability of ordered domains formed in bilayers composed of POPC, 7SLPC and PSM [26], probably due to preferential interaction between cholesterol and the saturated PSM leading to a more efficient phase separation [67] of PSM from the phospholipids favoring disordered domains (7SLPC and POPC). In the presence of the cholesteryl glycosides, ordered domains were formed, but the stabilizing effect seen with cholesterol was missing. In

previous fluorescence quenching studies, London and coworkers investigated the effects of several cholesterol head group analogs on the formation and thermostability of ordered domains in 12SLPC:DPPC:sterol bilayers [68,69]. All cholesterol head group analogs were observed to have a lower ability to increase the thermostability of ordered DPPC-rich domains when compared to cholesterol. Cholesteryl sulfate and 4-cholesten-3 β -one even seemed to destabilize ordered domains. The decreased ability of the cholesterol head group analogs to stabilize ordered domains was suggested to be a result of an unfavorable orientation or decreased depth in the bilayer [68]. An interesting example of large-scale effects of sterol head group modifications was presented by Bacia et al., who examined the effect of various sterol analogs, among them cholesteryl sulfate and cholesteryl sulfonate, on domain formation and bilayer curvature in giant unilamellar vesicles composed of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and *N*-stearoyl-*D*-erythro-sphingosylphosphorylcholine (SSM) [70]. In vesicles containing cholesterol, liquid-ordered domains showed positive curvature and spontaneously underwent fission which resulted in new vesicles outside the original one. In contrast, domains formed in the presence of cholesteryl sulfate and cholesteryl sulfonate gave rise to the budding of vesicles to the inside of the original vesicle. The findings of Bacia et al. suggest a mechanism for bilayer modulation which might be found to be relevant also for the cholesteryl glycosides.

4.2. Interactions between carbohydrate head groups

The glycosylation of cholesterol radically increases the size of the polar head group and the number of functional groups capable of participating in hydrogen bond formation. Hydrogen bonding between the carbohydrate residues of neighboring glycolipids has been speculated to stabilize the intermolecular interactions, but there is no clear evidence of hydrogen bonding taking place (discussed in [71]). The results obtained in this study showed that the cholesteryl glycosides destabilized rather than stabilized bilayers rich in PSM. A similar destabilizing effect of the cholesteryl glycosides were seen in bilayers in which half of the PSM was replaced by PGalCer. This indicates that the increase in the number of potential hydrogen bond donating and accepting groups in the head group region brought about by PGalCer incorporation was not of great importance for the ability of the cholesteryl glycosides to affect the bilayers analyzed in this study.

The average molecular area and molecular packing elasticity of glycolipids in monolayers have been found to vary depending on the hydrated bulk volume and average orientation of the polar head groups [72,73]. Increasing the head group size generally would be expected to increase the elasticity and limit the ability of the molecules to approach each other within the monolayer. However, for example the neutral glycosphingolipid lactosylceramide has been shown to exhibit tight lateral packing, which has been contributed to specific lactose–lactose interactions stabilizing the monolayer [74]. When the sterol glycosides included in this study were spread at the air/water interface, the stability of the resulting monolayer was

markedly increased as compared with a cholesterol monolayer. This stabilization could result from hydrogen bonding between sugar moieties in neighboring cholesteryl glycoside molecules and between the carbohydrate head groups and surrounding water molecules. The introduction of only a few mol% of cholesterol in a cholesteryl glycoside monolayer resulted in a considerable destabilization of the cholesteryl glycoside monolayer, as seen from the decrease in collapse pressure. The monolayer data show that cholesterol was fairly miscible in bulk cholesteryl glycoside, and also that cholesterol cooperatively interfered with the relatively weak interfacial stabilizing forces of the mixed monolayer.

4.3. Conclusions

We have observed that cholesteryl glycoside and cholesteryl galactoside clearly differ from cholesterol in their effect on glycerophospholipid and sphingolipid bilayers. It is noteworthy that the effect of the cholesteryl glycosides on the hydrocarbon chain order resembled the effect of cholesterol more closely in bilayers composed of POPC than in bilayers composed of PSM. We hypothesize that this is a result of the *cis*-unsaturation in the *sn*-2 chain of POPC, which increases the cross sectional area of the hydrophobic portion of the phospholipid and decreases the lateral packing density of the bilayer. A POPC bilayer is thus likely to allow for the incorporation of sterols having large polar head groups more readily compared to a tightly packed PSM bilayer. In phase separated bilayers containing both POPC- and PSM-rich domains, the cholesteryl glycosides showed a stronger tendency, compared to cholesterol, to affect the more disordered POPC-rich domains. These findings indicate that the cholesteryl glycosides, when compared to cholesterol, partition more efficiently into lateral domains rich in phospholipids having disordered hydrocarbon chains. Stress-induced glycosylation of cell membrane cholesterol can consequently be expected to result in a reorganization of sterols as well as other membrane components, and thereby possibly have an effect on the cellular signal transduction machinery.

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