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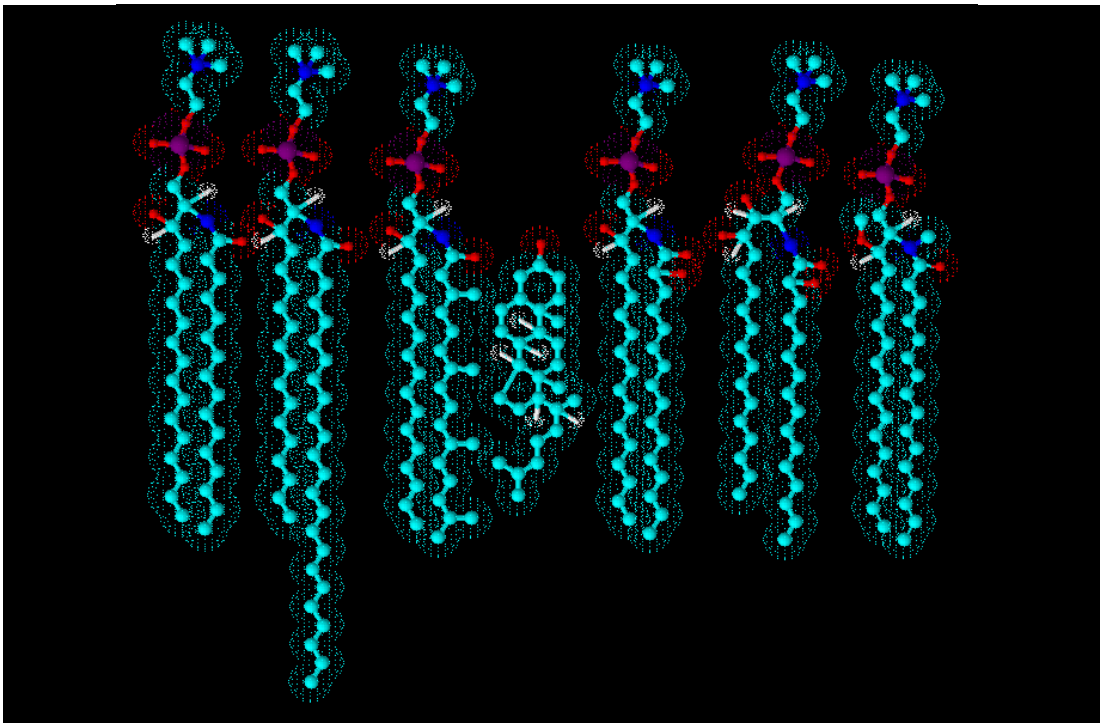

Effects of methylation and hydroxylation of sphingomyelins on their biophysical properties and interactions with cholesterol

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To my family

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Original Publications

This thesis is based on the following original publications which are referred in the text by Roman numeral (I-V).

I. **Effect of hydrophobic mismatch and interdigitation on sterol/sphingomyelin interaction in ternary bilayer membranes.** Shishir Jaikishan and J Peter Slotte. *Biochim Biophys Acta* 1808: 1940-5 (2011).

II. **Sphingomyelin analogues with differently branched *N*-acyl chains: the position of branching dramatically affects acyl chain order and sterol interaction in bilayer membranes.** Shishir Jaikishan, Anders Björkbom and J Peter Slotte. *Biochim Biophys Acta.*1798: 1987-94 (2010).

III. **Membrane bilayer properties of sphingomyelins with amide-linked 2- or 3-hydroxylated fatty acids.** Oscar Ekholm, Shishir Jaikishan, Max Lönnfors, Thomas KM Nyholm and J Peter Slotte**.** *Biochim Biophys Acta.*1808: 727-32. (2011)

IV. **Stabilization of sphingomyelin interactions by interfacial hydroxyls - a study of phytosphingomyelin properties.** Shishir Jaikishan and J. Peter Slotte. *Biochim Biophys Acta.* (*in press*)

V. **N- and O-methylation of sphingomyelin markedly affects its membrane properties and interactions with cholesterol.** Anders Björkbom, Tomasz Róg, Pasi Kankaanpää, Daniel Lindroos, Karol Kaszuba, Mayuko Kurita, Shou Yamaguchi, Tetsuya Yamamoto, Shishir Jaikishan, Lassi Paavolainen, Joacim Päivärinn, Thomas K.M. Nyholm, Shigeo Katsumura, Ilpo Vattulainen, J. Peter Slotte. *Biochim Biophys Acta.* 1808: 1179-86. (2011)

Contributions of the Author

The author contributions to the publications included in the thesis are as follows:

I. Participated in designing the study together with the supervisor. Synthesized the sphingolipid analogs and performed all the experiments. Contributed to the writing of the manuscript together with the supervisor.

II. Participated in designing the study together with the supervisor. Synthesized the sphingolipids analogs and performed all the experiments. Contributed to the writing of the manuscript together with the supervisor.

III. Synthesized the sphingolipid analogs along with the supervisor. Performed the experiments together with Oscar Ekholm.

IV. Participated in designing the study together with the supervisor. Synthesized the sphingolipid analogs and performed all the experiments. Contributed to the writing of the manuscript together with the supervisor.

V. Anders Björkbom participated in designing the study together with the supervisor. Sphingolipid analogs were synthesized by the research group of Professor Shigeo Katsumura. Performed part of the experiment and contributed to the writing of the manuscript together with Anders Björkbom and the supervisor.

Additional publication not included in the thesis

Phosphatidyl alcohols: effect of head group size on domain forming properties and interactions with sterols.

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Shishir Jaikishan
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Abbreviations

10MeSM	*N*-(10-methyl)hexadecanoyl-D-*erythro*-sphingosylphosphorylcholine
14:0 SM	*N*-tetradecanoyl-D-*erythro*-sphingosylphosphorylcholine
15:0 SM	*N*-pentadecanoyl-D-*erythro*-sphingosylphosphorylcholine
15MeSM	*N*-(15-methyl)heptadecanoyl-D-*erythro*-sphingosylphosphorylcholine
16:0 SM	*N*-hexadecanoyl-D-*erythro*-sphingosylphosphorylcholine, PSM
16MeSM	*N*-(16-methyl)heptadecanoyl-D-*erythro*-sphingosylphosphorylcholine
17:0 SM	*N*-heptadecanoyl-D-*erythro*-sphingosylphosphorylcholine
18:0 SM	*N*-octadecanoyl-D-*erythro*-sphingosylphosphorylcholine, SSM
19:0 SM	*N*-nonadecanoyl-D-*erythro*-sphingosylphosphorylcholine
2-OH-22:0 SM	*N*-(2-OH)-docosanoyl-D-*erythro*-sphingosylphosphorylcholine
2-OH-PSM	*N*-(2-OH)-palmitoyl-D-*erythro*-sphingosylphosphorylcholine
20:0 SM	*N*-eicosanoyl-D-*erythro*-sphingosylphosphorylcholine
22:0 SM	*N*-docosanoyl-D-*erythro*-sphingosylphosphorylcholine
24:0 SM	*N*-tetracosanoyl-D-*erythro*-sphingosylphosphorylcholine
3-OH-PSM	*N*-(3-OH)-palmitoyl-D-*erythro*-sphingosylphosphorylcholine
7SLPC	1-palmitoyl-2-(7-Doxyl)stearoyl-*sn*-glycero-3-phosphocholine
CTL	cholesta-5,7,9(11)-trien-3-β-ol, cholestatrienol
DPH	1,6-diphenyl-1,3,5-hexatriene
DSC	differential scanning calorimetry
l_d	liquid disordered phase
l_o	liquid ordered phase
L_α	liquid crystalline phase

$L_{\beta'}$	gel phase
MD	molecular dynamics
NMe-PSM	*N*(methyl)-palmitoyl-D-*erythro*-sphingosylphosphorylcholine
NMeOMe-PSM	3-hydroxymethyl-*N*(methyl)-palmitoyl-D-*erythro*-sphingosylphosphorylcholine
OMe-PSM	3-Hydroxymethyl-*N*-palmitoyl-D-*erythro*-sphingosylphosphorylcholine
PC	phosphatidylcholine
Phyto-2-OH(*R*)-PSM	*N*-[(2*R*)-Hydroxy]-palmitoyl-D-*ribo*-phytosphingosine phosphorylcholine
Phyto-2-OH(*S*)-PSM	*N*-[(2*S*)-Hydroxy]-palmitoyl-D-*ribo*-phytosphingosine phosphorylcholine
PhytoPSM	*N*-hexadecanoyl-D-*ribo*-phytosphingosine phosphorylcholine
PhytSM	*N*-Phytanoyl-D-*erythro*-sphingosylphosphorylcholine
POPC	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine
$P_{\beta'}$	ripple phase
SM	sphingomyelin
T_m	gel-liquid crystalline phase transition temperature

Summary

This thesis reports on the membrane properties of novel sphingomyelin (SM) analogs and their interactions with sterols. The study focuses on the biophysical properties of SM analogs in model membrane systems.

SMs are considered as major mammalian sphingolipids found mainly in the outer leaflet of plasma membranes and have many important biophysical and biological functions. SMs along with cholesterol and proteins are one of the important components of lipid rafts which are believed to play important roles in signaling. This study discusses in detail the effects of structural modifications (for example hydroxylations and methylations) in SM on their lateral distribution and interactions with cholesterol in lipid bilayers. The study was performed mostly with three component bilayer systems comprising unsaturated phosphatidylcholine, saturated SM analogs and cholesterol, mimicking the outer leaflet of biological membrane.

This thesis work shows that structural modifications in SMs affect their biophysical properties and interactions with cholesterol. The SMs were modified in several different ways. We measured the effects of *N*-linked acyl chains lengths on membrane properties of natural SMs (having the following saturated chains: 14:0-20:0, 22:0 and 24:0). The gel-liquid crystalline phase transition temperature (T_m) of SM analogs increased linearly with increasing chain length. The shorter acyl chain SM analogs showed a great increase in T_m for carbon number <16 followed by a small increase in T_m with the increasing chain length. SMs with long acyl chains induced hydrophobic chain mismatch and possibly interdigitation in SM bilayers. All short and long chain SMs formed sterol-enriched domains. Cholesterol favored interactions with 16:0 SM (both at 23°C and 37°C) over other chain length SM analogs. In another study we measured effects of methylations (branching) in the N-linked acyl chains of SMs. Methylations in the *N*-linked acyl chain of SM appeared to fluidize SM bilayers. T_m of *N*-hexadecanoyl-D-*erythro*-sphingosylphosphorylcholine (PSM) was reported to be 41.5 ºC, comparatively higher than distally branched SM analogs (methyl branching at carbon 15 and 16 of 17:0 acyl chains). SM species- phytanoyl SM (PhytSM, methyl at carbons 3, 7, 11 and 15) and *N*-(10-methyl)hexadecanoyl-D-*erythro*-sphingosylphosphorylcholine (10MePSM, methyl branching at carbon 10) did not show gel-liquid transition above 10ºC. Distally branched SM analogs formed ordered sterol-enriched domains in 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) bilayer whereas 10MePSM and PhytSM did not form sterol-enriched domains above 10ºC. Our results indicated that acyl chain branching of SM led to bilayers more fluid than those composed of unbranched SM and also weakened SM/sterol interactions due to interfered intermolecular interactions.

Further, we examined bilayer properties and interactions with cholesterol of *N*-acyl hydroxylated (2 or 3-OH) 16:0 or 22:0 SMs as compared to chain matched nonhydroxylated

SMs. Hydroxylation of acyl chain directly affected SM bilayer properties and interactions with cholesterol. 2OH-PSM bilayers showed higher T_m (~ +5-10 °C) than PSM, while 3OH-PSM bilayers had lower T_m (~ -5° C). 2OH-PSMs formed more stable sterol-enriched domains in model bilayers as compared to nonhydroxylated SMs, whereas 3OH-PSM gave decreased domain stability. The sterol affinity for hydroxylated SM bilayers was reduced compared to nonhydroxylated SM.

Another study reports on the effects of hydroxylation in the long-chain base of SM on SM membrane properties and interaction with cholesterol. The gel phase of palmitoyl phytosphingomyelin (phytoPSM, additional hydroxyl at carbon 4 in the long-chain base) bilayer was stabilized (T_m, 48ºC) compared to that of PSM. PhytoPSM analogs with *N*-linked 2-OH with *R* or *S* configurations have lower and higher T_m than phytoPSM respectively. Phyto-2-OH(*S*)-PSM bilayers showed higher T_m (~ +7°C) than phytoPSM, while phyto-2-OH(*R*)-PSM bilayers had lower T_m (~ -7°C), equivalent to that of PSM. All phytoPSM analogs formed sterol-enriched domains in a fluid POPC bilayer.

The effects of methylation in the interfacial region of PSM were examined where 2-NH was methylated to NMe, 3-OH was methylated to OMe, or both were methylated simultaneously. Methylation of the 2-NH had the largest destabilizing effect on the gel phase (T_m was lowered by 7ºC as compared to PSM). Atomistic simulation study showed that the methylated SM bilayers were more expanded but thinner as compared to PSM bilayers. 3-OH methylation dramatically attenuated hydrogen bonding via 2-NH group whereas 2-NH methylation did not similarly affect hydrogen bonding via the 3-OH group. Thus the 3-OH methylation effectively reduced ability to form sterol-enriched domains in fluid POPC bilayer whereas the 2-NMe formed less stable domains as compared to PSM. This study showed that the interfacial properties of SMs are very important for interlipid interactions and the formation of laterally ordered domains in complex bilayers.

Review of the Literature

Chapter 1

1.1 Introduction

Lipids have played a crucial role in structuring possibilities for life by providing compartmentalization of cell components. Possibly, simple fatty acids were the first biomolecules which could have self-assembled to form membranes [1]. Polar molecules like amino acids or ions might have been trapped into such assemblies [2]. These assemblies possibly were abiotic but could perhaps replicate due to thermodynamic instability. Many million years of evolutionary processes could have led to the first biotic cells. With the complexities in the functions of cells, cell components also evolved to survive competitively in harsh environments. Present cells – prokaryotes as well as eukaryotes – are much diversified and far superior in their structure and functions as compared to earliest possible cells.

Cell membranes are one of the most evolved and complex components of cells. Cell membranes play important roles in maintaining cell compartmentalization as well as in helping to perform vital cellular functions like cell-cell communications, cell division, signal-transduction, apoptosis, cellular fusions and many more. Membranes are highly organized and functional cell component containing thousands of lipid species and membrane proteins. Constituents of cell membrane serve many biophysical functions as well, like membrane curvature, membrane rigidity, bending, phase behavior, surface area and thermodynamics of the cell [3,4]. Each lipid molecule has unique biophysical properties and can have many cellular functions apart from being the structural unit of cell membranes. Membranes rapidly change their constituent lipids on the basis of functional requirements of the cell. The lipid variations are not only restricted to the cell type but are also manifested in the cell organelles, bilayer leaflets and the lateral plane of the membranes.

Phospholipids, proteins and sterols constitute the major bulk of the cell membranes. All mammalian plasma membranes contain glycerophospho-lipids, sphingolipids and sterols as vital lipid constituents [5]. SMs are key constituents of an important class of lipids - sphingolipids - which have many specific roles in cell membranes. Biologically, sphingolipids contribute to important aspects of cell signaling, and thus are important modulators of cell survival, growth and migration affecting *e.g.* cancer progression [6] The main focus of this thesis is to determine the effects of structural modifications, in

particular methylation and hydroxylation of SMs on their biophysical properties and interlipid interactions in bilayer membranes. The work in this thesis has been performed with the aid of model membranes and will help in understanding the role of SMs in cell membranes.

1.2 Biological membranes

The plasma membrane (thickness ~4 nm) is an integral part of prokaryotic as well as eukaryotic cells. The plasma membrane and other cellular membranes are highly dynamic semifluid assemblies of lipids and proteins that separate functional compartments. Membranes maintain integrity of cells while allowing the flow of matter, energy and information through lipid bilayer or protein channels aided by configuration changes in proteins [7]. Phospholipids, main building blocks of the membranes, are amphiphilic molecules arranged in bilayers with polar groups exposed to the aqueous environment and the hydrophobic part is formed by the acyl chains of phospholipids. In 1972, Singer and Nicholson's classical fluid mosaic model of the biological membrane described the cell membrane as two-dimensional structures in which proteins are dissolved homogenously in viscous phospholipid bilayers [8]k. Their representation of the cell membrane was much refined from previous postulations but was significantly oversimplified. Proteins do not randomly distribute in lipid bilayer as the free diffusion of protein are restricted in membrane [9].

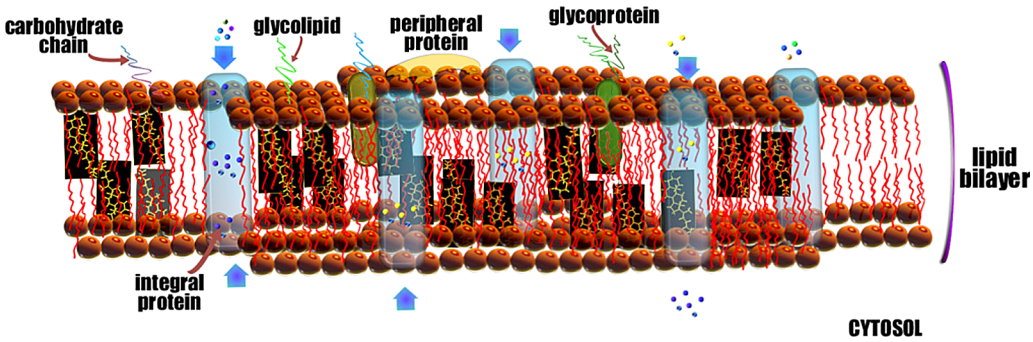


Figure 1. Schematic representation of cell membrane showing heterogeneity in structure

In 1997, Simons and Ikonen presented a new aspect of cell membrane structure based on the dynamic clustering of sphingolipids and cholesterol termed as 'lipid rafts' within fluid lipid bilayer [10]. Such clusters rich in SM and cholesterol may form spontaneously along with proteins leading to heterogeneity in membrane structure. There are many examples of protein activity in membranes which is assisted by the neighboring lipid matrix for example cardiolipin controlled mitochondrial ADP exchange, and cholesterol assisted Na^+/K^+-ATPase activity [11,12]. Membrane proteins are tightly bound to membrane lipids in bilayer by hydrophobic forces or ionic interactions and functions in

systematic manner [13]. In addition biological membranes are asymmetric in their lipid distributions across the bilayer [14,15].

In terms of composition, structure and dynamic complexities, our current understanding of biological membranes is much more diverse. Cell membranes should be considered as lipid-protein mixtures rather than as solution of proteins in a fluid lipid matrix. Both proteins and lipids are essential components of membranes. The lipid and protein composites in membranes are heterogeneously distributed forming lateral domains which have specific functions such as cell adhesion, cell signaling. Peptidoglycans, transmembrane proteins, and lipid-anchored proteins are also integral part of the cell membranes. Transmembrane proteins have hydrophobic membrane-spanning domains and the hydrophilic domain which is exposed in cytoplasm and extracellular environment. A hydrophobic domain of transmembrane proteins anchors within lipid bilayer whereas the hydrophilic domain interacts with molecules in polar environment [16]. Lipid anchored proteins such as GPI-linked proteins attach covalently to the cell membrane through single or multiple lipid molecules [17]. The cell membrane helps in providing shape to the cell by anchoring the cytoskeleton which lies beneath the cell membrane in the cytoplasm.

1.3 Structure of Lipids

In every cellular membrane, there are possibly thousands of structurally different lipid species [18]. Presence of a couple of hundreds of different lipid molecules per cell is very likely [19]. In mammalian cells, lipid constituents of the membranes mainly comprise glycerophospholipids, sphingolipids and sterols, which further varies in terms of net charge, head group and the chains (acyl, alkyl, alkenyl or long chain base). Important glycerophospholipids include phosphatidylcholine (PC), phosphatidylethan-olamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), and phosphotidylglycerol (PG). In mammalian cells, PCs, SMs and glycosphingolipids (GSLs) are abundant in the exoplasmic leaflet whereas charged PE, PI and PS are found mainly in the inner leaflet of the cell membrane [20-22]. Distribution of these lipids in the bilayer leaflets depends mainly on their chemical structures or physical properties like charge [23]. The distribution of lipids across the leaflet is also mediated by enzymes (such as flippases, translocases and scramblases) and is subject to change in response to external stimuli [24].

1.3.1 Sphingolipids

Sphingolipids are the second most abundant class of lipids containing a backbone of sphingoid bases. Sphingolipids are functionally important lipid molecules found in the outer leaflet of cell membranes. GSLs, SMs, ceramides, ceramide-1-phosphates, and sphingosine-1-phosphates are most common sphingolipids. The most typical example of sphingolipids present in mammalian cells is SM. Thudicum coined the

term ‘sphingomyelin’ derived from Greek word ‘sphinx’ for the enigmatic molecule he isolated from brain tissue in the late 19th century [25]. In 1927, its structure was proposed as N-acyl-sphingosine-1-phosphorylcholine [26]. In 1947, detailed structure of long chain base was characterized by Carter as 2*S*,3*R*,4*E*-2-aminooctadec-4-ene-1,3-diol [27] who also proposed the term “sphingolipides” for ‘lipids derived from sphingosine’.

Sphingolipids and glycerophospholipids differ structurally in their hydrophobic part: sphingolipids have a long-chain sphingoid base to which polar functional groups and an acyl chain are linked, whereas glycerophospholipids are built on L-glycerol. Sphingoid bases are structural units of sphingolipids which are long chain aliphatic amino alcohols with a variable chain length [28]. Amongst sphingolipid backbones there exists more than sixty structural variations in animals, plants or microorganisms [29].

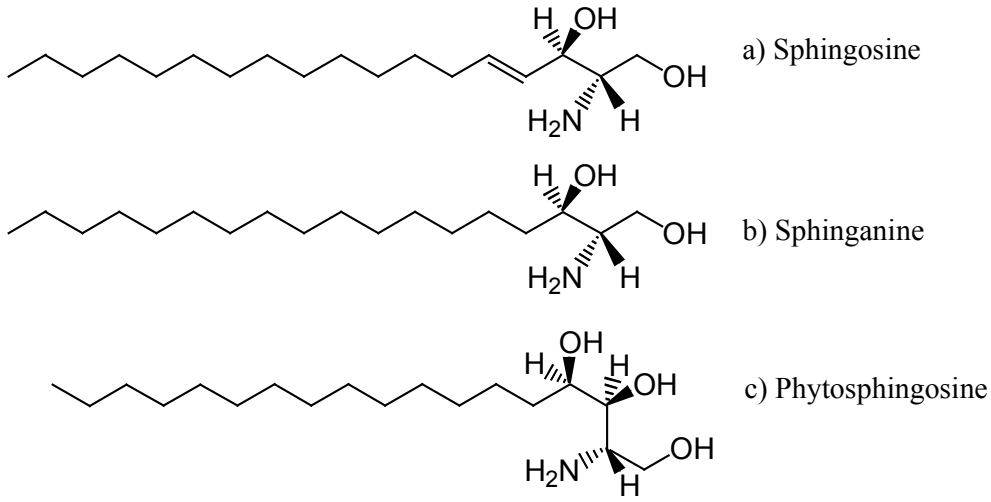


Figure 2. Chemical structures of important sphingoid bases: a) sphingosine, b) sphinganine, and c) phytosphingosine

The most common sphingoid base is sphingosine ((2S,3R)-2-amino-4-octadecene-1,3-diol), an unsaturated (trans-double bond between C4 and C5) amino alcohol with chain length of 18 carbons (Fig. 2). Sphingosines with varying chain lengths (C16:1, C20:1) are also present in substantial amount in plasma sphingolipids or brain gangliosides [30]. Mammalian kidney cells as well as protozoans also contain odd chain length variants of sphingosine due to branched alkyl chains [31,32]. Sphinganine or dihydrosphingosine ((2S,3R)-2-amino-4-octadecane-1,3-diol), another long chain sphingoid base is a saturated dihydroxy derivative of a sphingosine (Fig. 2). Sphinganine, reported to be less abundant in animal cells, is functionally an important constituent of yeast and plant cells. Another important sphingoid base is 4-hydroxy sphinganine or phytosphingosine ((2*S*,3*S*,4*R*)-2-amino-1,3,4 octadecanetriol) which has an additional hydroxyl group in the sphingoid base (Fig. 2). Originally isolated

from plants [33], phytosphingosine has been reported to be found in wide range of cells including mammalian kidney cells [34].

Structural variation of sphingolipids in biological membranes is partly based on different sphingoid bases. Complexity in sphingolipids may arise due to addition of functional groups to sphingoid base at C1 forming hydrophilic head group or at amine N forming hydrophobic acyl chains. The most common chemical moieties bonded to long chain base backbones in headgroup region include phosphate, phosphorylcholine or various carbohydrates. Further variations are possible in *N*-linked acyl chain with respect to degree and position of unsaturated bonds, substitution of hydroxyl group or methyl group etc. (one at a time or combined). Important examples of sphingolipids are shown in the Fig. 3.

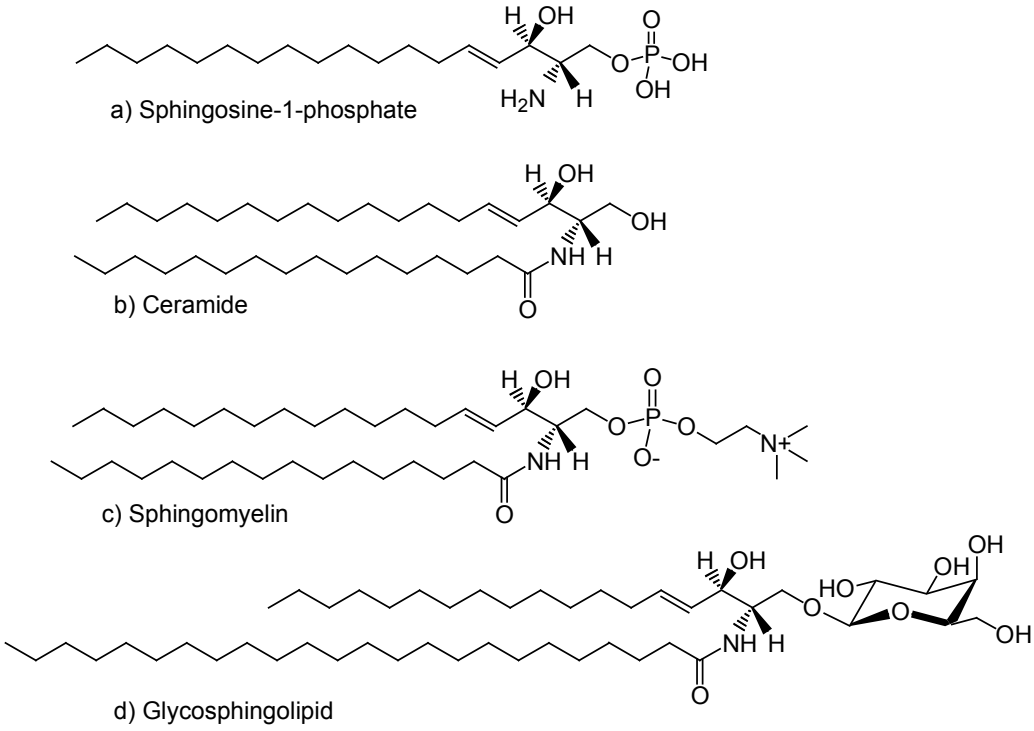


Figure 3. Chemical structures of complex sphingolipids: a) sphingosine-1-phosphate b) ceramides, c) sphingomyelins d) glycosphingolipids

a) **Sphingosine-1-phosphate**

Sphingosine-1-phosphate is a simple but an important sphingolipid metabolite. The evolutionary conserved functions of sphingosine-1-phosphate have been shown in yeast, plants and animals. It is formed in cells as a result of phosphorylation of sphingosine by two sphingosine kinase isoenzymes. Sphingosine-1-phosphate functions are mainly mediated by its binding to a family of five specific G protein-coupled receptors located on the surface of the cell [35]. Sphingosine-1-phosphate is a signaling molecule which plays

important role in cellular division, cancer, angiogenesis and immunity among others. Sphingosine-1-phosphate stimulates the growth factor and a series of signaling cascades. Sphingosine-1-phosphate enhances tumor growth by stimulation of cell proliferation, metastasis and cell survival. Consequently, reducing levels of sphingosine-1-phosphate may have anti-tumor role.

b) Ceramide

Ceramides, which comprise the hydrophobic part of complex sphingolipids, have an N-linked acyl chain attached to the long-chain base. Ceramides are formed by *de novo* synthesis, by degradation of complex sphingolipids (for example SM) or by acylation of sphingoid long-chain bases in response to specific stimuli like physiological stress. Long-chain bases in ceramide include more than 50 molecular species and mainly exist with double bond (ceramide), without double bond (dihydroceramide) or with a 4'-hydroxy sphingoid base (phytoceramide). Further these ceramide species can have 2-hydroxyl acyl chains, for example 2-hydroxy (α-hydroxy ceramide), 2,3-dihydroxy ceramide (mainly in plants) or hydroxyl groups in both sphingoid base and in *N*-linked acyl chains (α-hydroxy-phytoceramide). Ceramides have important roles in cell signaling, proliferation, differentiation, senescence and apoptosis [36]. In particular, the role of ceramides in apoptosis has received special attention. *N*-linked acyl chains of ceramide play important role in deciding biological as well as biophysical functions of ceramide. It has been shown recently that the balance between C16:0 and C24:0/C24:1 ceramides is important for induction of apoptosis in different cell lines [37]. C16:0 ceramide has pro-apoptotic effects and C24:0/C24:1 ceramides protective effects [37]. Similarly, C16:0 ceramide seems to play crucial role in apoptosis in non-neuronal tissues, while C18:0 ceramide has growth-limiting properties. Ceramides with specific acyl chain lengths are generated in different signaling pathways and any deregulation in chain length can cause severe diseases [38,39]. Ceramides are known to form lateral 'raft' like ceramide-enriched domains in chain-length specific manner due to self-aggregation or interaction with other sphingolipids, for example SM. Ceramides are also known to have an important biophysical property: the ability to replace cholesterol from SM-cholesterol domains at certain compositions [40].

c) Sphingomyelins

SMs are a ubiquitous component of eukaryotic cells. SMs, sphingolipid analog of PCs, consist of ceramide as the hydrophobic part with a polar headgroup, phosphocholine attached to C1. Sphingosine (C18) is the main long-chain base constituent of SMs along with sphinganine (most abundant in human lens membrane) and C20 homologs. Branching with odd numbered carbon chain in sphingosine base of SM was reported for simple eukaryotes, fungi and subsequently for bovine and murine kidney cells [34]. Natural SMs have mostly long saturated *N*-linked acyl chains, generally from 16 to 24 carbons [41-44]. Unsaturation in *N*-linked acyl chains of SMs are less common and if present, double bonds are located distally from the membrane/water interface [41].

SMs with 16:0 or 18:0 are more common in peripheral cell membranes, whereas SMs with 24:0 and 24:1$^{cis\Delta15}$ are enriched in myelin sheath of neural tissues. SMs of testes and spermatozoa contain very long chain polyunsaturated *N*-linked acyl chains (for example 28:4, 30:5) [45]. 2-hydroxy substitution in SM though rare, is present in testes and spermatozoa [46] and in kidney and intestinal mucosa [47]. The chain mismatch in the hydrophobic region of SMs is frequent as the long acyl chains extends beyond the length of the long chain base (sphingosine corresponds to an sn-1 acyl chain of about 13.3 carbons [48]).

d) **Glycosphingolipids**

GSLs are important constituents of central nervous system, especially in myelin where the amount of GSL can reach more than one half of total lipid content [49,50]. GSLs are formed by first attaching a single carbohydrate moiety on ceramide C1 (for example, galactosylceramide, and glucosylceramide). In more complex GSL, additional carbohydrates and acidic functional groups are incorporated into the polar head group. Number and nature of carbohydrate groups determine the class of GSLs, the most common ones being cerebrosides (monoglycosyl), gangliosides and globosides (neutral GSLs). The most common cerebrosides are glucosylceramide and galactosylceramide which constitute ~20% of the total lipids in myelin. Galactosylceramide is present abundantly in brain with long chain fatty acids (C22:0-C24:0) [51]. Gangliosides are negatively charged GSLs formed due to presence of one or more molecules of sialic acid linked to sugar residues of ceramide oligosaccharides. GSLs are enriched in the external leaflet of biological membranes [52]. Gangliosides present in myelin have lignoceric acid (24:0) as the most common fatty acid, to an astonishing amount of 70% of the total fatty acids [53]. The fatty acid composition of gangliosides present in human brain is mostly C18:0 (~80%) which remains unaltered throughout life [54].

1.3.2 Glycerophospholipids

GPLs are the largest phospholipid classes which compose the bulk of the membrane. Glycerophospholipids differ significantly from sphingolipids in terms of their hydrophobic backbone. The backbone of GPLs is glycerol to which two fatty acids are attached with ester or ether linkages at position *sn*-1 and *sn*-2 while different sets of possible headgroups are attached at *sn*-3 positions [55]. Phosphatidic acids (PA) are the simplest GPLs with a small phosphate group at *sn*-3 position. As the polar headgroup the phosphocholine, phosphoethanolamine, phosphoserine, phosphoinositol, are phosphoglycerol, are common. PCs are the most abundant phospholipids in biological membranes reaching roughly one half of the total. The acyl chains of PCs (mainly 16 to 18 carbons) are on average shorter than those of the SMs (16 to 24 carbons) and have a high degree of unsaturation when compared with the acyl chains of SMs. In addition to the chain length, the acyl chains may vary in branching, presence of cyclic chains [56,57]

or degree of unsaturation. The *sn*-1 acyl chains of PCs are commonly saturated while the acyl chains linked to sn-2 are often monounsaturated with a *cis* double bond (positioned in the center of acyl chain) or polyunsaturated. The most common unsaturated acyl chains found in mammalian cells are 18:1, 18:2, 20:4 and 22:6. It is interesting that most of the fatty acids in GPLs (and possibly in all lipid classes) are even numbered as they are synthesized from acetyl-CoA primer by sequentially adding two carbon units. GPLs have one noticeable property which helps membrane to interact with embedded proteins. Many of the GPL species are negatively charged (PA, PG, PI, PS) while PCs and PEs are zwitter-ionic in nature.

1.3.3 Sterols

Sterols are one of the most fascinating lipid species in biological membranes. Sterol composition differs in plant and animal cells. Cholesterol (animal cells), ergosterol (yeast cells), stigmasterol and sitosterol (plant cells) are the most common sterols in biological membranes. These sterols differ in structure either in their fused ring or in their aliphatic chain. Cholesterol is the most common sterol in mammalian cells.

The structure of cholesterol was determined in 1932 by Wieland [58]. Cholesterol (cholest-5-en-3β-ol) structure includes three functional components: a polar 3β-hydroxyl group, a rigid steroid (tetra cyclic fused ring with a double bond between C5 and C6) skeleton and a short iso-octyl chain attached at C17 (Fig. 4). The sterol ring of cholesterol is composed of three six member ring and one five member ring fused to each other in an all *trans* configuration. The fused ring structure of cholesterol is planar and rigid while the side chain exhibits a higher degree of conformational freedom [59,60]. Two methyl substituents are attached at position C10 and C13 and orients to the same side of the ring where side chain and hydroxyl group lies [61]. Due to this the cholesterol molecule is asymmetric, one side being flat (α-side) and the other side being more coarse (β-side) [62]. All these features allow cholesterol to interact with phospholipids in unique ways.

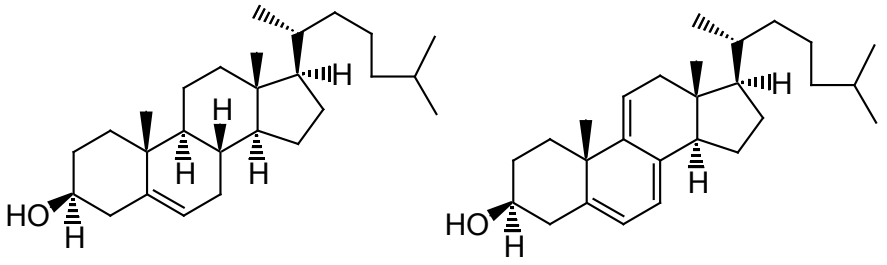


Figure 4. Cholesterol and its fluorescent analog CTL used in this study

Inclusion of cholesterol in membranes raises hydrophobic barriers for small polar molecules and reduces the permeability for non-polar molecules. Cholesterol present in the animal cells may amount to 20-50% of total membrane lipids. Mondal and

coworkers have shown that in CHO cells sterols reside mainly in the cytoplasmic leaflet of the plasma membrane and in the endocytic recycling compartments [63]. Membrane cholesterol exists either in stoichiometric complexes with other membrane lipids or free as active monomers [64]. Sphingolipids and cholesterol are found in unusually high levels in human lens membranes. Cholesterol/phospholipid ratios can vary from 1:1 to 2:1 in the cortex to even 3:1 to 4:1 in the nucleus of human lenses [65]. As much as of 35% of the total cell cholesterol is allocated in the plasma [66,67]. Cholesterol level in the membrane is always in homeostasis via active membrane cholesterol (the amount which is in excess than required by lipid bilayer) [68,69]. Increase in plasma membrane cholesterol beyond the threshold activates several feedback responses. Cholesterol may act as a substrate for its own esterification and for the synthesis of regulatory side-chain oxysterols [70]. Excess cholesterol has an elevated chemical activity and redistribute to plasma lipoproteins and intracellular organelles [71,72]. The excess of cholesterol forms pure cholesterol crystallite in membrane.

1.4 Lipid-lipid interactions

Phospholipid molecules aggregate and form bilayers in aqueous solutions to minimize their effect on the entropy level of water molecules at the phase boundary. Lipid-lipid interactions include lateral interaction within the layers or cross interaction between leaflets of bilayers. Such interaction in membrane arises mainly due to van der Waal interactions, hydrogen bonding and ionic interactions. Lateral interaction includes a short-range van der Waals attraction or hydrogen bonding [73] which helps in mutual adhesion or a repulsive hydration force between adjacent bilayers. Computational studies have shown that van der Waal interactions between saturated acyl chains are stronger than acyl chain/cholesterol interactions [62]. Lipid-lipid interactions lead to physical phenomena like cooperativity and interdigitation. Transfer of signal across the bilayers in gel state happens by interdigitations which means that long acyl chains of the lipids penetrates into the opposing leaflet [74]. It is possible that such an interaction can induce co-localizations in the opposite monolayer if the pairing effect is strong enough [75,76]. Two types of chain packing arrangements are possible for the asymmetric chain length lipids - partial interdigitation and full (mixed) interdigitation [77,78]. Interdigitation in SM bilayers has been prevalently shown earlier [79-81]. SM molecules have long chains and a relatively higher chain inequality so they are prone to form interdigitations. 24:0 SM can adopt two types of interdigitated gel- phases (*partial* and *mixed*) [81]. Cholesterol has been shown to decrease the interleaflet coupling instead of enhancing it, as shown in DOPC/cholesterol bilayers at 33 mol% of cholesterol [82]. This result is in contrast to the raft hypothesis where it was suggested that coupling between bilayer leaflets might occur due to cholesterol ordering effect on sphingolipids in the outer leaflets and glycerolipids in the cytoplasmic leaflet [83,84]. Biological implications of interdigitation are not well understood but it may regulate the function of proteins through membrane thickness.

1.5 Phase formations in membranes

Hydrated phospholipids show polymorphism depending on molecular structure and concentration. The most common arrangement of phospholipids is that of lamellar bilayer which forms the basic structure of biological membrane. Non-lamellar phases are equally important for biological processes involving membrane fusion, fission or vesicular transport [85] where normal lamellar topology of membrane is transiently disrupted. Phase behavior of lipids provides essential information on thermodynamic properties of membrane. All these aspects justify the detailed investigation of phase transitions in hydrated lipid systems.

1.5.1 Critical packing parameter

Physical dimensions like critical length of the hydrocarbon chain (*l*), area of the headgroup (*a*) and hydrocarbon volume (*V*) of an individual lipid outline the phase behavior of hydrated lipid bilayer. An intrinsic quantity, critical packing parameter (given by $S = V/al$) includes all these three quantities and defines the various aggregate forms of a lipid or properties of the membrane (Fig. 5).

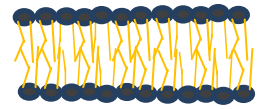
Phospholipids	Structures	Geometries	Representations
PE (S > 1)			
Lyso SM, lyso PC (0.33 < *S* < 0.5)			
SM, PC (0.50 < *S* ≤1)			

Figure 5. Phases of lipid: formation of non-lamellar and lamellar phases of lipids depends on their molecular geometry described by $S = V/al$, where '*S*' is critical packing parameter, '*V*' is hydrocarbon volume, '*a*' is the area of the headgroup and '*l*' is hydrocarbon chain length.

A lipid will form a spherical micelle if the value of $S \leq 0.33$. A non-spherical (globular or cylindrical) micelle shape is possible for the $0.33 < S \leq 0.5$. Planar bilayers will form if the *S* lies in between 0.5 to 1 and reverse micelle (inverted hexagonal phase) for $S > 1$. Single-chain phospholipids like lysoSM have $0.33 < S < 0.5$ and hence do not

form stable bilayers but micelles. SMs and PCs are rod shaped molecules with *S* value close to 1 and hence form stable bilayers. It is to be noted that the chain lengths in SM and PC must be greater than 8 carbons to allow bilayer formation. If the acyl chains of these lipids are shorter than 8 carbons, the term *a/l* is too high and hence S will have the lower value to allow bilayer formation. Cholesterol (hydrocarbon area, 400Å^3 and hydrophobic length, 17.25 Å [86]) will stabilize lipids which form hexagonal (H_{II}) phases. Unsaturated PEs (*a/l* is low) will have $S > 1$ and hence they will form hexagonal phases when hydrated. S is an additive property of a lipid and hence two lipids with different shapes (for example cone and inverted cone) can form a stable bilayer. For example, cholesterol and lysoSM can form a stable bilayer due to their complementary shapes and additivity of *S*.

1.5.2 Thermotropic transitions

The dimensions *V, l,* and *a* are extrinsic quantities so they will depend on temperature, pH, pressure or ionic strength, to name a few. Temperature plays important role in phase behavior of lipids or lipid cluster. At a single temperature (T_m) the maximum number of possible phases for a pure (single component) lipid system is two. Lipids exist in predominantly two phases in bilayers, gel phase or liquid crystalline phase but may show various phase behaviors depending on their chemical structure, temperature and composition [87]. With the increase in temperature, a series of different lipid phases can exist [88], but necessarily all phases may not appear for a single phospholipid [89]. Phases which are relevant for this study, are described below (Fig. 6) (for detailed discussions on phases, please refer to Cevc & Marsh [90] and Heimburg [3]).

Gel phase ($L_{\beta'}$)

In a gel or solid ordered phase, the acyl chains of bilayer lipids are arranged in mostly ordered all-*trans* configuration but tilted with respect to bilayer normal (represented by ‘ ’). Acyl chain ‘*tilt*’ is defined as a vector connecting the first and last atom of the chain. The tilt depends on headgroup structure. Saturated PCs may show a tilt of 30° whereas analogous PEs does not show a tilt deviating from the bilayer normal. In gel phase lipids are tightly packed with each other with less hydration as compared to fluid phase [91]. Such lattice like arrangement of acyl chains in the gel phase results in higher bilayer thickness as compared to other lipid phases. Upon chain melting, the hydrophobic thickness of gel phase is reduced due to a decrease in the *trans-gauche* ratio in the acyl chain [92]. Experimental studies have shown complex phase behavior for the SM bilayer with chain mismatch, for example four distinct gel phases have been reported for 18:0 SM [93].

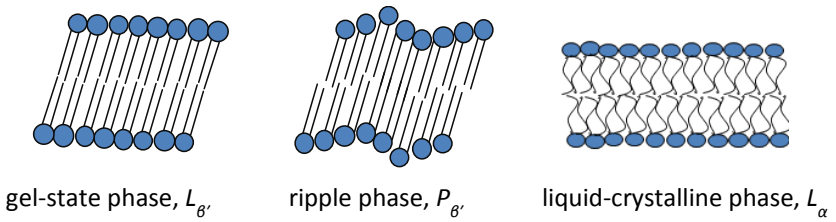


Figure 6. A general representation of phase transitions in lipid membranes in single plane. From left to right: the gel-state phase $L_{\beta'}$, the ripple phase $P_{\beta'}$ and the high-temperature fluid L_{α} phase. The ordering of the acyl chains decreases with temperature increasing from left to right.

Ripple phase ($P_{\beta'}$)

Upon heating, the gel phase forms ripple phase in which periodic arrangements of gel and fluid phase exists simultaneously [94]. $P_{\beta'}$ phase formation has been an interesting property of fully hydrated SM bilayers. Many theoretical models based on thermodynamics [95] or statistical mechanical approaches [96] have been proposed for the $P_{\beta'}$ phase formation. The interfacial area per lipid molecule is controlled by the cohesive hydrophobic interaction in the acyl chain region which tend to minimize the interfacial area and the repulsive headgroup interactions resulting in its expansion. Disorder in the hydrocarbon chains increases with temperature, which leads to enhanced hydration and lateral repulsion within the headgroups. As a result, there is an enhancement of water-hydrocarbon contact area, an effect strongly disfavored by the hydrophobic characteristics of the chains. A state of physical frustration develops as the compromised equilibrium area per molecule does not fully satisfy the packing preference of either headgroups or the hydrocarbon chains. This packing frustration leads to formation of $P_{\beta'}$ phase. Pure lipid has been found to form asymmetric ripple phases, whereas lipid mixtures adopt symmetric ripple phases. Pure hydrated bilayers of SMs or PCs form asymmetric ripple with sawtooth cross section having a wavelength of about 12-16 nm. Chain length of SM has been proved to be the driving force for the $P_{\beta'}$ phase formation. $P_{\beta'}$ phase formation in hydrated PC has been shown to depend on the relative acyl chain length and unsaturation [97]. Hydrated bilayers of combined and chain mismatched 14:0 SM and18:0-24:0 SM form $P_{\beta'}$ phases the physical properties of which (for example ripple periodicity) depends mainly on hydration level and rate of temperature change as well as duration of incubation period.

Liquid crystalline phase (L_{α})

The ripple phase is converted to a liquid crystalline or liquid disordered phase (l_d) upon further heating. In the L_{α} phase the lattice arrangement of lipid is completely lost and acyl chain becomes disordered due to an increased molecular motion. Acyl chains are further characterized by the loss of 'tilt' and *gauche* conformations are more frequent. In the L_{α} phase the cross sectional area of lipid molecules increases considerably at the expense of bilayer thickness. The hydration level at the interfacial level in fluid phase is higher than

in gel phase. Biological membranes are fluid at physiological temperature, which allows the diffusion of proteins or lipid molecules at a very high rate as compared to gel phase, making the membranes highly functional.

Liquid ordered phase (l_o)

Cholesterol has complex influence on the biophysical properties of the membrane bilayer due to its unique structure and possible high concentration in plasma membrane. A phospholipid bilayer which contained 22% cholesterol was reported to form cholesterol-poor and cholesterol-rich domains in which the physical properties of the cholesterol-poor domains resemble those of fluid phospholipid bilayer [92]. Cholesterol rich domains forms liquid ordered domains (l_o) inducing lateral heterogeneity in bilayers. NMR methods have shown that physical properties of l_o phase lies in between gel and fluid phase as acyl chain packing is tighter than in fluid phase and lateral mobility is higher than in gel phase [98]. Recently Mainali et al. showed that when the cholesterol content of a phospholipid bilayer is low, the properties of l_o phase coexisting with l_d or solid ordered phase lies between those of the l_d and solid ordered phases whereas at higher cholesterol content in the same bilayers, the properties of l_o phase aligns with solid ordered phase [99]. Zidar and coworkers also reported existence of two phases in cholesterol/SM bilayers [100]. Ordering of acyl chains in the presence of cholesterol or in l_o phase has been reported to be effective up to the carbon 9 whereas the high degree of disorder still prevails in the bilayer core [101]. However formation of l_o domains in membranes is essentially restricted to systems containing cholesterol and phospholipids with certain physical properties like high T_m and saturated acyl chains.

1.6 Biophysical properties of membrane components

Lipid-lipid interactions in lipid bilayer are largely determined by van der Waals interactions between neighboring lipids. Further, structural features in headgroup or interfacial region and acyl chains of the lipid distinctly define the biophysical properties of membrane for example T_m. This study focuses on observing how the chemical structures of the membrane components affect membrane properties as a whole.

1.6.1 Effect of headgroup structure on membrane properties

The phospholipid headgroup has important roles in determining the biophysical properties of the molecules. Headgroup structure, orientation, hydration, size and charge can significantly contribute to lipid-lipid and lipid-protein interactions. PC and SM have identical headgroups (zwitter- ionic phosphocholine) whereas PI, PE and PS have charged headgroups of different sizes and properties. The phosphocholine headgroup is highly hydrated and dynamically mobile [102]. Even though the headgroup charge is partially shielded due to hydration or counter ions, the partial charge is likely to cause lipid-lipid repulsion in the headgroup region [103,104]. When the number of methyl

groups in headgroup is decreased the polar head gets smaller and hydration in SM bilayers is reduced [105]. Different hydration pattern in headgroup region of SM leads to the formation of metastable gel phases as reported for 14:0 SM, 18:0 SM or 24:0 SM. Consequently these SM species shows complex gel-liquid phase transitions probably due to chain-mismatch.

Headgroup structures have an important effect on the T_m of phospholipids. Lipid species with identical acyl chains have different T_m due to different possible headgroup interactions. 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoserine (DPPS) have T_m of 41.7°C, 73.73°C and 55°C, respectively [106,107]. Similarly, T_m values of PSM and inositol phosphorylceramide having same acyl chain structure but different headgroup are 41°C and 48 °C due to higher number of bonded interactions in the inositol phosphorylceramide [108]. Hydrogen bonding in the headgroup and the ceramide interface region of GSLs increases favorable intermolecular interactions. Chain matched pure bilayers of palmitoyl galactosylceramide, palmitoyl glucosylceramide, palmitoyl lactosylceramide and palmitoyl galactosylceramide sultphate (GSLs with different sugar headgroups) have T_m of 85°C, 87°C, 78°C and 49°C respectively [109-112]. Degree of headgroup methylation has also noticeable effect on the T_m of lipid bilayer. Björkbom et al. have shown that corresponding decrease in the number of methyl group in SM bilayers increases T_m significantly [105].

Headgroup orientation is crucial for the formation of cholesterol-rich domains in biological as well as model bilayer membranes. Positively charged nitrogen in the choline moiety may make charge pairs with negatively charged oxygen atoms in the SM molecule (intra- or interlipid charge pairs). When compared with 16:0 SM, *N*-octadecanoyl-D-*erythro*-sphingosylphosphorylcholine (18:0 SM) bilayers have less frequent intramolecular charge pair as well as intermolecular H-bonds [113]. In lamellar bilayers, polar headgroups of phospholipids have been shown to orient approximately parallel to the bilayer plane [102,114]. These factors lead to an important increase in the ratio of headgroup size '*a*' to acyl chain critical length '*l*' giving an 'umbrella' shape to SM. The SM efficiently spaces cholesterol molecules in SM matrix and also inhibits unfavorable exposure of cholesterol to the hydrophilic environment. Additionally headgroup tilt in SM is more pronounced which makes SM-cholesterol charge pairing possible [62].

1.6.2 Effect of acyl chain structure on membrane properties

Each phospholipid class consists of a variety of molecular species due to different chain length, degree of unsaturation and number or nature of substitution. The van der Waals interactions between acyl chains depend on acyl chain length, functional groups (OH or Me) or double bond position or configuration (*cis* or *trans*) present in the acyl chain. Saturated acyl chains in SM or PC exists with high *trans/gauche* ratio asserting highly ordered packing of the lipids [3]. PSM and DPPC (chain matched) have similar gel-

liquid crystalline phase transition temperature of 41°C and 41.3°C with enthalpy values 6.8 Kcal/mol and 8.2 Kcal/mol respectively. The gel-liquid crystalline phase transition temperature of natural SMs shows nonlinear chain length dependency, whereas symmetric PCs have a fairly linear relationship between T_m and chain length [77,115]. T_m of pure 24:0 SM bilayers has been reported to be 48°C whereas symmetric di-24:0 PC and corresponding asymmetric 18:0/24:0 PC have T_m values of 80.3°C and 62.7°C respectively [116]. 14:0 SM, 18:0 SM and 24:0 SM show complex melting patterns with multiple phase changes. Positional isomerization (the acyl chains attached to *sn*-1 or *sn*-2 carbon in different order) also affects the physical properties of the lipid molecules. For example 16:0/18:0 PC have T_m value of 49°C, 5°C higher than 18:0/16:0 PC.

Acyl chain order is an important parameter for determining membrane properties. Saturated acyl chains are highly ordered due to increased cohesive interactions. In saturated PC or SMs, high order is restricted to carbons close to interfacial region only [117,118]. NMR studies have shown that in DMPC bilayers the acyl chain order parameter is relatively constant for the first nine carbons and then gradually decreases to a minimum for the terminal carbons [101]. Substitutions like unsaturation, methylation, and hydroxylation in acyl chains have substantial effects on the order profile of acyl chains in bilayers. Unsaturated acyl chains (*i.e.* the double bonds) make bilayers to pack loosely than the membranes comprised of with saturated acyl chains [119]. In terms of thermodynamics unsaturation of acyl chains leads to bilayer instability of but at the same time, to keep the biological membrane fluid, it is an essential requirement for lipid species. T_m and enthalpy of a phospholipid depend critically on the number, position and *cis-trans* configuration of the double bonds in acyl chains. Symmetric di-18:$1^{\Delta 9t}$ PC has T_m value 12°C as compared to -18.3 °C for di-18:$1^{\Delta 9c}$ PC (T_m of di-18:0 PC is 54°C).

Substitutions or branches are fairly common in the fatty acyl chains in biota. For example, brain SM contains α-hydroxy groups in their acyl chain [120]. Methyl branching of fatty acids in phospholipids of bacteria is also common. The methyl branching can interfere with bilayer packing of sphingolipids as the protruding methyl group will hinder the van der Waal interactions among acyl chains. Methyl branching lowers the T_m [121] and order in the acyl chain of PCs depending upon the position and number of the branches [122]. Hydroxylated SMs are less abundant but seem to be an important constituent of outer leaflet of myelin. In myelin 25% of fatty acyl chain moiety in galactosylceramide and sulphatide is hydroxylated at C2 position [120,123]. In contrast to branching effects, hydroxylation enhances the lipid-lipid interactions. 2-hydroxylated cerebrosides have been reported to have increased T_m as compared with nonhydroxylated chain matched species [124].

1.6.3 Effect of backbone/interface structure on membrane properties

In SM, sphingoid base forms the interfacial region and contains the nonpolar hydrocarbon chain as well. SMs have two hydrogen bond donor groups, amide and hydroxyl, in addition to single hydrogen bond accepting carbonyl group in the interfacial region. Functional

groups present at the interface or backbone of sphingolipids facilitate intramolecular and intermolecular interactions. Presence of 3-OH group and a 4,5-*trans* double bond in the interfacial region of SM is crucial for lateral interaction with neighboring lipid [41,125-127]. High T_m of ceramides and GSLs have been attributed to tight molecular packing in the interfacial region due to strong intermolecular interactions [128,129]. Presence of *cis* double bonds in SM acyl chains has smaller effect on the transition temperature than in chain matched PC [110]. This is possibly due to high hydration or hydrogen bonding in the interfacial region of SM bilayers [130,131].

SM restricts the transbilayer movement of galactosylceramide in POPC vesicles even though PC and SM have chemically identical polar head groups. Such difference in the biophysical properties of SMs and PCs might arise from difference in their backbone structure, which is also the basis of their classification. Mattjus et al. showed that galactosylceramide content which was 70% in inner leaflet of POPC vesicles decreased to 40% in POPC:SM (1:2) vesicles [132]. Geometric shapes alone cannot explain the transbilayer shift of galactosylceramides. SM and galactosylceramide interaction is preferred due to similarity in their interfacial structures. Interfacial structures, 3-OH, amide linkage and 4,5-trans double bond, were all required for the SM–galactosylceramides interactions. SM possibly formed intermolecular hydrogen bonding with galactosylceramide via bridged water molecules. In addition 4,5-*trans* double bond aided the intermolecular interactions. 3-deoxy PSM (3-OH group removed in SM) or dihydroPSM (saturated backbone in SM) completely abolished the change in transbilayer distribution of galactosylceramide induced by SM in POPC bilayer [133].

4,5-*trans* double bond in sphingoid base conserves order parameter in SM bilayer. When 4,5-*trans* double bond in SM was changed to 4,5-*cis* double bond, hydrogen bonding was considerably reduced in the interfacial region [134]. Disruption in the high ordering of 4,5-*trans* double bond in sphingosine base reduced the chain packing and hence bilayer thickness [134]. The lack of double bond in sphingoid base also increased the point of phase transition from the PSM value of 40.8°C to 46.9°C) [135].

1.7 Biophysical properties of Cholesterol

Cholesterol has diverse functional roles in eukaryotic membranes [61,125]. Considerable amount of work using various techniques has been done to understand lipid-cholesterol interactions. Cholesterol has been very selective in its interaction with membrane lipids. It is a well-known fact that cholesterol prefers to interact with a saturated PC or SM (high T_m lipids). Cholesterol has tendency to phase separate in presence of a high T_m and low T_m lipid (unsaturated) even though the two lipids do not phase separate in binary mixtures. Similar to unsaturated molecules like DOPC or POPC, cholesterol can phase separate with saturated but branched PCs or SMs [136]. Presence of 3β- hydroxyl group, a planar sterol ring system with two out of the plane protruding methyl groups, a double bond at Δ^5 and an iso-octyl chain are crucial for biophysical function and interactions

of cholesterol in membranes. Sterols with α-OH [62] or a ketone group [137] instead of β-OH lose their original cholesterol like properties. Sterol with ketone functional group (ketosterone) is found to be more tilted with respect to membrane normal than cholesterol. Ketosterone flip-flops faster across the membrane leaflet and promotes membrane fluidity [138]. The increased tilt angle and faster flip-flop ascertains the decrease in the polar interactions at the water-membrane interface. Similarly change in the structure in the iso-octyl chain [139] or the double bond position [140] dramatically changes the properties of cholesterol. Using cholesterol analogs, Leppimäki et al. demonstrated that the double bond position(s) (Δ^5 or Δ^7) of the sterol molecule failed to markedly stimulate [^{3}H] cholesteryl ester formation as well as unregulated PC synthesis in human skin fibroblast cells [140].

Most common sterol effect seen in fluid membranes is the 'ordering effect', which increases the membrane thickness due to higher *trans/gauche* ratio [141,142]. Degree of ordering depends on concentration of cholesterol as well as on the temperature of the system. Based on 1,6-diphenyl-1,3,5-hexatriene (DPH) anisotropy experiments Halling et al. showed that for the formation a uniform l_o phase a higher concentration of cholesterol is needed in POPC bilayers than in OSM bilayers [143]. Ordering effect of cholesterol is greatly reduced by the presence of unsaturation of acyl chains even though unsaturation alone has minor fluidizing effects on the membranes [144]. As calculated from NMR experiments, the ordering effect of cholesterol at the bilayer core is slightly more than at the interfacial region of the bilayer [101]. Cholesterol as compared to other sterols like desmosterol, ketosterol and 7-dehydrocholesterol has been shown to increase the packing and rigidity of acyl chains in SM and PC bilayers [62]. In the membranes of yeast and fungi the ordering effect of ergosterol is stronger than noticed in cholesterol-containing membranes, which is presumably due to higher stiffness of the ergosterol tail and tilt angle [145-147].

Cholesterol tilt, defined as angle between bilayer normal and the C3-C17 vector of cholesterol [148], is a major factor in ordering and condensing of SM bilayers. . The smaller the tilt angle, the higher will be the ordering effect and membrane thickness [149]. Cholesterol tilt is higher in unsaturated than in saturated SM bilayers [150]. Removing the two methyl groups from rigid ring protruding towards β-side remarkably increases the tilt angle from 20º to 25º [151] and hence increases the condensation effect of sterols in membrane bilayers.

Condensation effect is an extension of the interpretation of ordering effect of cholesterol in mixed lipid systems. It is defined as decrease in the surface area of a phospholipid molecule in presence of cholesterol as compared to pure phospholipid bilayer [61]. The membrane condensing effect of cholesterol depends on the chain length and the degree of unsaturation in bilayers [152,153]. Also the two protruding methyl groups on β-side of cholesterol are crucial as their removal has shown significant decrease in the condensation property of the cholesterol. The area of 16:0/16:0 PC and 14:0/14:0 PC in monolayer films decrease to about 0.41-0.42 nm^2 in presence of 50% cholesterol. In

fluid DMPC bilayers mixed with cholesterol at 50 mol%, area of 14:0/14:0 PC is close to that of gel phase of lipid bilayers of 0.42-0.47 nm^2 [154,155]. The area reduction per molecule in 18:0 SM monolayer in presence of 50 mol% cholesterol is of the order of 15-20%. However in *N*-2-OH- PSM monolayer, the cholesterol condensation effect was small due to 2-OH group of the N-linked chain which may interfere with van der Waals attractive interactions with cholesterol [156].

1.8 Phospholipid-cholesterol interactions

Interaction of cholesterol with phospholipids depends on various structural parameters of phospholipids including acyl chain length [129], degree of unsaturation in acyl chains [157,158], substitutions like branching or hydroxylation and headgroup variations [105,159,160]. Cholesterol has been shown to interact favorably with di-17:0PC due to maximum mean hydrophobic match of cholesterol with di-17:0PC bilayers. The direction and magnitude of change in cooperative phase transition temperature of PC and PG bilayers induced by cholesterol is dependent on hydrophobic chain length. For PE and PS bilayers, no hydrophobic mismatch-dependent effect of cholesterol on T_m is observed. For SM bilayers; no study has been performed so far. One of the major biophysical effects of cholesterol is elimination of cooperative gel-to crystalline phase transition of phospholipid bilayer at 50 mol% cholesterol. This property of cholesterol has been reported for various phospholipids including PC or PG [160] and SM as well [161].

Cholesterol induces fluid like macroscopic properties in a varied range of temperature [101]. Cholesterol, when present above 15% in phospholipid bilayer, is able to induce the liquid ordered phase which keeps acyl chains dynamic as compared with the gel phase [162]. A fluorescence correlation spectroscopy study showed that the molecular diffusion of lipids within l_o phase is intermediate to that of gel phase and l_d phase in raft-exhibiting model membranes [163,164]. At temperature lower than 30ºC, the liquid ordered domains of a raft system were larger in size and the lipid diffusion rates therein were 5 times slower than in the surrounding l_d phase. In cholesterol/egg SM (ESM) mixtures, at less than 33 mol% cholesterol, l_o and l_d phases are distinguishable as reported by spin labeled probes [99]. When the cholesterol concentration is equal to or higher than 33 mol% in ESM bilayers a homogeneous phase is obtained. These results are in correlation with solid state ^2H NMR spectroscopic data for cholesterol/ESM and cholesterol/PSM bilayers as shown by Bartels and coworkers [165]. On the basis of their molecular simulation studies Mihailescu et al. showed that the acyl chains in pure DOPC bilayers were very dynamic and 20% of the terminal methyl groups of the acyl chains were in intimate contact with the headgroups [82]. However adding of 33 mol% of cholesterol into the DOPC bilayers (formed l_o phase) dramatically reduced such interactions between headgroup and terminal methyl group. Due to the ordering effect of cholesterol the interactions were not entirely eliminated. This study validated earlier NOESY NMR results which also suggested contact between acyl-chain methyl

and the polar headgroups. It can be stated that even slight change in the structure of PC or SM at the acyl chain, interfacial region or the headgroup have noticeable effect on the biophysical properties or interactions with cholesterol.

1.8.1 Phase separation in complex systems

Cholesterol has preferential interaction with high-T_m lipid in ternary system of a high-T_m lipid and low-T_m lipid leading to lateral heterogeneity in the bilayers. Cholesterol forms with high T_m lipids l_o domains, which float in fluid bilayers. Orädd and Lindblom stated that the driving force for the lateral phase separation into l_o and l_d domains is the low solubility of unsaturated lipid species like 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) in highly ordered phase [166]. A number of experimental studies have been performed especially in POPC, PSM and cholesterol system reporting the formation of cholesterol-rich domains [143,162,167-170]. Several different types of l_o domains can be formed even with only three components in the membrane [171]. It has been shown that different proteins and lipids will have different partitioning properties into different types of liquid ordered domains [172]. The biophysical properties of l_o domains depend on the structure and composition of the included components. Domain size can range from 2 nm to few microns in lipid only mixtures. The size of cholesterol rich domains in lipid-only mixtures of 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC)/ DOPC/POPC/cholesterol or SM/DOPC/ POPC/cholesterol can be controlled by varying compositions and temperature [87]. Heberle and coworkers studied the domain size of l_o phase in ternary mixtures of DSPC and cholesterol together with either POPC or SOPC and reported it to be in the order of 2-8 nm, much smaller than the limit of optical resolution [173]. The diffusion rate of lipids depends on the packing order in a bilayer. The diffusion coefficient in DOPC/SM/water is slightly lower than in DOPC/water due to increase in the packing order in presence of SM. Diffusion coefficient of DOPC in DOPC/cholesterol is reportedly 3-5 times higher than in SM/ cholesterol systems [174,175]. The mean lateral diffusion coefficient of PSM is consistently 1.9 fold less than DPPC despite their similar phase properties in the temperature range 45ºC - 60ºC [176].

Partition coefficient also gives a good measure of diffusion as rate of diffusion is directly proportional to the partition coefficient. Partition coefficient for distribution of cholesterol between the methyl-β-cyclodextrin and POPC/PSM or POPC/DPPC bilayers was calculated to be 1.96 [143]. In ternary lipid mixtures of DOPC/PSM/ cholesterol, two diffusion coefficients is observed due to existence of two-phase regions of l_o and l_d enriched in SM/ cholesterol and DOPC/ cholesterol respectively [174]. Using pulse-field gradient NMR, diffusion in DOPC/SM/cholesterol was carried out with SMs from egg, brain and milk. The results showed that the ability to form domains differ among three SM due to difference in their acyl chain compositions [177]. Besides specific interactions between SM and cholesterol lateral packing (density) in the bilayer is also an important criterion for the cholesterol rich domain formation. The formation of cholesterol rich

domains in a bilayer containing egg SM (mainly 16:0 SM) was more extensive than in brain SM (mostly 18:0 SM) [177]. In the same study, cholesterol containing domains were found to be absent in bilayers containing milk SM.

1.8.2 Favored SM:cholesterol interaction

Cholesterol has proteins and many lipid species like PC, SM, PE, PS and so forth to interact with in the membranes. However propensity of cholesterol to interact with neighboring lipids varies and mainly depends on hydration, headgroup size and electrostatics, acyl chain composition as well as concentration of cholesterol in the bilayer membrane. PE bilayers are comparatively more tightly packed than PC (subject to similar acyl chain composition) because of less hydration in their headgroup [178]. PE headgroup also acts as hydrogen donor forming hydrogen bond with anionic phosphate oxygen of neighboring phospholipid [179]. Even though PC and SM have same headgroup structures, hydration in PC bilayers is higher than in SM bilayers, which makes SM bilayers pack more tightly [91].

The maximum threshold solubility of cholesterol (as molar ratio, cholesterol:X) for SM, PS, PC, and PE membranes has been reported to be 2:1 [180], ~2:1 [181], 1:1 [182] and 1:1 [183], respectively. Liu and Bitman has also shown that cholesterol has bilayer affinity in the order of SM>PS>PC>PE [184]. The cellular distribution of SM and sterols has also been found to be analogous [185]. SMs qualify best for cholesterol interactions because of the availability of hydrogen-bond acceptor and donor properties as well as likelihood of intra- and inter-molecular interactions. In raft like domains, egg SM and cholesterol form a stoichiometric molecular complex in a membrane [186]. Amide nitrogen in SM forms intermolecular hydrogen bonds with carbonyl oxygen of neighboring SM or other oxygen (for example cholesterol) with frequency of 0.41 [187] to 0.66 [100,113] H-bond per molecule. 3-OH group in SM acts as the main donor for intramolecular hydrogen bonds with the phosphate oxygen, and the number of H-bonding can go as high as 0.50 [113] to 1.08 [187] per SM molecule. The IR spectroscopy showed different amide bands for pure SM and SM-cholesterol bilayers [188] suggesting that the amide nitrogen in SM is important for SM-cholesterol interactions. Also when the oxygen linking phosphocholine and ceramide in SM molecule was replaced with S–atom, NH- or CH_2 group, the polarity and SM/cholesterol interaction were affected [189]. Cholesterol rich domains formed by such SM analogs were also found to lower thermostability as compared with chain matched SM in fluid POPC bilayers [189].

SM-cholesterol interactions can also be explained on the basis of two basic thermodynamic principles. At high local cholesterol concentrations, cholesterol molecules tend to move toward regions of low cholesterol concentrations, with a favorable gain in mixing entropy [100]. Above the maximum cholesterol concentration for lipid bilayers cholesterol precipitates as monohydrate crystal [183]. Alternatively cholesterol molecules are allocated to those regions of membrane where there is a possibility of hydrogen bond formation, a process with consequent gain in enthalpy [100]. Molecular simulation

work has also suggested that the headgroup orientations of SMs or PCs are different in bilayers. Headgroup of DPPC lies almost parallel to the bilayer whereas the orientation of headgroup (P-N orientation) in 16:0 SM is -15° towards the interior of bilayer and a significant number of SM molecules are oriented towards the aqueous phase [187]. This flexibility in the orientation of headgroup in the SMs has beneficial role in interaction with cholesterol. In POPC/SM/cholesterol system with dilute concentrations of SM and cholesterol, no H-bonding was reported but there were increased charge pair interactions between SM-cholesterol than PC-cholesterol. This is possibly due to flexible SM headgroup which can bend to form charge pairs with cholesterol molecules located deep in the membrane bilayer [190].

1.9 Membrane heterogeneity

Different lipid molecules, which have different structures and hence distinct physical and chemical properties, give rise to lateral heterogeneity. The different lipids tend to phase separate into clusters or domains. Lateral heterogeneity has been detected when mixing components of the complex biological membranes in simple artificial systems. Numerous model membrane studies have been implemented to understand biophysical properties of pure lipids or proteins and the lipid-lipid or lipid-protein interactions. The approaches include studies with monolayers, bilayers or liposomes, and computer simulations. Microscopic phase separation can be easily induced in such model systems with simple lipid mixtures at various compositions and temperatures [87]. Model membrane studies have shown that chain-length difference of four methylene group is sufficient to induce lateral demixing of lipids [191,192]. Recent experiments utilizing 'bleb' technique have revealed lateral heterogeneity in the complex plasma membrane. By treating the cells with formaldehyde and dithiothreitol, Baumgart et al. produced giant unilamellar vesicles from cell membrane which phase separated into l_o and l_d like domains below room temperature [193]. It was shown later that the phase separation in cell-derived giant plasma membrane vesicles was cholesterol and temperature dependent and mimicked simple model systems [194]. Presence of microdomains in artificial lipid membranes at low temperatures is a well-known fact, it is yet to be established if the formation of microdomains in model membranes and especially in cells is possible at physiologically relevant temperature [195,196].

1.9.1 The lipid raft hypothesis

The lipid rafts concept has changed the understanding of cell membranes. Lipid rafts are assumed to be submicroscopic dynamic assemblies of specific proteins and lipids and cholesterol in membranes, but still debatable [197]. Sphingolipids such as SMs and GSLs have major role in the formation of cholesterol-enriched domains in model and likely also in biological membranes. Lipid rafts were first reported in the Golgi complex of mammalian cells [198] but later they have been identified in yeast as well as plant

cells. Membrane rafts are defined as 10-200 nm highly dynamic, sterol- and sphingolipid-enriched domains heterogeneously distributed in a leaflet of cell membrane [199,200]. It has been proposed that the raft organization in membranes can be a spontaneous process driven by favorable intermolecular interactions including van der Waal interactions between saturated acyl chain and hydrogen bonding in the headgroups of neighboring lipids or proteins [84]. Rafts are considered to be present in exofacial leaflet of the membrane which contains sphingolipids with saturated acyl chains or big headgroups, for example SM and glycolipids. Theoretically rafts may also be present in the inner leaflet but such raft structures are not well defined [201]. Bakht et al. reported that even in absence of high-T_m lipids the cytofacial phospholipids of brain tissue, PE and PS have (borderline) tendency to form ordered domains with cholesterol [202].

Lipid rafts have major roles in many biological processes such as transmembrane signal transduction, membrane trafficking, protein sorting during endocytosis and exocytosis, apoptosis, cell adhesion, migration and synaptic transmission [83,203-207]. Membrane rafts may serve different function due to difference in their compositions. Many proteins like GPI-anchored proteins [208], Src family tyrosine kinases [209], cholesterol binding proteins like caveolins [210], phospholipid binding proteins such as annexins [211] and several types of ion channels [212] have been shown to partition into the rafts of cell membrane. Glycosynapses or glycosphingolipid signaling domains, membrane rafts not necessarily enriched with cholesterol, have been reported to be present in drug resistant cancer cells [213]. The glycosynapses are comparatively large and less dynamic domains important for tumor cell adhesions and signaling which is implemented through their extracellular carbohydrates [214,215]. Ganglioside and sulphatide can also be present in the microdomains with amounts 5 times those found in intact cells. Thrombin stimulated synthesis of phosphatidic acid and phosphatidylinositol 3,4,5-triphosphate can be found in cholesterol-enriched domains in human platelets [216].

Rafts are believed to be liquid ordered phases surrounded by l_d phase [217]. Rafts are insoluble in cold 1% Triton X-100, a non-ionic detergent [126,218]. Such detergent resistant membrane (DRM) fractions can contain as much as 32 mol% of cholesterol and 14 mol% of SM [218,219]. The DRM composition depends on the detergent used for extraction and the physicochemical conditions like temperature, detergent to membrane ratio, pH and ionic strength [220]. The solubilizing properties of different detergents are different for different classes of lipids and proteins. Dynamic properties of membrane rafts are also prone to change in the lipid or protein compositions after receptor stimuli [221]. The concept of lipid rafts has been controversial due to the submicroscopic size of the domains that cannot be resolved with present techniques. Conventional microscopes cannot distinguish membrane rafts in membranes due to their optical resolution limit, as given by Abbe's Law [222]. Recent advancements in techniques have tried to overcome such limitations. Stimulated Emission Depletion Microscopy (STED) and many other techniques are being used to get the insight into the raft properties like dynamics, size and composition and distribution of rafts in membranes.

Aims of the Study

Chapter 2

The main aim of the work summarized in this thesis was to find out how molecular details of SM influence their biophysical properties in membrane and assist in molecular interactions with cholesterol. The interactions of SMs with cholesterol were explored by inducing structural modifications in SM. The consequent alterations in the lateral organization of SM species and analogs along with cholesterol were studied in model bilayers.

More specifically, in **paper I** we examined the effect of varying chain length of SM on membrane phase behavior, sterol affinity and sterol-enriched domain formation. It is known that di17:0 PC has comparative preference for cholesterol amongst other symmetric saturated PCs due to its hydrophobic match with cholesterol. Similarly the effects of substituted methyl groups in the acyl chain were known for PCs but their effects of the substitutions in SM bilayers were not clearly understood. In **paper II** we studied the phase behavior, sterol affinity and domain forming properties of membranes containing SM analogs with different degree of methylation in the N-linked acyl chain.

2-hydroxylated sphingolipids are the major constituents of the myelin sheaths. We analyzed how hydroxylation contributed to the stability of SM containing model membranes and their interaction with cholesterol by virtue of the hydrogen bonding between the hydroxyl groups (**paper III**). Biophysical studies about effects of long chain base on SM membrane properties are few. Using phytoSMs, **in paper IV** we examined effects of an additional hydroxyl group in saturated long chain base of SM on membrane phase behavior and domain formation. 2-hydroxylated phytoSMs were also included in the study to examine the change in sterol affinity.

In **paper V**, we analyzed biophysical effects of methyl substitution in the interfacial region of SM on its domain forming properties. Using molecular dynamics (MD) simulations we analyzed how methyl substitution in the interfacial region hinders the formation of hydrogen bonds in SM bilayers.

Materials and Experiments

Chapter 3

Detailed description of material and methods can be found in respective articles (I-V). A very short description is given here for easy reference.

3.1 Chemicals (I-V)

Many of novel SM analogs 14:0-20:0 SM, 22:0 SM and 24:0 SM (**I**), 10MeSM, 15MeSM, 16MeSM and PhytSM (**II**), 2OH-PSM, 3OH-PSM and 2OH-22:0 SM (**III**), PhytoPSM, Phyto-2-OH(R)-PSM and Phyto-2-OH(S)-PSM (**IV**) were synthesized using protocols standardized in our laboratory [223,224]. N-Me PSM, O-Me PSM and N-Me-O-Me PSM (**V**) were synthesized by Professor Katsumura and group (Hyogo, Japan). c-Laurdan was kindly provided by Professor Bong Rae Cho (Department of Chemistry and Center for Electro- and Photo-responsive Molecules, Korea University, Seoul, Korea) and synthesized as described in [225]. Fluorescent probes CTL and quencher 7SLPC used throughout the study were synthesized in our laboratory [226]. PSM, used as reference molecule in all the study was purified from egg yolk SM. Precursors for lipid synthesis and lipids used in membrane studies without further modification were obtained mainly from Avanti Polar Lipids (Alabaster, Al, USA), Sigma-Aldrich (St Luois, MO, USA), Larodan Fine Chemicals (Malmö, Sweden), and Molecular Probes (Leiden, the Netherlands). Sources of all other chemicals and synthesis procedures are described in the original publications (I-IV).

3.2 Preparation of vesicles (I-V)

Lipid vesicles were used as model membranes to carry out the experiments in this thesis. When preparing the liposomes, a lipid concentration of 50 μM was used for fluorimetric studies and 1mM or 1.42 mM for differential scanning calorimetry (DSC) studies. For fluorescence spectroscopy and DSC measurements, required amounts of the lipids and/or probes were mixed and the solvents were evaporated under a constant flow of N_2 at 35˚C. The lipids were redissolved in chloroform to assure a homogeneous mixing of the lipids. Once the lipids were thoroughly mixed, the solvent was dried to yield a lipid film. After further drying under high vacuum for at least 3 hours at room temperature, the lipid mixtures were hydrated for 20 minutes at temperature above T_m of the lipid with the highest melting temperature in the sample. Vesicles for DSC studies were prepared by

bath sonication in a Bransonic 2510 (Branson Ultrasonics, Danbury, CT) bath sonicator for 15 min at T_m + 20˚C. For fluorescence measurements, multilamellar vesicles were prepared by probe sonication (sonicated for 2 min with 20 % duty cycle and 15 W power output) using a Branson probe sonifier (W-450, Branson Ultrasonics).

3.3 Differential Scanning Calorimetry

To study the thermotropic properties of pure SM analogs as well as various lipid mixtures, we performed DSC analysis of fully hydrated bilayers made from the SM analogs. DSC measurements were performed on a Calorimetry Sciences Cooperation Nano II DSC (Provo, UT). The software used for analysis of the DSC data was CpCalc (CSC, Provo, UT) and Origin 7 (Microcal, Northampton, MA). The lipid compositions studied were as stated in the articles. The sample cell of the calorimeter was filled with 0.4 ml of lipid suspension, whereas the reference cell was filled with PBS buffer. At least two consecutive heating and cooling scans from 0˚C to 80˚C were performed with a scan rate of 1˚C/ min. Thermograms reported in original articles are second heating scans.

3.4 Fluorescence Experiments

3.4.1 Fluorescence anisotropy

To study the bilayer packing properties and T_m of SM analogs, we measured the steady-state anisotropy of DPH as a function of temperature. Since DPH is located deep in the hydrophobic core, and also can partition into gel phases, the probe gives valuable information about phase transitions and acyl chain order [227]. The steady state fluorescence anisotropy of DPH was measured on a PTI Quanta-Master spectrofluorimeter (Photon Technology International, Lawrenceville, NJ) operating in the T-format. The wave lengths of excitation and emission of DPH were 360 nm and 430 nm, respectively. The steady state anisotropy was calculated as described in [228].

3.4.2 Fluorescence quenching

In order to follow the formation and melting of cholesterol-enriched or ordered domains, the steady-state quenching of CTL or DPH by the quencher 7SLPC was measured on a PTI Quanta-Master spectrofluorimeter operating in the L-format. The temperature was controlled by a Peltier element with a temperature probe immersed in the sample solution. The samples were heated from 10 ˚C to 70 ˚C at a rate of 5˚C/min. The measurements were done in quartz cuvettes with a light path length of 1 cm and the sample solutions were kept at a constant stirring (350 rpm) throughout the fluorescence measurement. Fluorescence intensity of CTL was detected with excitation and emission wavelengths at 324 nm and 374 nm and DPH was detected with excitation and emission wavelengths at 360 nm and 430 nm, respectively. The fluorescent probes were protected from light

during all the experimental steps. Fluorescence emission intensity was measured in the F-sample (quenched) consisting of POPC/7SLPC/SM analogs/cholesterol, (30:30:30:10, molar ratio) and in the F_0 sample (non-quenched), in which 7SLPC had been replaced with POPC. The fluorescence intensity in the F sample was divided by the fluorescence intensity of the F_0 sample giving the fraction of non-quenched CTL fluorescence plotted versus the temperature. CTL replaced 1 mol% of cholesterol and 1 mol% of DPH was added to the lipid solution.

3.4.3 Cholesterol partitioning between bilayers and cyclodextrin

The distribution of CTL between methyl-β-cyclodextrin (Sigma Chemicals, St. Louis, MO) and extruded large unilamellar phospholipid vesicles was determined as described by [229], a method significantly modified from the procedure reported by Niu and Litman [184]. The assay yields the molar fraction partition coefficient, K_x, for CTL. A high K_x indicates a higher affinity of CTL for the bilayer as compared with mβCD.

3.4.4 c-Laurdan emission spectra

The emission spectra of c-laurdan were determined in pure SM analog bilayers. The temperature for analysis was selected to be −5°C or +5°C from the T_m for each SM analog. The total lipid concentration in the assay was 50 μM and the c-laurdan concentration was 1 mol%. Excitation was at 365 nm and emission spectra were recorded between 390 and 550 nm.

3.5 Molecular simulations of lipids

In atomistic simulations, 100 ns long MD studies using the GROMACS package [230] were performed at 323 K (50 °C, which is above the main transition temperature of all individual lipid types included in the model system) on systems composed of pure SM analogs and SM analog/cholesterol molecules (8:2 molar ratio) parameterized with the OPLS force field [231] to study interactions in fluid bilayers at an atomistic level. OPLS stands for optimized potentials for liquid simulations. OPLS force fields represent a complete set of intermolecular potential functions developed for use in computer simulations of lipids or proteins in their native environment. The temperature was selected so that all lipids would be in the fluid phase state during the simulation, and so that comparisons could be made to previously performed simulations of analogous bilayer systems.

3.6 List of methods involved in papers I-V

Experiment	I	II	III	IV	V
DSC: pure component		x		x	
DSC: mixed component		x		x	
DPH Anisotropy: phase transition	x	x	x	x	x
DPH Anisotropy: cholesterol ordering		x			
CTL Quenching	x	x	x	x	x
DPH Quenching		x			x
c-Laurdan			x	x	x
CTL Partitioning	x	x	x	x	x
Molecular Dynamics					x
Confocal Microscopy					x

Results and Discussion

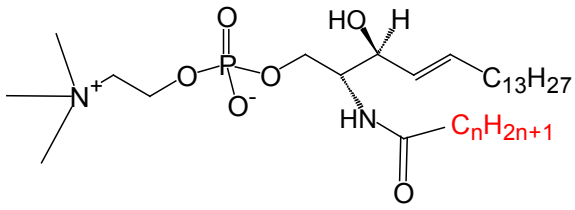
Chapter 4

The five articles included in this doctoral thesis present the results on the membrane properties of SM analogs and their interaction with sterol in model membranes. The main emphasis was to observe the biophysical effects of structural modifications in SM.

4.1 Effect of differing acyl-chain lengths on SM membrane properties

SMs with acyl chain lengths from 14 to 24 carbons are common in SMs from egg, brain or milk. The long and saturated nature of SM is expected to have significant effect on interactions with neighboring lipids, notably-sterols and also interactions with proteins are affected. The headgroup and interfacial structure will be of comparatively less significance as these are same in all SM with varying acyl chains. However, chain mismatch or interdigitation can play major role in deciding membrane properties of SMs. Structurally, it has been shown earlier that with the increase in the acyl chain length in SM, the average area per lipid slightly decreased and bilayer thickness increased for saturated as well as unsaturated acyl chains [80]. SM with differing N-acyl chain lengths have been observed to provide varying degrees of chemosensitization [232]. Nevertheless, systematic studies including the important biophysical functions of SM with differing acyl chain lengths have not yet been performed [233].

A monolayer study showed that cholesterol is able to interact with mismatched SM analogs [223]. The monolayers were composed of equimolar mixtures of SM and cholesterol, and cholesterol was enforced to interact with the only available SM analog. In complex bilayers, however, cholesterol can choose to interact with either mismatched SM analogs or for example PC. Thus, it is not astonishing at all that the present study employing bilayer systems provides results that contradict those obtained from monolayer studies. However we consider that bilayer studies should be considered more relevant than monolayer studies as the bilayers are structurally close to cell membranes.



n :	13	14	15	16	17	18	19	21	23
Compound :	14SM	15SM	16SM	17SM	18SM	19SM	20SM	22SM	24SM

Figure 7. Molecular structure of SM analogs used to study effect of differing acyl-chain lengths on SM membrane properties.

4.1.1 Gel phase stability of SM with differing acyl chains

Steady state anisotropy of DPH in SM analog bilayers with varying acyl chain length (Fig. 7) was measured as a function of temperature. Since DPH can partition deep into the gel phase of bilayer, valuable information about acyl chain order and phase transitions can be inferred [234]. The resulting data reported both the T_m and acyl chain order in the respective SM bilayers.

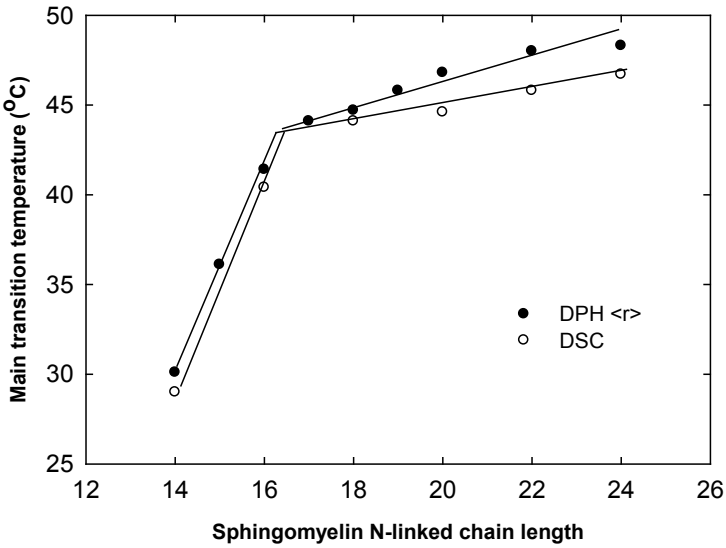


Figure 8. DPH- and DSC-reported gel-phase melting in pure SM analog bilayers. The T_m for the gel-phase melting in SM analog bilayers was determined from the DPH anisotropy curves (filled symbols). Corresponding T_m values determined by DSC are shown as open symbols (DSC data taken from Reference [235]). The figure is adapted from paper I with permission from Elsevier.

Acyl chain order obtained from the anisotropic measurements suggested that chain mismatch did not noticeably affect packing in the gel phase or in the fluid state (T_m+10°C) for all the SM analogs.

The T_m values obtained from anisotropy of DPH added into SM analog bilayers were similar to those reported previously by several groups [235]. A good correlation was found for the T_m values obtained with DPH anisotropy and DSC measurements (Fig. 8). Anisotropic measurements showed that the T_m values increase with the increase in chain length of SM analogs, but not linearly as they do in the case of symmetric saturated PC bilayers with increasing chain length [115]. SM analogs with short acyl chain (<16) showed a sharp increase of T_m per added carbon (Fig. 8) in contrast to the relatively mild increase in T_m of SM analogs with long acyl chains (>16). Such a disparate increase in T_m in asymmetric SM analogs was likely due to hydrophobic chain mismatch. A previous study has shown that the gel phase in SM bilayers with longer acyl chain is destabilized due to increased possibility of *gauche* configurations in the terminal part of the N-linked acyl chain which extends beyond the length of the long chain base [235]. Additionally the chain mismatch might result into partial interdigitation in non-tilted hexagonal-like packing [236,237] in which the longer acyl chain of the one lipid in one leaflet will pack with shorter sphingosine chain on another lipid in the opposing leaflet of the bilayer. X-ray diffraction study [238] and MD simulations [80] suggested the presence of interdigitation in at least 20:0 SM, 22:0 SM and 24:0 SM bilayers.

4.1.2 Formation of ordered domains in ternary bilayer systems

To study the interaction of SM analogs with varying acyl chains with sterol and hence the formation of sterol-enriched ordered domains, we measured fluorescence quenching susceptibility of CTL [239]. CTL is considered to be closest mimic of cholesterol [240]. CTL interacts favorably with SM analogs in ordered domains where its fluorescence will be protected against collision-induced quenching by 7SLPC, present mainly in the fluid phase rich in POPC. F(quenched)/F_0(unquenched) ratio as a function of temperature was plotted to observe CTL-enriched domains and their thermostability in the ternary bilayers. F/F_0 ratio provides the quenching susceptibility of CTL and reports how CTL is protected from the quenching by 7SLPC in bilayer. All SM analogs with differing acyl chain lengths were able to form CTL-enriched domains (Fig. 9A). We concluded that sterols are able to associate with long-chain SMs in bilayers irrespective of the hydrophobic mismatch between the cholesterol molecule and the long chains of SM. A previous study has also shown that cholesterol interacted with mismatched long-chain SM when present in equimolar concentrations in monolayers [223]. The present study included ternary bilayers where the cholesterol was free to interact with either POPC or the SM analog. Nevertheless interaction of cholesterol with SM analogs showed preferential interactions as deduced from $\Delta F/F_0$ value (Fig. 9B). The $\Delta F/F_0$ value depends on the amount of CTL in the domain in which CTL is protected from the quenching by 7SLPC. The lower value of $\Delta F/F_0$ due to increased solubility of 7SLPC is very unlikely in SM rich domains because partitioning of bulky doxyl group is excluded into gel-like phase of saturated long chain SMs. Thus, $\Delta F/F_0$ value reflects the amount of CTL and hence cholesterol in the ordered domains.

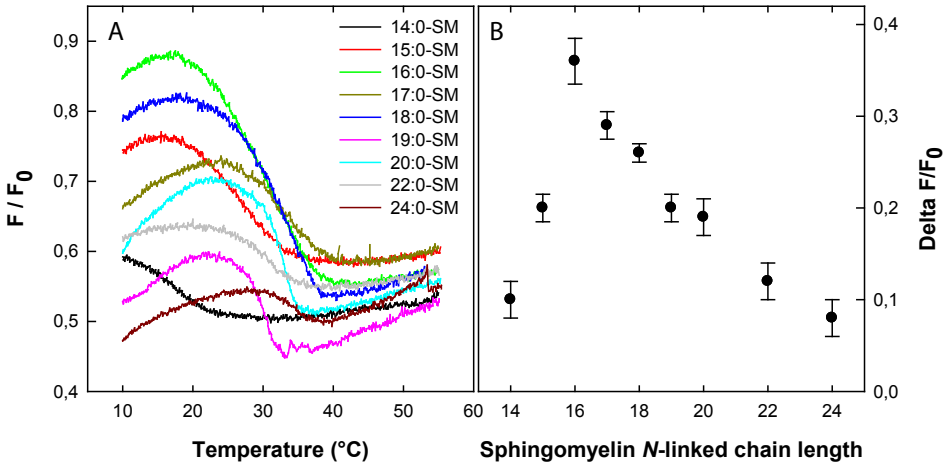


Figure 9. Melting of ordered domains observed from the quenching of CTL fluorescence. (A, left panel) The melting profile is shown as F(quenched)/F_0(unquenched) ratio plotted against temperature. The F-sample consisted of POPC/7SLPC/SM analog/cholesterol/CTL (30:30:30:9:1 mol%) and in the F_0-sample 7SLPC was replaced with POPC. (B, right panel) Delta F/F_0 is the difference in F/F_0 before and after melting of the ordered domains. The figure is adapted from paper I with permission from Elsevier.

The $\Delta F/F_0$ value was calculated to be highest with 16:0 SM and was lower for SM analogs with shorter or longer acyl chain. Long *N*-linked acyl chains for example 22:0 SM or 24:0 SM were less prone to include cholesterol into the ordered domains surrounded by fluid phospholipid bilayer.

Results from CTL partition assay were in line with those from the CTL quenching experiments, showing the highest affinity for 16:0 SM containing bilayers and decreased affinity for smaller as well longer acyl chain SMs. These data are consistent with previous DSC experiments which showed that for symmetric PCs with varying chain length cholesterol interacted preferentially with symmetric 17:0 PC having the best hydrophobic match [115]. Similarly, based on desorption rate from monolayers to cyclodextrin in the subphase, symmetric 14:0 PC was found to be the best match for androsterol (a sterol which lacks the iso-octyl side chain as in cholesterol) [241]. In addition, several previous studies have supported our interpretations of favorable molecular interactions on the basis of hydrophobic matching [242,243].

4.2 Effect of *N*-linked methylated acyl chain on SM membrane properties

Bovine and murine kidneys contain sphingolipids with hydroxylated or methyl branched long chain bases [34]. Methyl branching of PC acyl chains has been shown to make PC bilayers more fluid as compared to their non-branched analogs [244], an effect similar to that caused by the *cis*-double bonds in the chains.

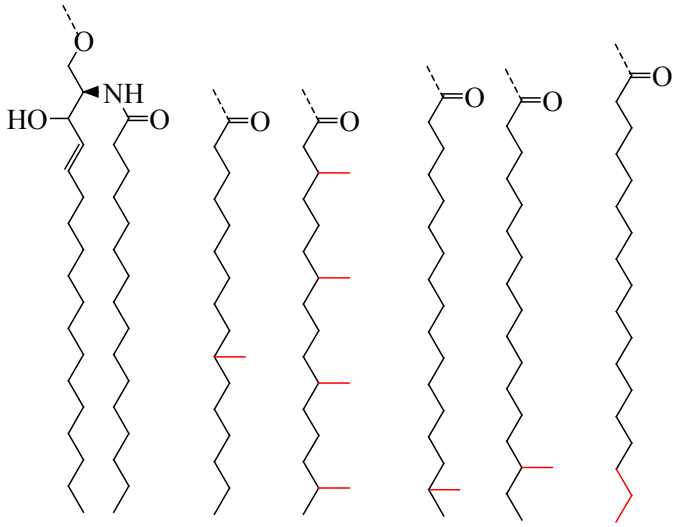


Figure 10. Molecular structure of SM analogs used to study the effects of branching in SMs on their bilayer properties. From left to right: PSM, 10MeSM, PhytSM, 16MeSM, 15MeSM, SSM.

Harderian glands (present in eyes) contain methylated lipid species instead of unsaturated ones but the reason to employ the branches instead of double bonds is not known. One possible explanation could be that methylation instead of *cis* double bond might be chemically less reactive modification of the lipid molecule. Sphingolipids have also been found to have methyl branching in their long chain base and in their N-linked acyl chains [34,245-248]. Methyl protrusions in sphingolipids might be useful for lipid-protein connections in membranes through spatial interactions. Nonetheless, effects of methyl branching on the membrane properties of SM bilayers have not been studied. We included PSM and SSM as the reference molecule for the reason that chain length of some of the methylated analogs matched to PSM whereas others have 18 carbon atoms in total in their acyl chain, equivalent to that of SSM (Fig. 10).

4.2.1 DPH anisotropy in pure methylated SM analog bilayers

Using anisotropy of DPH (Fig. 11) and DSC, the T_m values of the methylated SM analogs studied were found to be lower than the values of non-branched SMs. The T_m of SSM occurred at a slightly higher temperature (44ºC) than the T_m of PSM (41.5ºC) consistent with our other studies [249]. The lowering of the T_m was dependent on the position of branching in SMs. As compared to SSM, 16MeSM (*iso*-branching) bilayers showed gel-to-liquid phase transition at 36ºC whereas 15MeSM (*anteiso*-branching) showed melting at 28ºC, a notable decrease of 8ºC and 16ºC from SSM T_m values, respectively. Methyl branched PCs are also supposed to be more fluid than their unbranched analogs. DSC studies revealed that PC with two *anteiso* 17:0 chains had gel-to-liquid phase transition at 18.7 ºC [244]. These results suggest that even distal methyl groups in the acyl chain can hinder the lateral packing in the SM bilayers. Although, the effects of methylation in the long chain bases of SM have not been studied, one can expect similar effects

on lateral interaction and molecular packing. The T_m of 10MeSM and PhytSM were not observed through anisotropic measurements in the temperature window examined. NMR measurements also failed to report gel-fluid transition of symmetric phytanoyl PC bilayers, in a wide temperature range of -120ºC to 120ºC [250]. The biophysical effects of the branches are similar in nature to the *cis* double bonds of unsaturated SM as methyl protrusions showed effects comparable to a *cis* double bond induced at the site of branching [143].

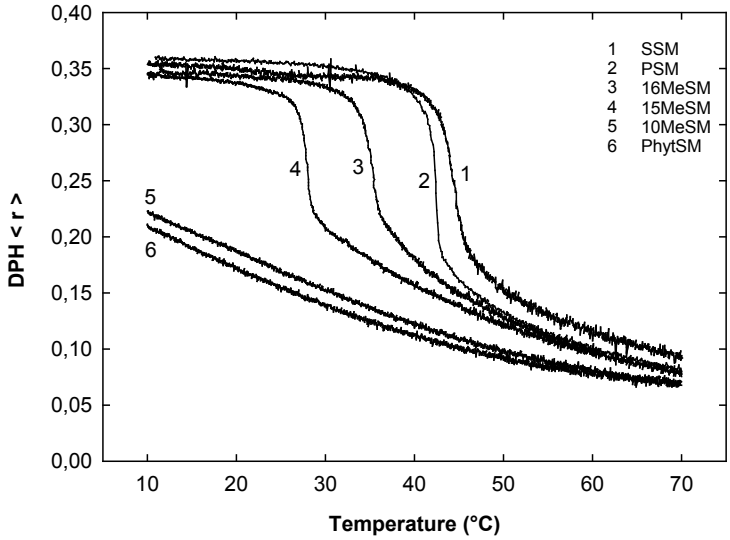


Figure 11. Steady-state anisotropy of DPH in pure SM bilayers as a function of temperature. Experiments were performed with multilamellar vesicles at a scan rate of 2 °C/min with a lipid concentration of 50 μM and 1 mol% of DPH. The figure is reprinted from paper II.

10MeSM and PhytSM bilayers showed a continuous decrease in the anisotropy value with the increase in temperature and in the fluid phase their acyl chain disorder was surprisingly similar. However DSC studies (data presented in the paper II) showed that methyl branching in SMs did not affect markedly the miscibility with PSM. It was concluded that miscibility with SM is determined mainly by hydrogen bonding properties and less by polar headgroup structure and linkages [189] or acyl chain branching.

4.2.2 Interactions of methylated SM analogs with cholesterol

The interaction of sterol with SM analogs in ternary bilayers including fluid POPC was determined by fluorescence quenching of CTL by 7SLPC. PSM and SSM were able to form sterol-enriched domains which melt between temperature ranges of 30-40ºC. Both 16MeSM and 15MeSM were able to form sterol-enriched domains, with 16MeSM domains end melting temperature similar to PSM and SSM. 15MeSM ordered domains melted at lower temperatures. When 10MeSM and PhytSM analog were inserted into fluid POPC bilayers, no CTL enriched-domains were formed in the temperature range studied.

Although methyl branching decreased the lateral packing and phase transition temperature, distally methylated SM analogs were able to form sterol enriched ordered domains (Fig. 12A). It has been shown that the normal position of cholesterol in bilayers allows it to interact mostly with the top 10 carbons of adjacent acyl chains [251]. For 10MeSM, which has a methyl group at position 10 and PhytSM, no ordered domains were reported because packing will be hindered greatly due to methyl protrusions present on cholesterol molecule as well as close to interfacial region of SM analog in the SM bilayers.

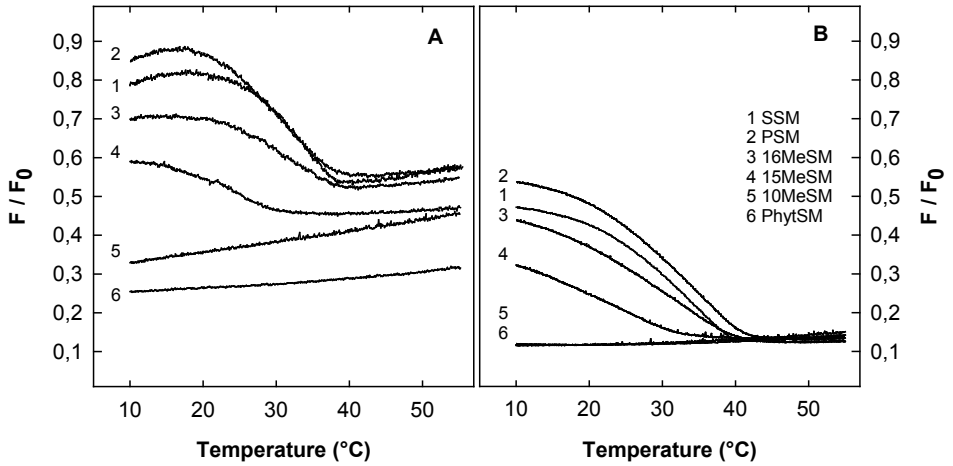


Figure 12. Melting of ordered domains observed from the fluorescence quenching of CTL (A) and DPH (B). The total lipid concentration was 50 μM with 1 mol% CTL or DPH and samples were heated at 5 °C/min. The melting profile is shown as the F(quenched)/F_0(non-quenched) ratio plotted versus temperature. The figure is adapted from paper II.

To study whether the methylated SM analogs formed ordered domains not necessarily containing sterol, we measured the quenching susceptibility of DPH in the model membranes (Fig. 12B). DPH has advantage over CTL since it partitions more equally between ordered and fluid phases without showing any special preference for SM. SSM ordered domains indicated by DPH were slightly less stable as compared with CTL, perhaps due to that SSM phase behavior is metastable in low temperature. In compliance to CTL quenching results, 10MeSM and PhytSM failed to report any ordered domains.

4.2.3 Sterol affinity for SM analog containing bilayers

The sterol affinity for SM analog containing bilayers was assessed at 23ºC and 37ºC by calculating the partition coefficient (K_x) of CTL between mβCD and bilayer vesicles composed of POPC/SM analogs (8:2, molar ratios). The higher value of K_x means higher sterol affinity for the bilayers. POPC or POPC/PSM (8:2, molar ratio) were used as a controls.

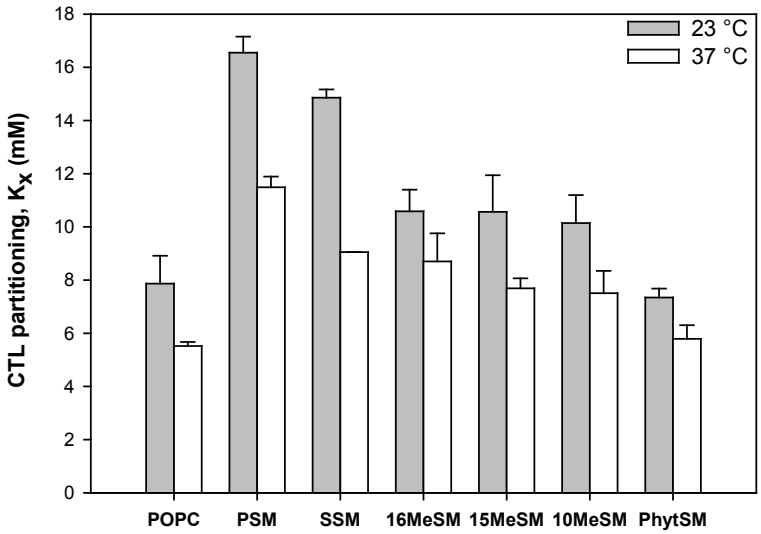


Figure 13. Partitioning of CTL into unilamellar bilayer vesicles containing either POPC or POPC:SM analogs (4:1 molar ratio) at 23°C (grey columns) and 37°C (unfilled columns). A high K_x indicates a higher affinity of CTL for the bilayer as compared with methyl-β-cyclodextrin. The figure is adapted from paper II with permission from Elsevier.

The sterol affinity for bilayers containing a mono methyl-branched SM analog was always lower than for PSM containing bilayers but higher than for POPC (Fig. 13). The K_x value was lowest for PhytSM containing bilayers suggesting that CTL interactions with PhytSM were similar to POPC. Evidently the sterol affinity for the fluid SM analog 10MeSM was not qualitatively very different from that of an unsaturated SM, oleoyl SM [143].

4.3 Membrane properties of SM with 2 or 3-hydroxylation in *N*-linked acyl chain

Sphingolipids containing 2-hydroxylated fatty acids are one of the most abundant lipids of myelin. The high content of hydroxylated fatty acids in myelin seems to have an important role in the formation or function of the myelin sheath [252]. Hydroxyl groups in interfacial region of the sphingolipids have been shown to stabilize the interlipid interactions in model membranes through hydrogen bonding between neighboring molecules or to interfacial water molecules [124,253]. In present study phase behavior of hydroxylated SM bilayers and sterol interactions were characterized. We synthesized 2-OH-PSM and 3-OH-PSM, having D-configuration in both analogs (Fig. 14).

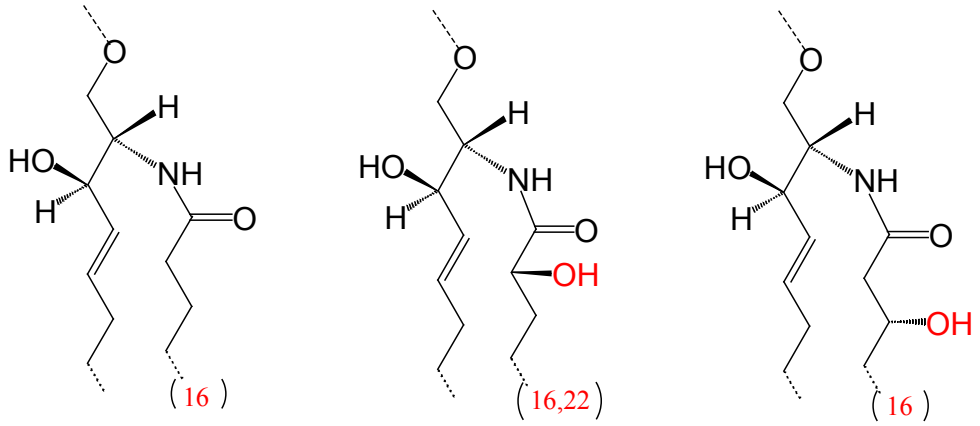


Figure 14. Structure of SM analogs used to study the effect of hydroxylations in *N*-linked acyl chain on SM membrane properties. The number in parenthesis shows the acyl chain length. Molecules include from left to right PSM, 2-OH-PSM and 2-OH-22SM, 3-OH PSM.

Pasher has shown for ceramides that the natural form 2-OH with D-configuration involves intermolecular hydrogen bonding whereas L-form 2-OH prefers intramolecular hydrogen bonding [254].Difference in their hydrogen bonding pattern enabled the separation of the two conformers on reverse-phase C18 HPLC column.

4.3.1 Gel phase stability and interfacial polarity of pure hydroxylated SM bilayers

2-Hydroxylation in sphingolipids has been reported to increase T_m as compared to their non hydroxylated chain-matched analogs [124,254]. We also observed that 2-hydroxylated SM analogs, 2-OH- PSM and 2-OH- 22:0 SM showed higher gel-to-fluid transition as compared to their chain matched non-hydroxylated SM analogs, with reported increase in T_m by 10°C and 5°C respectively (Fig. 15A). The lower increase in T_m for 2-OH-22SM is possibly due to chain mismatch and interdigitation effect, which diminished the stabilization induced by 2-OH group. Interestingly DPH anisotropy reported biphasic gel-to-liquid crystalline phase transition in 2-OH-PSM bilayers. In contrast to 2-OH-PSM, 3-OH-PSM bilayers were found to be less stable (T_m, 34°C) than PSM. Since in both analogs the hydroxyl group was in D configuration the position of the hydroxylation in SM relative to interface determined the stability of the SM bilayers.

Due to its polar nature in hydrophobic environment of acyl chains the 3-OH group might cause steric hindrance or intermolecular repulsion in the gel phase. The gel phase stabilization in 2-OH-PSM could arise from the increased possibilities of hydrogen bonding in neighboring molecules and with interfacial water molecules.

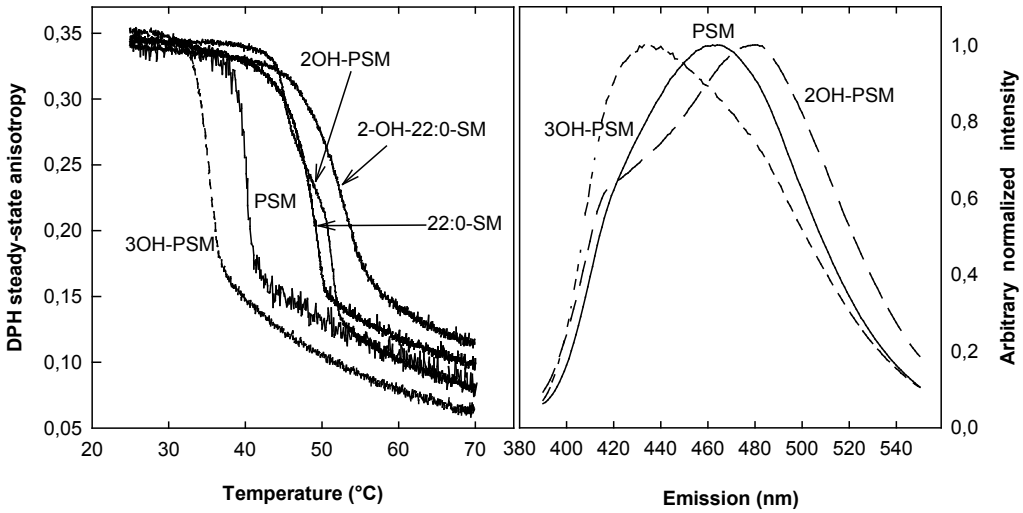


Figure 15. (Left panel) Steady-state DPH anisotropy in multilamellar vesicles as a function of temperature. (Right panel) c-Laurdan emission spectra of multilamellar vesicles containing PSM, 2-OH-PSM, or 3-OH-PSM. Excitation was at 365 nm and emission spectra were recorded between 390 and 550 nm. Data were obtained from the gel phase at T_m minus 5°C, which corresponds to 36°C for PSM, 42°C for 2-OH-PSM, and 30°C for 3-OH-PSM. The figure is adapted from paper III with permission from Elsevier.

The emission spectra of c-laurdan in gel phase (T_m-5°C) and fluid phase (T_m+5°C) of the hydroxylated analogs were recorded to observe the effect of hydroxyl group substitutions on interfacial hydration (Fig 15B). c-Laurdan is sensitive to the changes in the interfacial hydration and lateral packing of lipid bilayers [255]. In the gel-phase, emission spectra of 3-OH-PSM were blue shifted relative to PSM bilayers, indicating that the 3-OH-PSM bilayers were less hydrated. This may be explained by the position of 3-OH group, which lies in a more hydrophobic environment away from the interfacial region. It was also possible that the position of 3-OH group could have interfered with the position of c-laurdan probe in the bilayer. Emission spectra for 2-OH-PSM were red shifted in gel phase and hence concluded more hydrated than PSM bilayers. In fluid phase, the maxima of the emission spectra of hydroxylated SM analog were found close to 470nm but differed from the curve of 3-OH-PSM that had the maxima at 410nm.

4.3.2 Stability of cholesterol and hydroxylated SM containing domains

The presence of sterol-enriched domains was determined in ternary bilayers containing POPC (disordered lipid), SM analogs (ordered lipid) and cholesterol. Quenching curves were obtained by fluorescent quenching of CTL by 7SLPC (Fig. 16). PSM formed cholesterol(CTL)-enriched ordered domains in fluid POPC matrix with end-point melting at ~39-41°C [239].

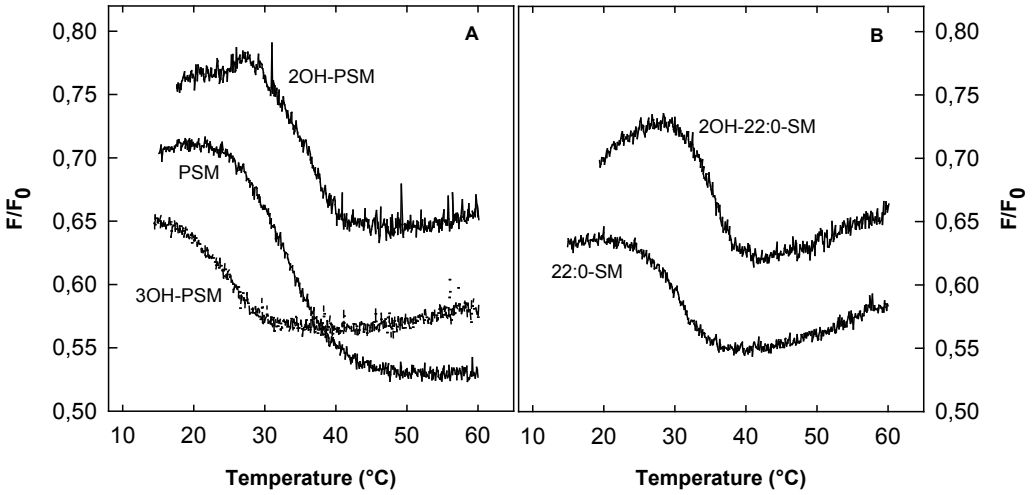


Figure 16. Melting of ordered domains containing sterol observed from the quenching of CTL fluorescence. The melting profile is shown as the F (quenched)/F_0 (non-quenched) ratio plotted vs. temperature. (A) Panel shows results for PSM and its hydroxylated analogs, whereas (B) panel shows results with 22:0-SM and 2OH-22:0-SM. The figure is adapted from paper III with permission from Elsevier.

2-OH-PSM formed CTL-enriched domains with end-point of melting at ~42°C, which indicates that these domains were slightly more stable than PSM domains. 3-OH-PSM also formed CTL-enriched domains, but their thermostability was markedly diminished, with end-point of melting at ~30°C. 22:0SM and 2-OH-22:0SM formed CTL-enriched liquid-ordered domains, but 2-OH-22:0SM domains were more stable than the chain length matched nonhydroxylated SM.

4.4 Effect of hydroxylation in the sphingosine backbone on SM membrane properties

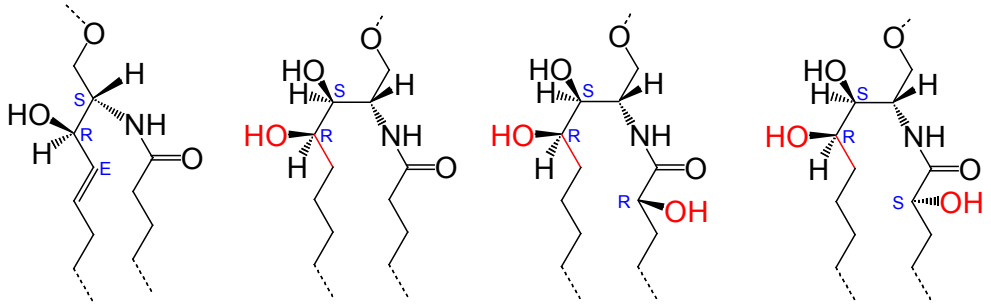


Figure 17. Structure of SM analogs used to study the effect of hydroxylation in the SM backbone as well as in the *N*-linked acyl chain on SM membrane properties. From left to right: PSM, PhytoPSM, Phyto-2-OH(*R*)-PSM, Phyto-2-OH(*S*)-PSM.

D-*ribo*-phytosphingosine is one of the biologically significant long chain bases. Structurally different from sphingosine, these long chain bases lack a double bond and have two hydroxyl groups at positions 3 and 4 in the backbone. These bases are abundant in yeasts, fungi, plants and mammalian tissues. Since *N*-acyl chain hydroxylation showed marked stabilization of the gel phase, we aimed to see if an additional hydroxyl group in the long-chain base also affected gel phase stability and interlipid interaction. In this study, we synthesized PhytoPSM along with their N-linked hydroxylated analogs. In addition we also incorporated α-hydroxylation in N-linked acyl chain of PhytoPSM to yield two conformers (*R* and *S*) of Phyto-2-OH-PSM (Fig. 17).

4.4.1 Gel phase stability of phytoSM analog bilayers

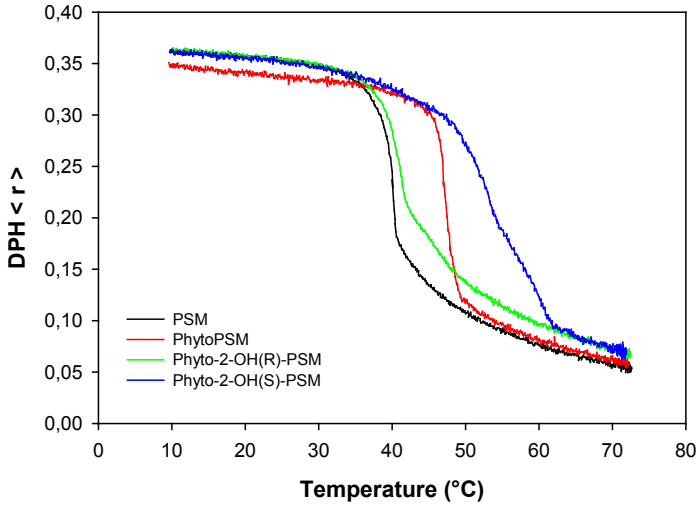


Figure 18. Steady-state anisotropy of DPH. Measurement of fluorescence anisotropy was used to study the order in acyl chain of the SM-analogs in the membrane interior.

Based on steady-state anisotropy analysis of DPH in binary bilayers containing pure SM analogs, acyl chain order and gel-to-liquid phase properties were observed (Fig. 18). The T_m of PhytoPSM (48°C) was shown to be markedly higher as compared to the T_m of PSM (41°C). The hydroxylated SM analogs showed distinct phase behavior, markedly depending on the stereo- specific orientation of the α-hydroxy group. For Phyto-2OH(*S*)-PSM, the gel phase was stabilized compared to phytoPSM, whereas with Phyto-2OH(*R*)-PSM (natural isomer), it was destabilized.

4.4.2 Formation of sterol-enriched ordered domains

All tested phytoPSM analogs were able to form sterol-enriched ordered domains in the POPC bilayers (Fig. 19A). The stability of the domains varied slightly; so that phytoPSM domains were more stable than PSM domains (end melting temperature about 52°C and 38°C, respectively (Fig. 19A).

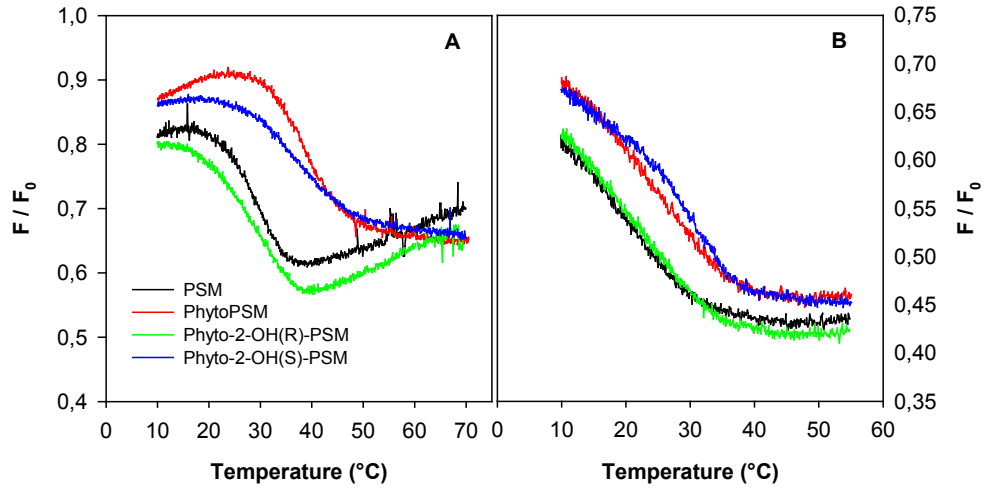


Figure 19. Cholesterol-rich ordered domain formation and sterol interaction with SM analogs was determined from the quenching susceptibility of CTL **(A)** and DPH **(B)** by the quencher 7SLPC. DPH reports on ordered domains in general not necessarily containing sterol.

For α-hydroxylated phytoPSM analogs, the ordered domain stability varied depending on the orientation of the α-hydroxy group, and correlated with the transition temperature of the pure bilayers. Phyto-2OH(*R*)-PSM and Phyto-2OH(*S*)-PSM containing domains had the end melting temperatures about 39°C and 49°C, respectively (Fig.19A). The delta F/F_0 (F/F_0 ratio before and after melting) was similar for all analogs, suggesting that the CTL (and thus sterol) content in the ordered domains were similar for the SM analog domains. Fig. 19B shows quenching susceptibility of DPH demonstrating formation of ordered domains not necessarily containing sterol. All phytoPSM analogs included in the study formed gel-like ordered domains. The end melting temperatures of the ordered domains indicated by DPH were similar to the domains detected by using CTL.

4.4.3 Sterol affinity for SM analog containing bilayers

To further analyze the interaction between cholesterol/CTL and the phytoPSM analogs, we determined the CTL equilibrium partitioning coefficient for POPC bilayers containing 20 mol% of one of the PSM analogs and methyl-*β*-cyclodextrin as acceptor. The presence of PSM in POPC bilayers is known to increase the sterol affinity markedly, and this was confirmed both at 23°C and 37°C (Fig.20).

The CTL affinity for POPC bilayers containing phytoPSM was higher than for bilayers with PSM (both at 23°C and 37°C, Fig. 20), suggesting that the additional hydroxyl group in the long-chain base was able to stabilize sterol/SM interaction. The *N*-linked 2-hydroxylated palmitoyl acyl chains in phytoSM did not markedly alter sterol affinity. The CTL affinity results show a good agreement with the CTL quenching data (Fig. 19A). The 4-OH in phytoSM stabilized SM/SM as well as SM/sterol interactions in complex bilayer membranes.

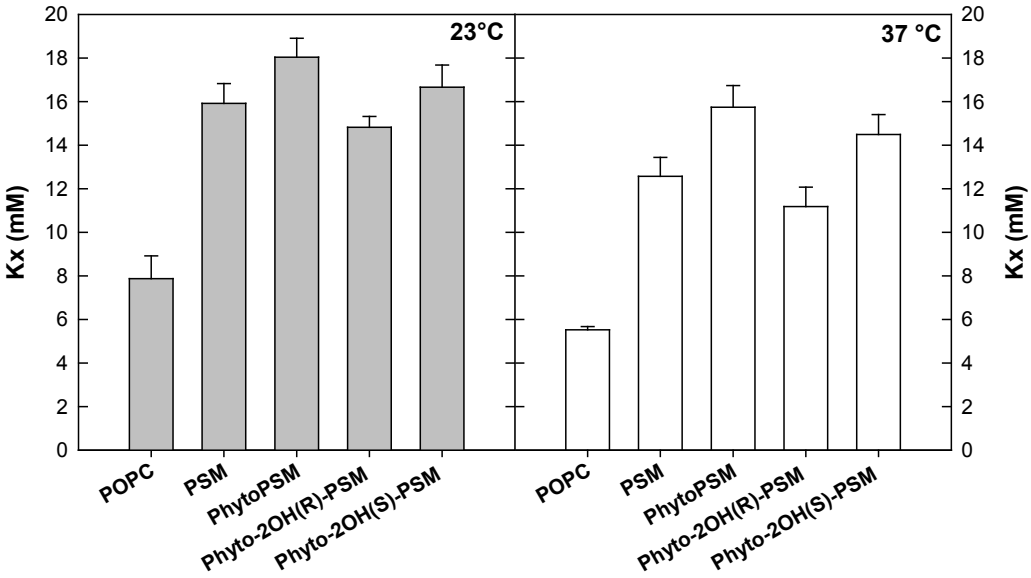


Figure 20. Sterol affinity for membranes containing SM-analogs. The sterol affinity was determined from an equilibrium distribution of CTL between methyl-β-cyclodextrin and unilamellar vesicles containing each of the phytoSM-analogs in mixtures with 80 mol% POPC. The CTL and thus sterol concentration was 2 mol% in all partitioning experiments.

4.5 Effect of methylation in interfacial region on SM membrane properties

In this project the importance of hydrogen bonding in SM bilayers for stabilizing the lipid-lipid interaction was studied experimentally and via atomistic MD simulations. We studied PSM analogs with 2-NH methylated to NMe, the 3-OH methylated to OMe, or both were methylated simultaneously (Fig. 21). DPH anisotropy experiments showed that methylation in the interfacial region (at amide nitrogen and 3-O as well) affected interlipid interactions , decreased T_m by 5-10°C and increased fluid phase disorder in SM bilayers.

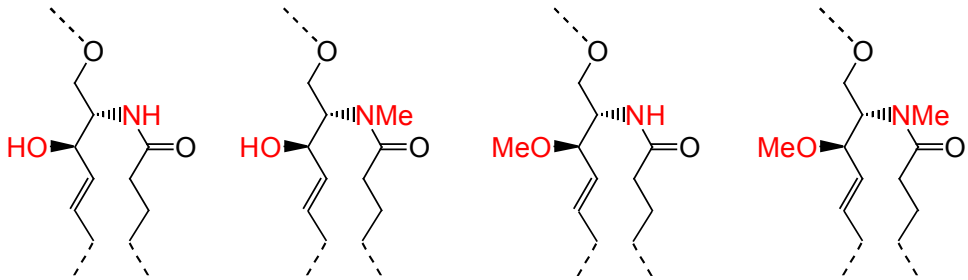


Figure 21. Structure of SM analogs used to study the effects of methylation in interfacial region on SM membrane properties. From left to right: PSM, NMe-PSM, OMe-PSM, and NMeOMe-PSM.

4.5.1 Interaction of sterols with N- and O-methylated SM analogs

Sterol/SM analog interaction was addressed by studying the formation and thermostability of CTL-enriched SM domains in a fluid phospholipid bilayer (Fig. 22A). The melting of CTL-enriched domains containing PSM appeared to melt completely above 40°C. After replacing PSM with double methylated SM analog, no sterol rich domains were observed. The bilayer with NMe-PSM contained domains but they were thermally unstable and melted at lower temperature than those formed with PSM. OMe-PSM also formed sterol-rich domains, which however seemed to contain very little CTL. These data suggested that 2-NH or 3-OH methylation had adverse effect on sterol–SM interaction. As evident from the CTL quenching curves, methylation of 3-OH hindered more sterol–SM interaction as compared with 2-NH. The outcome that OMe-PSM failed to form sterol-enriched domains was unexpected. It has been reported that removing the 3-OH group from SSM does not affect the cholesterol exchange rates between the membrane and cyclodextrin [256]. Cholesterol exchange rate from OMe-SSM vesicles was found to be similar for SSM, signifying that the methyl substitution on 3-O did not enact steric effects that would affect cholesterol exchange kinetics. Possibly, 3-O analogs are less effective in shielding cholesterol from unfavorable interactions with the water, but the interpretation remained inconclusive. Sterol MD simulation studies also showed that the 3-O significantly affects the headgroup dynamics, whereas no such effect was observed for NH methylation.

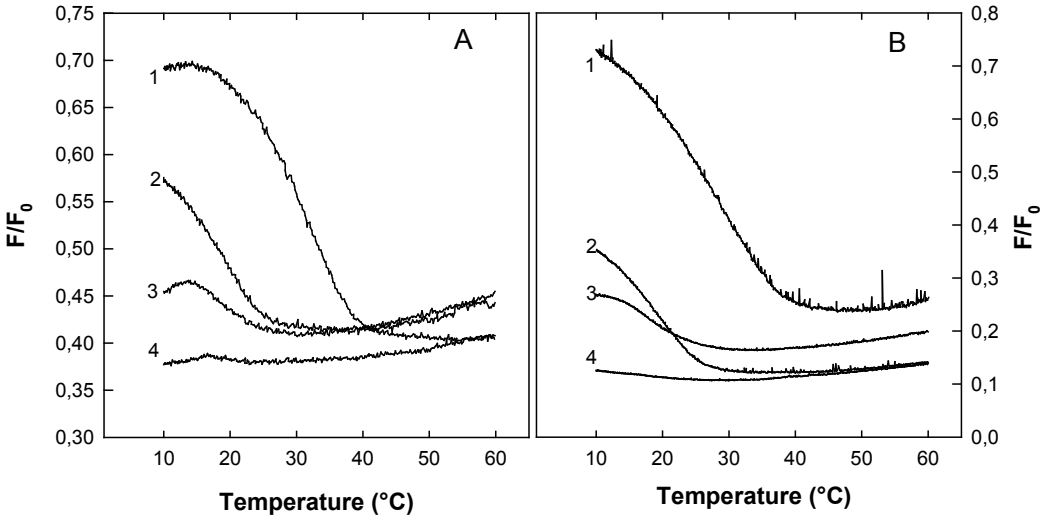


Figure 22. Detection of ordered domains in ternary bilayer membranes. Sterol-enriched domains were determined by CTL quenching (panel A), and ordered domains in general from DPH quenching (panel B), Curve identification: 1 is PSM, 2 is NMe-PSM, 3 is OMe-PSM, and 4 is NMeOMe-PSM. The figure is adapted from paper V with permission from Elsevier.

The ability of the SM analogs to form ordered domains not necessarily containing sterol were tested by DPH quenching (Fig. 22B). The DPH quenching results complemented well the information obtained by CTL quenching and confirmed the inability of methylated SM analogs to form such ordered domains as detected when using PSM. Sterol partition study also showed that sterol affinity to bilayers containing methylated SM analogs is much weaker than the affinity for PSM bilayers.

4.5.2 Simulation results

MD simulation of bilayers containing 80 mol% SM analog and 20 mol% cholesterol was performed. All studied bilayers showed decreased area per lipid (Table 2) and were more ordered in the presence of cholesterol, consistent with the known ordering effect of cholesterol. The condensation effect was strongest for PSM, followed by NMeOMe-PSM, OMe-PSM and finally NMe-PSM. The cholesterol tilt seemed to correlate well with surface area and was the highest for cholesterol containing NMeOMe-PSM bilayers (Table 2). The bilayer thickness increased in presence of cholesterol as reported by P-P distance for all of the SM studied. The bilayer thickness in the l_d phase also decreased when PSM was replaced with methyl modified SM analogs.

Table 2. Structural properties of lipid bilayers as deduced from atomistic simulation. Area per lipid, membrane thickness (measured as a distance between average positions of phosphate atoms in opposite leaflets (P–P distance)) and cholesterol tilt (characterized by an angle between the bilayer normal and the vector linking the cholesterol atoms C3 and C17). The table was adapted from paper V with permission from Elsevier.

	Area [nm^2] ±0.001	**P–P distance** [nm]±0.005	**Cholesterol tilt** ±0.03
PSM	0.6093	3.853	-
PSM-CHOL	0.5694	4.028	22.26
NMe-PSM	0.6201	3.832	-
NMe-PSM/CHOL	0.5932	3.943	23.71
OMe-PSM	0.6490	3.754	–
OMe-PSM/CHOL	0.6185	3.907	25.38
NMeOMe-PSM	0.7121	3.478	–
NMeOMe-PSM/CHOL	0.6744	3.655	27.91

The packing order of the long chain bases and the acyl chains was observed to be lower in the modified SM bilayers. Positioning of the hydroxyl group of cholesterol in bilayer was also affected by the structural modifications of SM. Both 3-OH and 2-NH methylations

enforced cholesterol to be positioned closer to the interface of the SM analog bilayers than they were in the PSM bilayers. The cholesterol hydroxyl group in modified SMs was placed approximately 0.20-0.25 nm farther from the bilayer center than in SM bilayers. The number of hydrogen bonds with the phosphate groups and water had increased and the number of charge pairs with the choline group increased as well for all the modified bilayers. MD simulations further showed that the N- and O- methylations have no effect on the orientation of the headgroup. However the O-methylation increases the rotational speed of the headgroup whereas the effect of N-methylation was relatively small. The charge pair interactions were weaker for all the methylated SM analogs than for PSM, which suggests reduced interlipid interactions (Table 3).

Table 3. Number of hydrogen bonds between lipids and with water (H_20), charge pairs (CP), hydrogen bonds via the amide group (NH), and via the hydroxyl group (OH) between lipids per lipid molecule. Hydrogen bonds were evaluated on the basis of geometrical criteria: the donor (D)–acceptor (A) distance is less than 0.325 nm and the angle between vectors D–H and D–A is less than 35°. Charge pair was characterized by electrostatic interactions between the positively charged choline methyl groups and the negatively charged oxygen atoms in lipid molecules whose distance from one another is less than 0.4 nm (see [62] and references therein). The figure is adapted from paper V with permission from Elsevier.

	H_2O±0.1	**CP**±0.02	**NH***±0.005	**OH***±0.005
PSM	5.9	4.15	0.335	0.410
PSM-CHOL	6.2	3.69	0.352	0.513
NMe-PSM	5.4	4.13	–	0.341
NMe-PSM/CHOL	5.7	3.43	–	0.254
OMe-PSM	5.7	4.07	0.051	–
OMe-PSM/CHOL	6.0	3.52	0.072	–
NMeOMe-PSM	5.5	3.11	–	–
NMeOMe-PSM/CHOL	5.7	3.10	–	–

* Only those H-bonds where the group acts as a donor are considered here.

The most important group required for hydrogen bonding between SM and cholesterol was the hydroxyl, with almost 46% of cholesterol involved via this group. The second most important functional group involved in hydrogen bonding was carbonyl (25%), followed by amide and phosphate oxygen, which contributed 10% of the total hydrogen bond. In bilayers containing modified SM analogs the number of hydrogen bonds was reduced with. One important effect of methylation on the hydrogen bond interaction between SM and cholesterol was that modification of one functional group affected the bonding ability of another group. When NH was substituted with NMe,

the number of hydrogen bonds between OH and cholesterol was reduced by 40% and with carbonyl group was lessened by about 25%. Hydrogen bonding was affected by the positioning of hydroxyl group of cholesterol in the modified SM bilayer. Methylations at both 2-NH and 3-OH forced cholesterol closer to the interface with increased tilt angle in SM analog bilayers. Such arrangement of cholesterol resulted in lowered van der Waals attraction and an easy efflux of cholesterol to methyl-β-cyclodextrin "barrels". CTL partition experiments also showed lower bilayer affinity of cholesterol for SM analogs with interfacial methylations, as evident from low value of partition coefficients.

Conclusions and Implications

Chapter 5

Scientists are still challenged to understand the diversity of lipid molecules as well as their role in a biological membrane. Apparently the structurally different lipids have specific functions in the membrane. One of the significant roles of SMs in membrane may be to provide local order into the otherwise fluid and disordered membranes. Small alterations in the structure of SMs affect their biological role as well as properties like lateral distribution and localization with cholesterol in the membrane. The main aim of this thesis was to study how the substituents like methyl or hydroxyl groups and differing acyl chain lengths of SMs affect membrane properties. Since cholesterol also modulates the fluidity of the sphingolipid domains we studied interactions of SM analogs with cholesterol in complex model membranes.

Important conclusions drawn from this study are:

1. Due to the best hydrophobic match with cholesterol, PSM is the most preferred SM studied in this work for interaction with cholesterol.

2. The presence of methyl group in SM affects SM membrane properties in position dependent manner. Methyl branching in *N*- linked acyl chain interfered with molecular packing and induced thermo-instability in SM membranes. Methylation at position 10 as in 10MePSM conferred fluidity for the bilayer, an effect similar to the presence of *cis*-unsaturation in phospholipid acyl chain moiety.

3. Position as well as orientation of hydroxylation in N-linked acyl chain of SM is important for determining their membrane properties and formation of sterol-enriched domains.

4. PhytoPSM have higher thermostability than PSM indicating that the presence of an additional hydroxyl group in long chain base enhanced inter-molecular interactions. Additional hydroxyl group as well as the orientation of 2-hydroxyl group in N-linked acyl chain of PhytoPSM is crucial for determining their thermotropic properties and formation of sterol-enriched domains.

5. Methylation of 2-NH and 3-OH groups of PSM decreased the gel phase stability of the membrane and the interactions between SM and sterols were markedly attenuated. The 2-NH and 3-OH methylations abolished hydrogen bonding, not only at the methylated positions but also affected additional potent hydrogen bonding via functional groups in the interfacial region of SM.

SMs are essential for a number of basic structural functions and for a number of processes of physiological importance, such as raft formation, cellular signaling, among others. Our findings clearly demonstrated that structural modifications in SM significantly affect their biophysical properties and lateral interactions in the membrane. The findings of this work were obtained in studies using model membranes but the molecular interactions clarified apply also to more complex biological systems. In future, it will be interesting to introduce the novel SM molecules into cells and observe their properties and functions in such complex systems. Nevertheless, the findings of this study will help to understand the significance of SM structure for their interaction with sterols and role in inducing lateral heterogeneity in biological membranes.

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