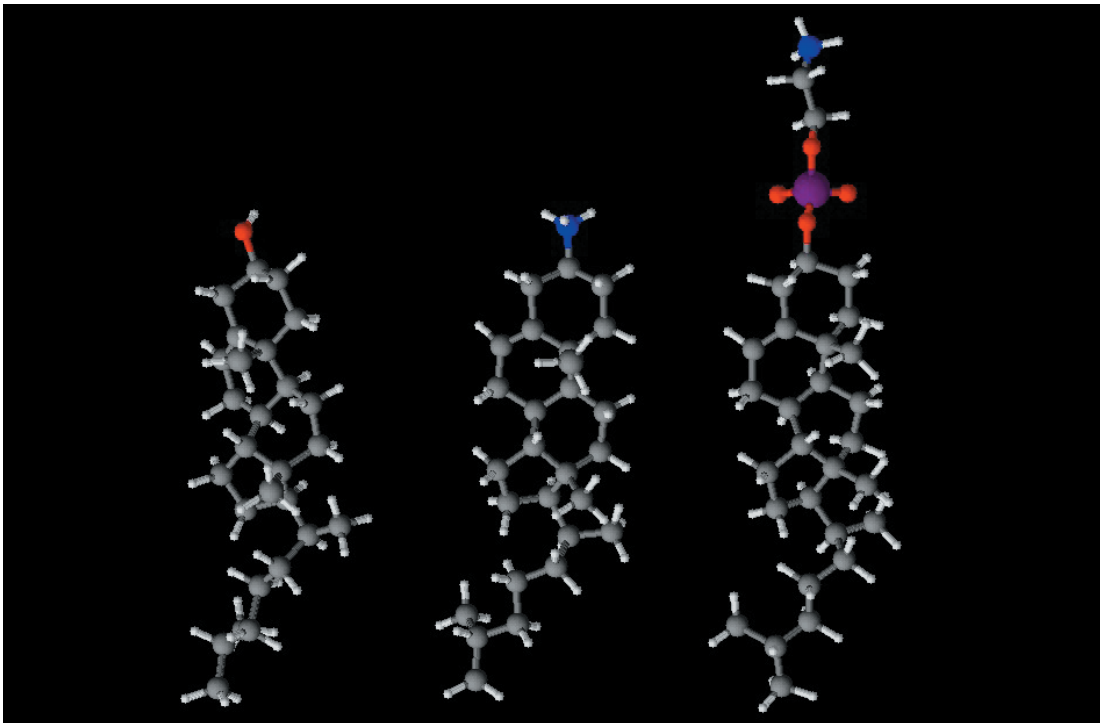
Åbo Akademi University

Max Lönnfors

Studies on Membrane Properties of Cholesterol and 3-beta Modified Sterol Analogs



Studies on membrane properties of cholesterol and 3-beta modified sterol analogs

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Biochemistry, Department of Biosciences
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To my family

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications referred to by the Roman numerals I-IV throughout the thesis. The original publications have been reprinted with the permission of the copyright holders.

I) M. Lönnfors, J.P. Doux, J.A. Killian, T.K. Nyholm, J.P. Slotte, **Sterols have higher affinity for sphingomyelin than for phosphatidylcholine bilayers even at equal acyl-chain order,** Biophysical Journal 100 (2011) 2633-2641.

II) M. Lönnfors, O. Engberg, B.R. Peterson, J.P. Slotte, **Interaction of 3β-amino-5-cholestene with phospholipids in binary and ternary bilayer membranes,** Langmuir 28 (2012) 648-655

III) M. Lönnfors, O. Långvik, A. Björkbom, J.P. Slotte, **Cholesteryl phosphocholine - a study on it interaction with ceramides and other membrane lipids,** Langmuir 29 (2013) 2319-2329.

IV) P. Sukumaran and M. Lönnfors, O. Långvik, I. Pulli, K. Törnquist and J.P. Slotte, **Complexation of C6 ceramide with cholesteryl phosphocholine - a potent solvent-free ceramide delivery formulation for cells in culture,** PLoS. One. 8 (2013) p.e61290.

CONTRIBUTIONS OF THE AUTHOR

The author contributions to the included original articles were as follows:

I) Designed the experimental setups together with the supervisor and T. Nyholm. Synthesized the lipids 14:0-SM, 14:0/14:0-PC, 14:0/15:0-PC and 15:0/14:0-PC and performed all the experiments with the exception of the NMR studies which were conducted in the Netherlands. Contributed to the writing of the manuscript together with the supervisor and T. Nyholm

II) Designed the experimental setups together with the supervisor and performed the initial experiments. Roughly half of the experimental work was performed by O. Engberg under the author's supervision. Participated in writing the manuscript together with the supervisor.

III) Designed the experimental setup together with the supervisor and A. Björkbom and performed all experiments except for GUV preparation and imaging which was performed by A. Björkbom. Contributed to the writing of the manuscript together with the supervisor and O. Långvik.

IV) Designed the experimental setups together with the supervisor, P. Sukumaran and K. Törnquist. Prepared the complexes used to load the cells with ceramide and performed lipid extraction and TLC analysis. Contributed to the writing of the manuscript together with the supervisor, K. Törnquist and P. Sukumaran.

Additional publications not included in the thesis

J.H. Nyström, M. Lönnfors, T.K. Nyholm, **Transmembrane peptides influence the affinity of sterols for phospholipid bilayers,** Biophysical Journal 99 (2010) 526-533.

A. Björkbom, T. Rog, K. Kaszuba, M. Kurita, S. Yamaguchi, M. Lönnfors, T.K. Nyholm, I. Vattulainen, S. Katsumura, J.P. Slotte, **Effects of sphingomyelin headgroup size on molecular properties and interactions with cholesterol,** Biophysical Journal 99 (2010) 3300-3308.

O. Ekholm, S. Jaikishan, M. Lönnfors, T.K. Nyholm, J.P. Slotte, **Membrane bilayer properties of sphingomyelins with amide-linked 2- or 3-hydroxylated fatty acids,** Biochim. Biophys. Acta 1808 (2011) 727-732.

H.K. Ijäs. M. Lönnfors, T.K. Nyholm, **Sterol affinity for phospholipid bilayers is influenced by hydrophobic matching between lipids and transmembrane peptides,** Biochim. Biophys. Acta 1828 (2013) 932-937

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ABBREVIATIONS

7SLPC	1-palmitoyl-2-steraoyl-(7-doxyl)-*sn*-glycero-3-phosphocholine
CD	cyclodextrin
Chol-PC	cholesteryl-3-beta-phosphocholine
CTL	cholestatrienol (cholesta-5,7,9(11)-trien-3-beta-ol)
DOPC	1,2-dioleoyl-*sn*-glycero-3-phosphocholine
DPH	1,6-diphenyl-1,2,5-hexatriene
DPPC	1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine
DSC	differential scanning calorimetry
GP	generalized polarization
GUV	giant unilamellar vesicle
HPLC	high-performance liquid chromatography
K_x	molar fraction partition coefficient
Laurdan	6-lauroyl-2-(*N*,*N*-dimethylamino)naphtalene
L_d	liquid disordered phase
L_o	liquid ordered phase
L_β	lamellar gel phase
$P_{\beta'}$	rippled phase
mβCD	methyl-β-cyclodextrin
MD	molecular dynamics
NMR	nuclear magnetic resonance
OCer (18:1-OCer)	*N*-oleoyl-D-*erythro*-sphingosine
PC	phosphatidylcholine
PCer (18:1-PCer)	*N*-palmitoyl-D-*erythro*-sphingosine
POPC	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine
PSM	*N*-palmitoyl-D-*erythro*-sphingosylphosphorylcholine
S_{CD}	deuterium order parameter
SM	sphingomyelin
TLC	thin layer chromatography
Tm	gel-liquid-crystalline phase transition
***t*Pa**	*trans*-parinaric acid

ABSTRACT

Cholesterol (Chol) is an important lipid in cellular membranes functioning both as a membrane fluidity regulator, permeability regulator and co-factor for some membrane proteins, *e.g.* G-protein coupled receptors. It also participates in the formation of signaling platforms and gives the membrane more mechanical strenght to prevent osmotic lysis of the cell. The sterol structure is very conserved and already minor structural modifications can completely abolish its membrane functions. The right interaction with adjacent lipids and the preference of certain lipid structures over others are also key factors in determining the membrane properties of cholesterol. Because of the many important properties of cholesterol it is of value to understand the forces and structural properties that govern the membrane behavior of this sterol.

In this thesis we have used established fluorescence spectroscopy methods to study the membrane behavior of both cholesterol and some of its 3β-modified analogs. Using several fluorescent probes we have established how the acyl chain order of the two main lipid species, sphingomyelin (SM) and phosphatidylcholine (PC) affect sterol partitioning as well as characterized the membrane properties of 3β-aminocholesterol and cholesteryl phosphocholine. We concluded that cholesterol prefers SM over PC at equal acyl chain order, indicating that other structural properties besides the acyl chain order are important for sphingomyelin-sterol interactions. A positive charge at the 3β position only caused minor changes in the sterol membrane behavior compared to cholesterol. A large phosphocholine head group caused a disruption in membrane packing together with other membrane lipids with large head groups, but was also able to form stable fluid bilayers together with ceramide and cholesterol. The Ability of the large head group sterol to form bilayers together with ceramide was further explored in the last paper where cholesteryl phosphocholine/ceramide (Chol-PC/Cer) complexes were successfully used to transfer ceramide into cultured cells.

1. INTRODUCTION

For life to be able to form there was a need for compartmentalization, the molecules of life had to concentrate and form defined spaces. The cells that formed had to be able to distinguish the outside from the inside, one space from the other and to create environments with different properties enabling energy to be harvested and stored. The barrier could not be impermeable, since life is constantly evolving, changing and adapting, sensing its environment and sending out signals as well as acquiring energy. This semi-permeable compartmentalization was achieved with the formation of lipid bilayers, cellular membranes, composed of amphiphilic lipids clustering together to form a bilayer. As evolution progressed, the membranes developed into macromolecular structures comprising the lipid bilayer and various membrane proteins, attached to or embedded in the bilayer. The cytoskeleton connected the inner monolayer to interior parts of the cell and the glycocalyx outer leaflet served as a cell-cell recognition site (*1*). Although at first thought to be a rather static structure, it is now known that the lipid bilayer has to be highly dynamic, constantly changing structure and composition in order to support cell viability. Interactions between lipids will determine the biophysical properties of the membrane, and the cell regulates many of its membrane functions by regulating the lipid composition. The properties of the membrane can affect the function and the conformational state of membrane proteins and vise versa. The physiological state of the cell is thereby partly determined by the physical state of the lipid bilayer (*2;3*). The membrane lipids can also function as "lipid second messengers" or as a source for the release of signaling molecules (*4;5*). In mammalian cells one of the key players in membrane regulation is cholesterol. It has the ability to improve packing of loosely packed membranes as well as make highly dense regions more fluid to accommodate lateral transport. It can also regulate ion permeability and help to maintain the electrochemical gradient across the plasma membrane (*6*). By regulating the cholesterol content in its membranes the cell can regulate the fluidity, thickness and permeability of these membranes. The understanding of how cholesterol is able to perform these tasks is largely contributed to the molecular structure of cholesterol and the lipids with which it interacts. One way of studying these interactions is to alter the chemical structure of cholesterol and then investigate how the alterations affect the molecular behavior. The results can then be used to evaluate which properties are crucial and by which molecular mechanisms cholesterol interacts with other lipids in present in the membrane.

2. REVIEW OF THE LITERATURE

2.1. Biological membranes

Most cell membranes are built up of a double layer of amphipihilic molecules, called lipids (Fig. 1). Because these molecules have both a hydrophobic and a hydrophilic part they will tend to self aggregate into structures where the hydrophobic parts are shielded from water. The amphiphilic nature of the lipids will cause them to aggregate into a double layer structure, a bilayer where the hydrophobic acyl chains face each other and the hydrophilic polar head groups face the aqueous phase on both sides of the bilayer. By adopting this structure, the unfavorable disruption of the hydrogen bond network between water molecules by the hydrophobic parts of the lipids is minimized. Thus these structures will form spontaneously at physiological temperatures. The lipids form the bulk membrane and the functional groups of the participating lipids will determine the membrane properties of both the micro- and macroenvironment of that membrane.

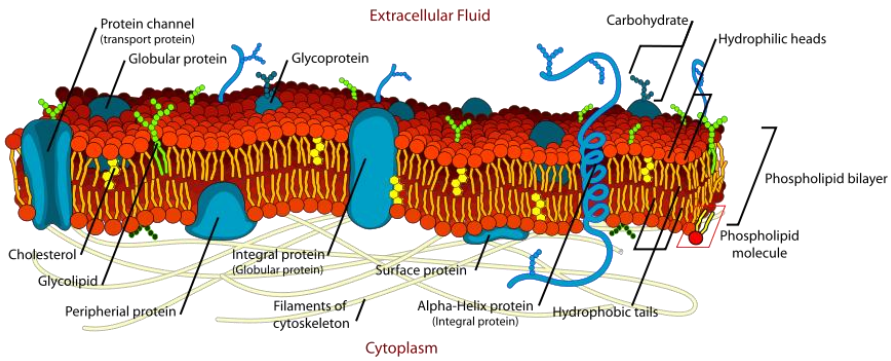

Figure 1. *Schematic picture of the cell membrane structure, lipids in red/yellow and proteins in blue (en.wikipedia.org, reprinted by permission of the author Mariana Ruiz).*

A biological membrane also contains a large number of proteins (Fig. 1). The proteins can span the bilayer completely (integral proteins) or can be embedded or attached to only one of the leaflets (peripheral proteins). They can also form channels or pores in the membrane through which a controlled efflux and influx of small molecules and nutrients can take place. The proteins as well as the lipids can be decorated with carbohydrates to form glycoproteins and glycolipids,

important *e.g.* in cell to cell recognition. In the cytosolic leaflet some proteins are anchored to cytoskeletal filaments, e.g. actins giving the cell its shape and structural strength. The plasma membrane, the largest organelle of the cell is present both in eukaryotes and prokaryotes and has a thickness of ~4 nm (*1*).

2.2. Lipid structure

Apart from being amphiphilic, the properties of membrane lipids can vary significantly. A cell membrane is composed of hundreds to thousands of different lipid species and different subcellular compartment display variations in their lipid composition (*7*). Lipids can be classified based on their structural features. The major lipid classes present in mammalian cell membranes are glycerophospholipids, sphingolipids and sterols (cholesterol in mammalian cells). Sterols share little resemblance with the other lipids but like the other lipids it is an amphiphilic molecule with a hydrocarbon backbone.

2.2.1. Glycerophospholipids

Glycerophospholipids, the most abundant lipid species in mammalian cells, are members of the larger glycerolipid family and shares the common backbone of L-glycerophosphate. The three hydroxyl groups of glycerol, named *sn-1*, *sn-2* and *sn-3*, are coupled to a polar phosphate group (*sn-3* position) and two acyl chains of varying length and degree of saturation (*sn-1* and *sn-2* position) (*8*). The phosphate can bind additional chemical groups ranging from the small hydroxyl group to very large and complex oligosaccharides, forming an array of different lipids with markedly different membrane properties. The most common group is a choline group, forming phosphatidylcholine or PC for short. PCs with different acyl chain lengths and saturation make up the bulk membrane in eukaryotic cells, the most common being 1-palmitoyl-2-oleoyl-*sn*-glycero-phosphatidylcholine (POPC, Fig 2A.). This molecule has also been used in the model membrane work in this thesis to represent the bulk membrane. Other common phospholipids in mammalian membranes include phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylglycerol (PG).

2.2.2. Sphingolipids

Sphingolipids are the second most abundant phospholipid species in mammalian cells, composing 20-25% of the total phospholipid content in the

outer leaflet of the plasma membrane (*9*). Apart from their structural role they also have a functional role as precursors for intracellular messengers and are also able to, together with cholesterol, function as modulators of membrane fluidity. Sphingolipids were first described in the late 19th century by Thudicum (*10*), who also gave them their name, derived from the mythical Sphinx in Luxor, Egypt. Later in 1947 Carter determined the first structure of a sphingolipid and also suggested that all lipids derived from sphingosine should be classified as sphingolipids (*11*). Sphingolipids differ from glycerophospholipids in that they have a sphingoid long-chain base with functional groups and amide linked acyl chains attached as the structural backbone. The structure of the sphingoid backbone can vary and there are more than sixty different variations known amongst the cells of animals, plants and microorganisms. The most basic sphingoid backbone structure is sphinganine whereas the most abundant in human cells is the unsaturated derivative of sphinganine, sphingosine ((2S, 3R)-2-amino-4-octadecene-1,3-diol), an unsaturated 18 carbon long amino alcohol with a *trans* double bond between C4 and C5 (*12*). There are exceptions, in human lens tissue for example it is sphinganine, not sphingosine, which account for more than 50% of the sphingolipid backbone structure (*13*).

The structural variation among sphingolipids is caused by a difference in the sphingoid base, the hydrophilic head group attached to the C1 position and the difference in length and unsaturation level of the hydrophobic acyl chain attached to the amine. The most common head groups are phosphate, phosphorylcholine and different carbohydrates, and the most common acyl chains are 16:0, 18:0, 24:1 and 24:0. The acyl chain length of sphingosine corresponds to an about 13.5 carbon chain long *sn-1* chain in a glycerophospholipids (*14*), leading to acyl chain mismatch in most sphingolipid species (*14*). Sphingolipids can also be hydroxylated at various positions in the hydrocarbon chain, for example about half of the glycosphingolipids found in human brain tissue have a hydroxyl group at the alpha position in the N-acyl chain (*15*).

Sphingomyelin (SM) is the most abundant sphingolipid in mammalian cells. The backbone of SM consists of sphingosine, and a phosphatidylcholine head group attached to the C1 carbon. Although the head groups of PC and SM are identical, the interface regions are different. Due to the amide- and the 3-hydroxy groups, SM is able to act both as a hydrogen bond donor and acceptor, whereas PC only can act as an acceptor (*16*). This may also lead to a different orientation and

mobility of the SM head group compared to that of PC (*17*). SM also has a *trans* double bond between C4 and C5, making the sphingosine backbone more rigid (*18;19*). The amide linked fatty acid of SMs are usually long, between 16 and 26 carbons and are more saturated than those present in PC lipids (*12;20;21*). If double bonds are present, they are often found further away from the interface region than in PC causing less disturbance in the acyl chain packing (*22*). The most common acyl chains are 16:0 (Fig. 2B) and 18:0, mostly found in peripheral cell membranes whereas longer 24:0 and 24:1 acyl chains are abundant in the myelin sheets of neural axons (*22-24*).

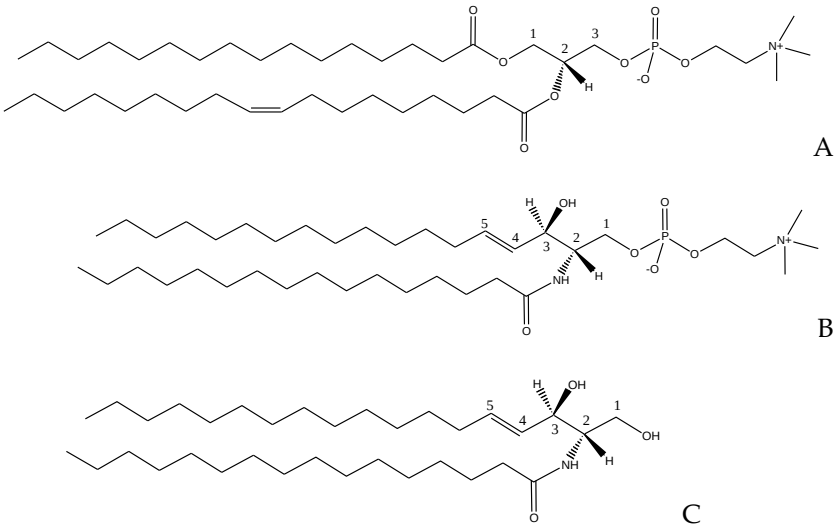

Figure 2. *Molecular structures of: 1-palmitoyl-2-oleoyl-phosphatidylcholine (A), palmitoyl-sphingomyelin (B) and palmitoyl-ceramide (C)*

Ceramide (Cer) is a hydrophobic lipid and a biosynthetic precursor for SM. It lacks the head group at C1 and therefore has a considerately smaller molecular area than SM or PC. Ceramide can be generated via *de novo* synthesis, degradation of SM by sphingomyelinases or via the salvage pathway which re-uses long-chained sphingoid bases from the lysosomal degradation pathway of sphingolipids. In addition to the sphingosine long-chain base (also present in SM) ceramides can be formed from over 50 different long chain bases with varying structure. For example the double bond between C4 and C5 in the sphingoid base can be saturated to form dihydroceramide. Hydroxylations at

various positions of the long-chain base are also common. The N-linked acyl chain is usually between 14 and 26 carbons long (Fig. 2C) but ceramides with an acyl chain longer than 36 carbons has been reported (*25*). The structure of the N-linked acyl chain has been shown to be important for the function and location of ceramide. Ceramide is a very versatile molecule and important both as a structural component and a bioactive molecule as well as a precursor for more complex sphingolipids. It has been proposed that ceramide is able to form pores in the plasma- and mitochondrial membranes as well as ceramide rich platforms, which are thought to be important in signal transduction. Ceramide is also, under certain conditions, able to displace cholesterol from SM/Chol ordered domains (*26*).

2.3. Sterols

Sterols are a unique class of membrane components, which are almost completely hydrophobic and has a unique structure compared to other membrane lipids. The common structural feature for all sterols is the ring structure. Different organisms have adopted different carbon ring arrangements with differences also to the decoration of these rings. Cholesterol (mammalian cells), ergosterol (yeast) and stigmasterol and sitosterol (plant cells) are the most abundant sterols in biological membranes. Apart from being important constituents of membranes, sterols also have a regulatory role, for example many human sex hormones are synthesized with cholesterol as the precursor.

2.3.1. Cholesterol

Cholesterol was first isolated from gallstones in 1789. Cholesterol is synthesized from acetyl CoA in the endoplasmic reticulum (ER) and to some extent in peroxisomes. All human cells with the exception of the red blood cells are capable of producing cholesterol. Cholesterol consist of a sterol part with four hydrocarbon rings, a hydrocarbon chain attached to C17, a double bond between C5 and C6, two methyl groups at C10 and C13 and a polar hydroxyl group at C3 (Fig. 3). The effective length of a cholesterol molecule in the membrane has been estimated to correspond to a 17 carbon long, all-*trans* acyl chain (*27*). Most of the cellular cholesterol (40-90%) is found in the plasma membrane whereas mitochondria and ER have the lowest cholesterol content of all cellular membranes (*28;29*).

Cholesterol is a key player in maintaining the electrochemical gradient over the plasma membrane in animal cells. Animal cells mainly use sodium to maintain the gradient and the fused ring structure of cholesterol is thought to prevent sodium ion leakage through the plasma membrane (*6*). Cholesterol has also been shown to directly affect the function of some membrane proteins involved in membrane trafficking and cell signaling including the G-protein coupled receptors (*30*). Cholesterol is also used as a building block, functioning as a precursor for steroid hormones, vitamin D and bile acids (*31;32*). Many diseases have been directly linked to cholesterol homeostasis, the best recognized ones being cardiac and brain vascular diseases and dementia (*33;34*).

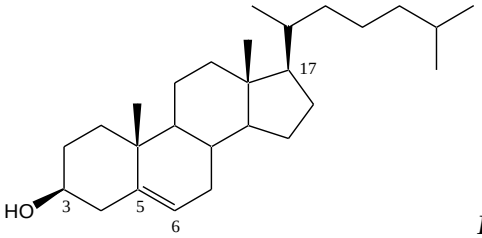

Figure 3. *Molecular structure of cholesterol.*

2.4 Membrane structure

The first proposed models for biological membrane structures came from Langmuir monolayer studies performed in the early 20th century. In 1925, Gortel and Grendel proposed the bilayer structure composed of two layers of amphiphilic molecules arranged with the polar head group facing water on both sides of the bilayer (*35*). Since this first description the membrane model has been refined several times (*36*). In 1972, Singer and Nicolson proposed the fluid mosaic model introducing the concept of peripheral and integral membrane proteins capable of diffusing laterally within the membrane (*37*). During the following decades this model has been revised and updated a number of times, both regarding lipid distribution and protein concentration (*36*). The abundance of transmembrane proteins and their importance has also been of growing interest. Some research estimate that protein content in membranes can reach as high as 50%. Transmembrane and lateral distribution of lipids has been shown to be heterogeneous. Simons and Ikonen introduced the term "lipid rafts" in 1997, proposing that lipids do not diffuse freely as proposed in the fluid mosaic model.

Instead there is a clear difference in segregation of different lipids between different areas in the membrane (*38;39*). Regions with high sphingolipid and cholesterol contents described as microdomains or "lipid rafts". These domains have since been suggested to have a higher degree of order than the surrounding areas and also that they could cause specific proteins to cluster within these domains. Because of the difference in lipid affinity between different lipid species and the formation of the lipid rafts, lateral movements in the bilayer will be restricted, at least to a certain degree. Single lipid tracking has shown that the rate of collision between a labeled lipid and oxygen is fast in the lipid bulk membrane and is slowed down in protein-rich membrane domains containing cholesterol (*40*). These findings are consistent with the lipid raft hypothesis, but can also be explained by the high protein content in cells and the underlying cell matrix structures such as actin networks and intermediate filaments (*41*).

2.4.1. The entropy effect and lipid aggregations

The driving force behind the formation of lipid membranes is the hydrophobic effect, based on the second law of thermodynamics (the entropy of an isolated system never decreases) (*22;36;42*). When lipids are mixed with water they will disrupt the hydrogen bonding network between the water molecules leading to a decrease in entropy which is not thermodynamically favorable. The decrease in entropy can be minimized if the lipids and water separate into two phases, minimizing the contact between water and the hydrophobic parts of the lipids (*36;42*). Depending on the structure of the lipids, the temperature and the composition, different forms of lipid aggregates are adopted (*36*). In biological context four different arrangements are relevant; the micelles, hexagonal and inverted hexagonal phase and bilayers. The parameters that defines the aggregation behavior are the critical length of the acyl chain (l), the head group area (a) and the acyl chain volume (V) (*22;43*). These can be combined in the critical packing parameter, $S = V/al$, defining which type of lipid aggregate that is formed (Fig. 4). Ion concentration, pH, temperature etc. of the solution will affect the packing parameter by affecting V, a and l. If $S \leq 0.33$ spherical micelles will be formed, an S value between 0.33 and 0.5 will form non-spherical micelles, if $0.5 < S \leq 1$ planar bilayers are formed and if $S > 1$, an inverted hexagonal structure is be formed. One important property of S is that it is additive, *i.e.* a lipid with an S value < 0.33 and one with a value > 1 can form bilayers when mixed together (*44*). The most relevant biological aggregate is the bilayers which are also the lipid aggregate studied in this thesis. Lamellar bilayers will be discussed in more detail in section 2.4.2.

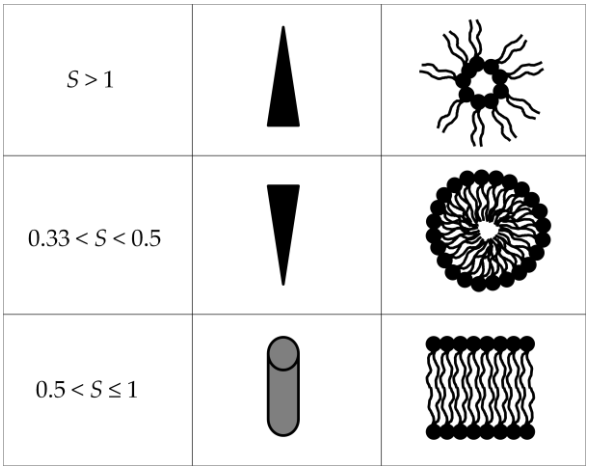

Figure 4. *The lipid aggregate formed dependents on the molecular geometry of the lipid and can be described by S = V/al (see text).*

Both the lateral and transmembrane distribution will also be affected by the overall structural shape of the lipid. Lipids with a large head group compared to their acyl chain volume *e.g.* lysophospholipids ($S \leq 0.33$) will induce what is called positive membrane curvature (*45*), *i.e.* the acyl chains will curve inwards towards one another to facilitate the large head groups without disrupting the hydrophobic packing. These cone-shaped lipids primarily form micellar structures (*46*). Lipids were the head group size and acyl chain size match will not induce any membrane curvature and thus form planar bilayers ($0.5 < S \leq 1$). Lipids with small head groups compared to the acyl chain size will induce a negative membrane curvature ($S > 1$) where the head groups will curve inwards to facilitate the large acyl chains. These inverted cone-shaped lipids form hexagonal phases, one example being the hexagonal phase induced by high ceramide concentration (*45;47*). The structure of ceramide promotes the formation of hexagonal phases and membrane vesiculation (*48*). Vesicle leakage and thermotropic lamellar-to-inverted non-lamellar phase transition was first shown by Ruiz-Argüello et al. (*49*) and later studies *in situ* (*50;51*) have confirmed the vesicle leakage of SMase treated SM-containing mixed membranes. In most cellular membranes lipids that induce both positive and negative curvature as well as bilayer forming lipids will be present. Since most membranes can be considered flat the lipids inducing positive and negative

curvature gives rise to membrane tension (*52*) which could activate or inactive some transmembrane proteins (*53*).

2.4.2. Bilayer phases

The lateral segregation of lipids leads to the formation of distinct domains with different compositions. The segregations is however never absolute nor is it static but the domain components are constantly interchanging with those of the surrounding membrane. These domains form different types of lamellar phases, *i.e.* the gel phase (L_β), liquid disordered (L_d), liquid ordered (L_o) and ripple phase ($P_{\beta'}$). The phase that will be adopted depends both on the structure and chemical properties of the lipid as well as on external factors such as temperature, hydration, ionic strength, pressure and pH. A temperature dependent phase behavior is the most studied one and techniques such as differential scanning calorimetry (DSC), x-ray diffraction and ^{31}P-nuclear magnetic resonance (NMR) spectroscopy have been used in such studies.

At subzero temperatures most SM and PC bilayers are in a lamellar gel phase, L_β (Fig. 5A). In the L_β phase the head groups of the lipids are hexagonally arranged at the membrane surface and the acyl chains have restricted rotational mobility and are in an extended, all-*trans* configuration. The acyl chains are also tilted in respect to the bilayer normal (*1;36;54*). When the bilayer is heated, it will undergo a phase transition from the gel phase to a liquid crystalline phase, also called a disordered phase, L_d (Fig. 5B). The temperature at which this transition occurs is called T_m and dependens on the lipid structure. Above the T_m the lipid bilayer will be in the L_d phase where the molecular motion is substantially increased and where *gauche* conformations in the acyl chains dominate (*1;36;54*). The molecular cross-section area is also substantially increased, thus leading to the lateral expansion of the bilayer as well as an increase in hydration. (*54*).

Because of the more saturated acyl chains allowing for stronger van der Waals interactions SMs have higher T_m compared to PCs. Possible double bonds in the SM acyl chain are also often further away from the membrane-water interphase, causing a smaller disordering effect than those in the acyl chains of PC which are closer to the interphase. SMs could also form a more extensive hydrogen bonding network compared to PC, thus increasing the amount of energy needed to give the system its fluid nature. The effect of the seemingly small structural differences becomes clear when looking at the T_m. The most abundant SMs (C16-24) have a T_m of 40-50 °C whereas the most abundant PCs (C16-20) have a T_m of ≤

0 °C (*55;56*). Another crucial parameter determining the T_m is the acyl chain length. Both for SM and PC there is a curvy-linear relationship between T_m and the number of acyl carbons (*55;57*).

When cholesterol is added to a bilayer it can induce a third phase, the liquid ordered phase (L_o) phase (Fig. 6). The rigid ring structure of cholesterol will promote stronger van der Waals interactions between the acyl chains, thus increasing their effective length and decreasing the acyl chain volume. This will lead to an increase of the membrane thickness. Addition of cholesterol will cause the acyl chains to pack more densely, similar to that in the L_β phase. The rotational freedom, however, will be less hindered resembling that of the L_d phase (*58-60*). The L_o phase can therefore be considered an intermediate between the L_d and L_β phases, something which is also evident from the molecular diffusion rates, *i.e.* L_o displays intermediate diffusion rate as compared to the L_β and L_o phases (*61;62*).

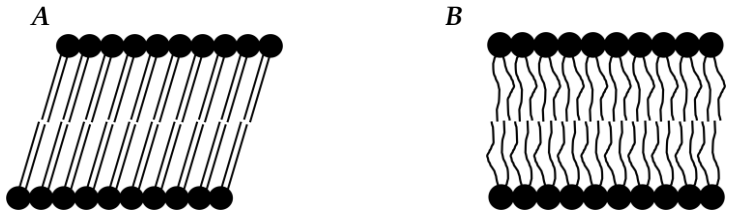

Figure 5. *Schematic picture of a gel phase (A) and a liquid disordered phase (B)*

2.4.3. Liquid ordered phase

Addition of cholesterol to a bilayer will promote lateral phase separation resulting in a coexistence of the L_o and L_d or L_o and L_β phases. The exact nature and formation of such a phase separation dependens both on the lipid structure as well as the physiological environment such as temperature and membrane curvature. In model membranes cholesterol preferably forms L_o phases together with saturated phospholipids (*63-65*)(Fig. 6). Since SM is usually the most common saturated lipid in biological systems it is highly likely that SM-cholesterol ordered domains are the most common ordered structure in cellular membranes. The strength of interaction between cholesterol and SMs in the L_o phase is dependent on the lipid structure, head group properties, hydrogen bonds, acyl chain length and degree of unsaturation (*66-73*). By measuring the

cholesterol desorption rate from monolayers by the compound cyclodextrin (CD) one can obtain an approximate value for the interaction strength of cholesterol and different lipids. From such studies it has been observed that the desorption decreases linearly with increasing acyl chain length of saturated PCs, while the chain length of SMs does not have a major effect on cholesterol desorption from monolayers (*66*). The affinity of sterol for membrane lipids can also be assessed by comparing the distribution of cholesterol between unilamellar vesicles and CD (*74;75*) (for details see Methods, section 4.3.5.).

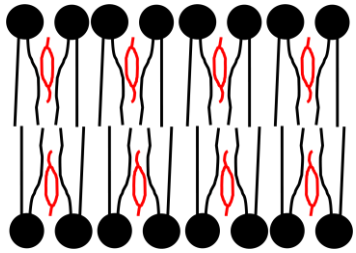

Figure 6. *Schematic picture of a liquid ordered phase where cholesterol is shown in red.*

2.4.4. Model membranes

Lipid vesicles were first described by Bangham and co-workers in the 1960s (*76;77*) and has since then been widely used as models for cellular membranes. Lipid vesicles have been prepared with both distinct lateral and transmembrane distribution. It is also possible to insert transmembrane peptides to the bilayer and even purified protein complexes have been successfully incorporated into vesicles. Cargo molecules can be loaded into the vesicles in the preparation stage to monitor membrane leakage for example. Common methods of vesicle preparation will yield large multilamellar vesicles of different sizes than can then be further processed by sonication and membrane extrusion to control both their lamellarity and size (*78-80*). It is important to keep in mind that the size of the vesicle will determine bilayer curvature and can affect enzymatic reactions and lateral diffusion (*81-83*), lipid miscibility and thermotropic behavior (*84-87*). The vesicles in this thesis were generally 100 to 200 nm in diameter (GUVs being an exception with μm size) and thus the local membrane curvature experienced by the lipids is relatively low.

2.5. Membrane properties of cholesterol

2.5.1. Sphingomyelin-cholesterol interactions

As already mentioned, cholesterol is able to increase the acyl chain order in the L_d phase and decrease it in the L_β phase, creating what is known as the L_o phase. This property of cholesterol is vital, enabling mammalian cells to regulate membrane thickness, fluidity and leakage and also makes the membrane stronger and resistant to osmotic pressure (*88-91*). Although it is almost impossible to directly image L_o domains in living cells because of the size of the domains and their dynamic nature, there are indirect proof of the possible interactions between sphingolipids and cholesterol. Cholesterol and SM distribution in cells correlate (*69*) and it has been observed that plasma membrane SM levels affect both the distribution and *de novo* synthesis of cholesterol (*92;93*). It has been proposed that sphingolipid-cholesterol lateral microdomains, also containing particular proteins, are formed on the membrane of cellular organelles from where they then bud off to form transport vesicles heading for the plasma membrane (*38;39;94;95*). Indeed, secretory vesicles enriched in cholesterol and sphingolipids has been observed to bud off from the *trans*-Golgi network destined for the plasma membrane (*96*). A recent definition of membrane rafts stipulates that they are "small (10-200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes" (*97*). Rafts are thought to be involved in cell signaling, cell recognition, apoptosis and endocytosis and are implicated in many diseases (*39;98-100*). In recent years, scientist have been able to make the first direct observations of sphingolipid-cholesterol microdomains in the plasma membrane of living cells (*101*). By combining the high temporal resolution of fluorescence correlation spectroscopy (FCS) and high nanoscopic resolution of stimulated emission depletion (STED) it was shown that fluorescently labeled sphingolipids and glycosylphosphatidylinositol anchors in the bilayer were hindered in their motions in a cholesterol concentration dependent manner (*101*).

In model membranes it is possible to observe L_o domains of cholesterol and SM. The reason for this interaction is not yet fully understood. The superior hydrogen bonding properties of SM compared to other lipid classes (see Section 2.2.2) and the presence of a hydrogen bond donor (hydroxyl group) in cholesterol has suggested that a direct hydrogen bond link between the C3 hydroxyl group of SM and the hydroxyl group of cholesterol exists (*16;102;103*). IR and monolayer studies on SM analogs with modified functional groups affecting hydrogen

bonding have, however, indicated that the amine group of SM is of greater importance for SM/Chol interactions than the C3 hydroxyl group (*104-107*). Molecular dynamics (MD) simulations in binary systems support a direct SM/Chol hydrogen bonding (*108-110*) but in ternary systems where POPC is added such hydrogen bonds can no longer be observed (*111*). What is observed however is the formation of charge pairs between the hydroxyl group of cholesterol and the positive charge in the choline group of SM (*109;111*). The head group of PSM also tilts down perpendicular to the bilayer normal, which was more than observed for 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) (*112*). The tilt increased with the effective head group surface area and may help SM shield cholesterol from unfavorable interactions with water molecules, a phenomenon known as the umbrella effect (*113*). The umbrella effect may be at least partially the reason for SM-Chol interactions.

As mentioned in Section 2.4.3. the presence of double bonds and methyl branches in the N-linked acyl chain decreases the interactions between SM and cholesterol (*55-62*) as indicated by that the thermal stability and sterol content of the sterol-rich domains decrease if unsaturation and branching is increased (*114;115*). This is true for both SM and PC. Methyl branches display the greatest effect if they are in the center of the acyl chain or close to the interphase, while methyl groups at the end of acyl chains have little effect on SM/Chol interactions (*116*). The lipid head group and electrostatics also have a large effect on sterol interactions. A lipid with a large head group, *e.g.* PC or SM solubilizes more cholesterol than a lipid with a smaller head group, such as PE or N-palmitoyl ceramide phosphoethanolamine (*72;117*). This could be explained by the umbrella model mentioned earlier. Studies in bilayer vesicles have shown that SM has the highest affinity for cholesterol, followed by PS > PC > PE (*75*). Monolayer studies contradict these findings having the lipid species in the order SM > PC > PS ≈ PE (*118*), perhaps a more logical order, since SM and PC shares the same head group structure. Unpublished work from our group has shown that both SM and PC do form sterol rich domains whereas PS failed to do so, indicating that PC is a better partner for cholesterol in the L_o phase compared to PS. The difference between SM and PC can be explained by the difference in head group tilting causing the effective head group area to be larger for SM (*119*). Although the results are still not solid for all the lipid species SM always displays the highest affinity for cholesterol. The varying results obtained in different studies show that the sterol interactions with phospholipids depend on many modes of interaction and involve several structural elements. The contribution of

each structural element also depends on the contribution of the other elements, the adjacent molecular structures and the environment.

2.5.2. Structural modifications of cholesterol

A number of studies have been carried out to try to resolve which functional groups of cholesterol give it its membrane properties. The planar ring system (*120*), an acyl chain with similar structure to that of cholesterol (*121;122*) and the hydroxyl group in the β position at C3 (*123-125*) are all properties that have been deemed important for proper membrane function. These studies have all been done using sterols present in small amounts in cells; precursors or degradation products of some sterol synthesis pathway, and their membrane properties, such as effects on membrane permeability, have been examined and compared to those of cholesterol. The structure of the surrounding lipids will also be of importance and many studies about how the structure of the phospholipids and sphingolipids affect interactions with cholesterol have been carried out.

A number of naturally occurring 3β-analogs of cholesterol is present as intermediates in sterol synthesis or as sterol degredation products. The epimer of 3β-cholesterol, 3*α*-cholesterol or epicholesterol, is a rare cholesterol species in nature. Although structurally differing only in the stereochemistry of the C3 carbon the two epimeres have markedly different membrane properties (*126*). Thus, replacing cholesterol with epicholesterol will inhibit cell growth and survival (*127*). The altered stereochemistry will also lead to a change in membrane location. MD studies of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) bilayers showed that the OH of epicholesterol is located near the phosphate head group of a DMPC molecule whereas the OH of cholesterol was located close to the ester carbonyl groups (*128*). Studies on pure epicholesterol monolayers have shown that it occupies a larger cross-section area and has a reduced collapse pressure compared to cholesterol (*129;130*). It is also less effective in condensing saturated PC monolayers (*131*). Reduction of enthalpy and cooperativity of the gel-liquid crystalline phase transition of PC bilayers observed with the addition of cholesterol was observed to be less obvious when epicholesterol was used instead of cholesterol (*126;132*).^{2}H-NMR studies have also indicated that epicholesterol is less effective in ordering the acyl chains of saturated PCs than cholesterol (*133*). However, C13-NMR and ESR spectroscopy studies on unsaturated PCs showed no difference in the ordering effects of the two sterols (*134-136*). Epicholesterol has also been shown to be less effective in decreasing the passive permeability of PC bilayers than cholesterol (*137;138*). The

flip-flop rate of epicholesterol between the leaflets of a PC bilayer is also much faster than cholesterol (*139;140*). Despite these differences, the L_o domain forming properties of epicholesterol is rather similar to those of cholesterol (*141*).

Oxidation of the sterol 3-OH group yields cholest-5-en-3-one or 3-keto-cholesterol. Studies regarding membrane properties of this cholesterol metabolite are not as extensive as those regarding epicholesterol. In pure monolayers 3-keto-cholesterol occupies a cross-section area similar to that of cholesterol (*142*). The collapse pressure of a 3-keto-cholesterol monolayer is however much lower than that of cholesterol (*142*). The condensing effect in sterol/DPPC monolayers is also somewhat lower for 3-keto-cholesterol (*143*) while in sterol/POPC monolayers the two sterols display a similar condensing effect (*142*). MD calculations have indicated that 3-keto-cholesterol is somewhat less effective in increasing the acyl chain order and the membrane thickness of a DPPC bilayer than cholesterol (*144*). The 3-keto-cholesterol is less polar than cholesterol, leading to an increase in the tilt angle and in the rate of motion in bilayers (*144*). 3-keto-cholesterol will also penetrate deeper into a DPPC bilayer and, unlike most other sterols studied, it occasionally enters the hydrocarbon core of the bilayer and flip-flop to the other leaflet (*144*).

3-keto-cholesterol can be further methylated or ethylated to form cholesteryl methyl ether and cholesteryl ethyl ether respectively. Both of these sterols are able to condense the packing in 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC) membranes (*129*). Cholesteryl methyl ether can also support the growth of *Mycoplasma capricolum* in the absence of cholesterol (*145*). Cholesterol can also be acetylated forming cholesteryl acetate, whose membrane condensing e is only marginal compared to the ether analogs (*129*). It is however capable of sustaining the growth of *M. capricolum (145)*. Other studied natural 3β analogs of cholesterol are for example cholesterol sulfate, a negatively charged cholesterol analog present in several human tissue types (*146*).

2.6. Ceramide as a bioactive molecule

2.6.1. The formation of ceramide rich signaling platforms

Ceramide is both a structural element of cellular membranes as well as an important molecule in cell signaling events. Ceramides have for example been shown to affect cell proliferation, differentiation, migration and apoptosis (*147-150*). The structure of ceramide determines its membrane properties. The saturated acyl chains of ceramide form strong van der Waals interactions with

saturated neighboring lipids and the sphingosine back bone can act as both a hydrogen bond donor and acceptor. The double bond between C4 and C5 has also been shown to be important for the membrane packing of ceramides (*151*). Although little direct evidence of inter-lipid hydrogen bonds between ceramides and other lipids exist, it is known that ceramides form hydrogen bonds to neighboring ceramides. Shah et al. were the first to suggest such hydrogen bond patterns (*152*). Using DSC and X-ray diffraction they proposed that the hydroxyl hydrogen of a ceramide hydrogen bonded with the amide of an another ceramide (*152*). A more recent study proposed that the hydroxyl hydrogen would bond to the carbonyl oxygen instead of the amide (*153*). Ceramides can be generated by *de novo* synthesis, by a salvage pathway or SM degradation by sphingomyelinase (SMase). Out of these the one mostly used by cells in quickly generating larger quantities of ceramide is degradation of SM by SMase. When composed of POPC/PSM were treated with SMase the acyl chain order increased followed by a slower formation of ceramide rich gel domains (*154*). When cholesterol was added to the vesicles presence of phase separation enhanced the SMase activity and that the cholesterol displacement by the ceramide formed only took place at lower cholesterol concentrations (*155*).

The structural properties of ceramide make it very hydrophobic. It has a high T_m and a restricted miscibility with other lipids (*156-159*). Ceramide impose negative membrane curvature and thus curvature stress in bilayer membranes (for more details see section 2.4.1.). Addition of ceramide to a phospholipid bilayer increases the order of the lipid acyl chains similarly to the effects seen with addition of cholesterol (*160-162*). Ceramide has also been shown to, under certain conditions, displace cholesterol from SM/Chol rich ordered domains to form SM/ceramide rich gel domains (*163*).

Ceramides can also induce the formation of lateral microdomains without SM (*164*). Such domains are thought to play a role as signaling platforms. Increase in ceramide content in the membrane could lead to the formation of small ceramide-rich domains that could then cluster together and form larger assemblies (*165*) leading to a spatial and temporal reorganization of membrane components including receptors and signaling molecules and thus facilitating the signal transduction (*166*). Ceramide may not only facilitate formation of a platform were signaling molecules can cluster but it can also recruit molecules to transfer the signal by interacting with the activated receptor as well as exclude unwanted molecules that would interfere with the signal transduction (*166*). The

tight packing of ceramide rich domains might also help to stabilize large signaling complexes so that the signal can be properly transducted trough the membrane (*166*). The most studied case of ceramide platform signal transduction is the clustering and activation of cell death receptors such as Fas/CD95 and CD40 (*167-169;169;169-173*). Ceramide-rich domains have also been implicated in infections by bacteria and viruses (*174-178*), bacterial mediated hot-cold hemolysis (*179*), the death of cancer cells by anti-tumor drugs and radiation (*180-183*) as well as in control of intracellular traffic (*184;185*). There is also evidence from both model and natural membrane studies that ceramide is able form well organized transmembrane channels to accommodate protein and ion diffusion (*186;187*). Long chained ceramides (C24-Cer) have also been shown to form tubular structures when mixed with POPC in giant unilamellar vesicles (*188;189*).

3. AIMS OF THE STUDIES

The main aims of the studies presented in this thesis were to investigate how cholesterol interacts with membrane phospholipids and to understand some of the structural features of both the lipids and cholesterol that modulate these interactions. We also studied a new ceramide delivery system for cultured cells. The specific aims of each paper are listed below.

In **paper I** the aim was to study if the acyl chain order of SM and PC lipids has an effect on sterol bilayer affinity. The acyl chain order of 14:0-SM *versus* 14:0/14:0-PC, 14:0/15:0-PC or 15:0/14:0-PC was determined using fluorescence spectroscopy and NMR. Partitioning of the fluorescent probe cholestatrienol was also measured with single component bilayers. The order and partitioning data were combined in order to determine if the acyl chain order had an effect on the sterol bilayer affinity.

In **paper II** the aim was to compare the effects of cholesterol and the cholesterol analog, aminocholesterol in order to see how the partial positive charge on aminocholesterol affects sterol bilayer behavior. We measured the phospholipid acyl chain order, bilayer lateral domain formation, liquid-ordered domain formation and detergent solubilization protection of both sterols in model membranes.

In **paper III** the aim was to study the biophysical properties of the cholesterol, Chol-PC, in binary and ternary bilayer systems in order to obtain more information about how the sterol shape affects bilayer properties. We examined the interactions of Chol-PC with both saturated and unsaturated ceramides as well as saturated and unsaturated phospholipids to determine how the large phosphocholine (PC) head group affects sterol behavior.

In **paper IV** the aim was to compare the availability of N-hexanoyl ceramide (C6-Cer) to HeLa and rat thyroid FRTL-5 cells when presented either in DMSO as a Chol-PC/C6-Cer lipid complex. Ceramide uptake and effects on cell proliferation, apoptosis and Ca2+ homeostasis were used as indicators of C6-Cer transfer into cells.

4. MATERIALS AND METHODS

Here follows a brief description of the methods used in our studies. This chapter is only meant as an overview and a more detailed description of the methods used can be found in the original publications. Papers I, II and III contain only model system studies whereas paper IV is focused on studies in cultured cells.

Table 1. *List of methods involved in the original papers I-IV*

	Paper			
Technique	**I**	**II**	**III**	**IV**
c-Laurdan emission		x	x	
Fluorescence Anisotropy	x	x	x	
Fluorescence Quenching		x	x	
Fluorescence Lifetime		x	x	
Sterol Partitioning	x			
Differential Scanning Calorimetry		x	x	
Nuclear Magnetic Resonance	x			
Cell Culture				x
TLC			x	x

4.1. Materials

Lipids and lipid precursors used in the studies were purchased from Avanti Polar Lipids (Alabaster, Al, USA), Sigma-Aldrich (St Louis, MO, USA) and Molecular Probes (Leiden, the Netherlands) and used without further purification. In paper I all the lipids used were synthesized in-house according to standard protocols (*66;190*) using the coupling of lyso-SM or lyso-PC to selected fatty acids. c-Laurdan was kindly provided by Professor Bong Rea Cho (Department of Chemistry and Centre for Electro- and Photo-Responsive Molecules, Korea University, Seoul, Korea), synthesized according to (*191*). PSM was purified from egg yolk SM (Avanti Polar Lipids) using reverse phase HPLC (*116*). The fluorescent probes cholestatrienol (CTL) and *trans*-parinaric acid (tPa) were synthesized in-house (*192;193*). The source of other chemicals and molecules used in the experiments can be found in the original publications (I-IV).

4.2. Vesicle preparation

In all of the papers synthetically prepared model membrane vesicles of known lipid composition was used. In brief, the desired lipids and fluorophores were mixed at indicated concentrations from organic solvent stock solutions and then dried under a stream of nitrogen at 40 °C. To insure proper mixing the formed lipid film was then re-dissolved in chloroform and dried again. The lipid films were then hydrated in buffer or milli-Q water (final lipid concentration 50 or 100 μM, see figure legends) at a temperature higher than the T_m for the highest melting lipid species (typically 55-65 °C) to create multilamellar vesicles (MLVs). To produce more uniformly sized MLVs (all experiments except CTL partitioning and LUV size measurements, papers I and II respectively) the solution was sonicated in a Bransonic 2510 (Branson Ultrasonics, Danbury, CT) bath sonicator for 5-10 min at $T > T_m$. For the experiments requiring large unilamellar vesicles, LUVs, (CTL partitioning and LUV size measurements, papers I and II) the hydrated solutions were extruded (at $T > T_m$) through a 200 nm membrane filter to form LUVs. All vesicle solutions were used within 48h of preparation.

4.3. Fluorescence spectroscopy

4.3.1. c-Laurdan emission

To determine the degree of hydration in the bilayer interface (paper II) we used c-laurdan emission measurements. The emission spectra for c-laurdan were measured at the indicated temperatures in MLVs (total lipid concentration 50 μM, c-laurdan present at 1 mol%). The excitation was set to 365 nm and emission spectra recorded between 400 and 550 nm. From the data a generalized polarization value (GP-value) vas calculated according to: (I_{440} – I_{480} nm) / (I_{440} + I_{480} nm).

4.3.2. Fluorescence anisotropy

To study acyl chain order, steady-state fluorescence anisotropy measurements were performed in MLVs using the fluorophore diphenylhexatriene, DPH (*194*). Samples containing 1 nmol of DPH and 100 nmol of lipid were hydrated in 2 ml of milli-Q water or buffer (final lipid concentration 50 μM) and sonicated to create MLVs. The fluorescence intensity was recorded as a function of temperature on a PTI Quanta-Master spectrofluorimeter (Photon Technology International, Lawrenceville, NJ) operating in the T-format. Temperature was increased at a rate of 2 °C/min and the wavelengths used were 360 nm for

excitation and 430 nm for emission. The G-factor was determined prior to each measurement and the measured emission intensity was converted to anisotropy using the FeliX32-software according the formula described in (*195*).

4.3.3. Fluorescence quenching

To obtain information about domain thermostability quenching of the ordered domain fluorophore *trans*-parinaric acid, tPa by the quencher 7SLPC was performed. MLVs at a final lipid concentration of 50 μM were prepared in milli-Q water or buffer in sets of two; the quenched sample and the non-quenched sample. The quenched F-sample contained both tPa at 1mol% and 7SLPC, replacing half of the disordered lipid POPC that was used as the bulk lipid in these experiments. The non-quenched F0-sample contained only tPa (1mol %). The fluorescent emission for both samples was recorded on a PTI Quanta-Master spectrofluorimeter as a function of temperature. Excitation was set at 305 nm and emission at 405 nm and the temperature was ramped at 5 °C/min. The emission intensity was combined as F/F_0 (using FeliX32-software), giving the fraction of non-quenched tPa and plotted against temperature.

4.3.4. Fluorescence lifetime

To obtain information about the degree of order in our model bilayer systems we measured the time-resolved fluorescence decays of the fluorophore tPa. MLVs containing 1 mol% of tPa were prepared to a final lipid concentration of 100 μM. The fluorophore was excited using a 298 nm PLS300 led laser source and the emission data collected at 405 nm using a FluoTime 200/ PicoHarp 300E TCSPC time-resolved spectrofluorimeter (PicoQuant GmbH, Berlin, Germany). The obtained data was fitted with a non-linear least squares iterative reconvolution method, based on the Marquardt-Levenberg algorithm, to obtain the decays. The reduced χ^2 and the random distribution of the weighted residuals were used as justification for the number of exponentials.

4.3.5 Sterol partitioning

To determine the sterol affinity for PC and SM bilayers we measured the distribution of CTL between methyl-β-cyclodextrin (Sigma Chemicals, St. Louis, MO) and large unilamellar phospholipid vesicles according to Nyholm et al. (*196*). Briefly, a buffer solution of LUVs containing the fluorophore is subjected to increasing concentrations of mβCD and the anisotropy of CTL is monitored. From the anisotropy values it is then possible to calculate the amount of CTL left

in the vesicles and further processing of the values yield the molar fraction partition coefficient, K_x, for CTL, a high value indicating strong affinity.

4.4. Differential scanning calorimetry (DSC)

To study the thermotropic properties of SM and PC bilayers with added sterol derivatives we performed DSC analysis on fully hydrated bilayers (composition as stated in papers II and III). The measurements were performed on a Calorimetry Sciences Cooperation Nano II DSC (Provo, UT) and the data was analyzed using CpCalc (CSC, Provo, UT) and Origin 7 (Microcal, Northampton, MA). At least two consecutive heating/cooling scans (0 to 80 °C, rate 1 °C/min) were performed for all samples.

4.5. Nuclear magnetic resonance (NMR)

The NMR experiments in paper I were performed at the University in Utrecht, The Netherlands, by Jacques P.F. Doux and J. Antoinette Killian. These experiments were done with two deuterium labeled molecules 14:0/14:0(d27)-PC and 14:0(d27)-SM prepared in our lab (for detailed synthesis protocol se paper I, Materials and Methods). Two to three milligrams of either lipid was dissolved in deuterium free water and hydrated for 3 hours at 40 °C. After this the samples were freeze-thawn three times and moved to 4mm o.d. ZrO2 NMR tubes and sealed with Kel-F. The NMR measurements were performed with a wide-bore 11.7 T Ultrashield magnet (Bruker Biospin, Billerica, MA, USA) equipped with an Advanced III consol (Bruker, Biospin).

4.5. Cell culture

For the cell experiments in paper IV two cell types were used: Rat thyroid FRTL-5 cells (Interhyr Foundation, Bethesda, MD) and HeLa cells. The FRTL-5 cells were grown in Coon's modified Ham's F12 medium supplemented with 5% calf serum and six hormones in a water saturated atmosphere of 5% CO_2 and 95% air at 37 °C (*197*) and HeLa cells in Dulbecco's Modified Eagle Medium supplemented with fetal bovine serum (10%), L-glutamine (1%) and penicillin-streptomycin (1%) at 37 °C and 5% CO_2. For further reference regarding the performed cell experiments ([^{3}H] labeling, thymidine incorporation, cell counting, cytosolic Ca^{2+} measurements, mitochondrial Ca^{2+} measurements and FACS) please see the Experimental section in paper IV.

4.5.1. Thin layer chromatography (TLC)

To study [^{3}H]-C6-Cer incorporation in HeLa and FRTL-5 cells cellular lipid content was analyzed by TLC. Lipids were extracted using hexane followed by solvent evaporation. After the evaporation the samples were dissolved in hexane/isopropanol for application onto a TLC plate (TLC PE SIL G Flexiplate, Whatman Ltd, Maidstone, Kent, England). Standard lipids were co-eluted together with the cell samples. After elution the spots were dried and visualized using iodine vapor. The desired spots were cut out and placed in scintillator tubes together with 3 ml of scintillator fluid (Optiphase 3, PerkinElmer/Wallac, Turku, Finland) and incubated over night. The next day radioactivity was counted with a LKB Wallac 1216 Rackbeta liquid scintillator counter (Wallac Oy, Turku, Finland).

5. RESULTS

5.1. Effect of acyl chain order on cholesterol partitioning

Cholesterol is an important membrane fluidity regulator but it does not interact equally well with all membrane lipids (*6;65;118;198-200*). For example, given a choice between saturated and unsaturated phospholipids it will prefer the saturated ones (*65;200*). The most abundant saturated phospholipid in biological membranes is SM with a long chain base and an N-linked saturated or monounsaturated C16-24 long acyl chain (*201*). SM and PC share the same head group but the interface region is different. SMs have more possibilities to form hydrogen bonds compared to PCs. When comparing acyl chain matched PCs and SMs, cholesterol will partition with SM (*66;200;202*). The reason for this is not fully understood. NMR and MD studies indicate that although important for SM/SM interactions, hydrogen bonding seems not to play a role in SM/Chol interactions (*203;204*). Another reason has been thought to be the difference in acyl chain order. NMR and MD studies of DPPC and PSM bilayers have shown that although these two lipids have an almost identical acyl chain structure and transition temperature, their acyl chain order profiles are markedly different, that of SM being more ordered (*17;205*). It was therefore of importance to investigate how much the difference in acyl chain order is affecting sterol partitioning behavior. To determine this we investigated the partitioning of the fluorescent cholesterol analog cholestatrienol in membranes composed of myristoyl SM and corresponding PCs under fluid conditions.

5.1.1. The effect of temperature on the acyl chain order

Because of the temperature sensitivity of the probe used (CTL) the lipids were chosen so that they were fluid below ~45 °C (the upper limit for reliable CTL measurements). Because of this limitation we used 14:0-SM which has a T_m of ~24 °C (*54*). The chain matched PCs, 14:0/14:0-PC, 14:0/15:0-PC and 15:0/14:0-PC have T_m of 27, 32 and 31 °C respectively. Since the *sn*-1 chain and the long chain base of SM are not identical in length we chose to include PCs with a moderately longer acyl chain both in the *sn*-1 and *sn*-2 position to see if this had any affect. To determine the relative acyl chain order of these different lipids in the fluid phase we measured the DPH anisotropy at the temperatures indicated in Fig. 7. As expected, 14:0-SM showed the highest degree of order whereas 14:0/14:0-PC showed the highest degree of disorder, the other two PCs giving intermediate values (Fig. 7). Such a difference has been shown previously for other acyl chain

lengths and can be explained by the difference in hydrogen bonding properties between SM and PC (*103;206*).

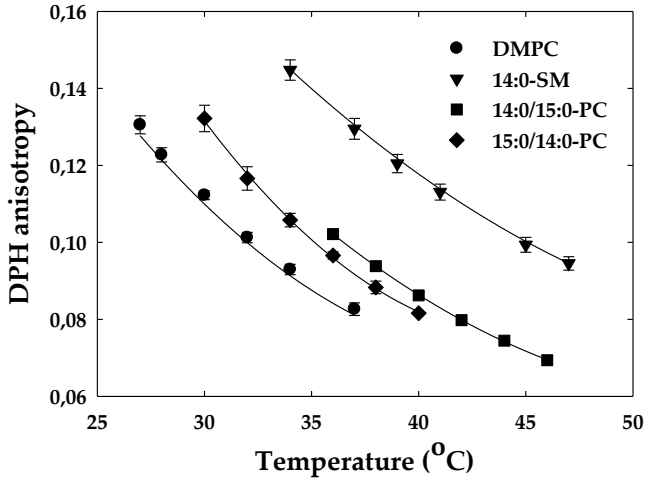

Figure 7. *DPH Anisotropy in pure phospholipid bilayers. Steady-state anisotropy was recorded as a function of temperature for bilayers of the indicated lipids. Each value is the average of 2-3 different measurements (mean ± SEM), the lines are polynomial fits to the data. The figure is adapted from paper I with permission of Cell Press.*

The acyl chain order was also measured by a more direct method, ^{2}H-NMR spectra were measured for perdeuterated tetradecanoate (d_{27}) acyl chains linked to the N- or *sn*-2-position in SM and PC, respectively. This technique allowed for measurements in a pure SM or PC membrane without the addition of probes, and it also provides a more detailed view for the individual acyl chain segments than DPH which only reported an average chain order. The measured spectra were deconvoluted using dePaking algorithms (*207-209*) and from these spectra the order parameter for the acyl chain carbons were obtained (Fig. 3 paper I). For the 14:0-PC carbons 2-8 had no difference in the order profile, only more distant carbons experienced more disorder, consistent with previous studies (*210;211*). For SM no clear plateau was detected, instead the order parameter decreased with decreasing distance from the interface. The average acyl chain order parameter reported by NMR was calculated for carbons 2-8 for PC and 3-8 for SM since these segments are the ones most likely to interact with cholesterol (Fig. 8). The NMR results were in agreement with the DPH anisotropy data, *i.e.* showed a higher order for SM versus PC and displaying the same temperature dependence (lower order at higher temperatures).

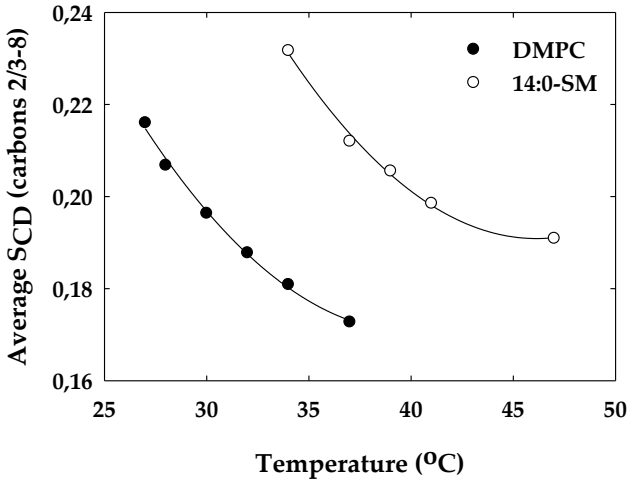

Figure 8. *Average deuterium order parameter (S_{CD}) as a function of temperature. The average order parameter was calculated for acyl carbons 2-8 in 14:0/14:0-PC and 3-8 in 14:0-SM. The lines are polynomial fits and given for visual aid. The figure is adapted from paper I with permission of Cell Press.*

5.1.2. Equilibrium partitioning of cholestatrienol

To determine how the PC or SM acyl chain order affects the CTL partitioning coefficient, K_x, the partitioning of CTL between unilamellar LUVs and methyl-β-cyclodextrin was measured (see Materials and Methods section). The measurements were performed at the same temperatures as the DPH and NMR measurements for each lipid and the obtained CTL partitioning values were correlated with the DPH anisotropy data (Fig. 9A). As evident from the graph there was a significant difference between PC and SM in the affinity of CTL for the bilayer, even at equal acyl chain order. The difference also became larger as the bilayer order was increased (temperature lowered). Since CTL is more restricted in its movements and resides closer to the interface in the bilayer compared to DPH it was of importance to also measure CTL anisotropy and correlate these values with the partitioning data. Data in Figure 9B differed only moderately from figure 9A indicating that even when the probe was different the affinity of CTL was higher for SM than PC. The partitioning data was also plotted against the ^{2}H-NMR data and the same trend was observed (Fig. 6 in paper I).

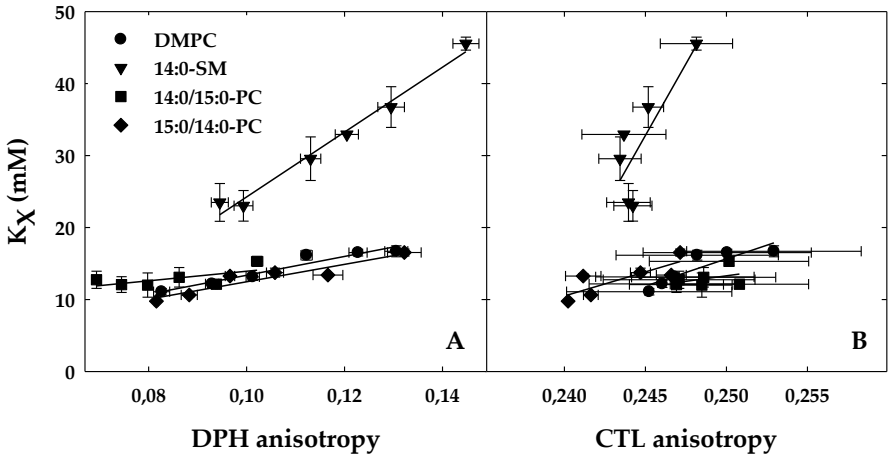

Figure 9. *CTL partitioning into phospholipid bilayers plotted against DPH (A) or CTL anisotropy (B). K_x value of CTL is plotted against DPH or CTL anisotropy. Each value is the average of 2-3 measurements ±SEM. Linear regression fits are added for visual aid. The figure is adapted from paper I with permission of Cell Press.*

5.2. Membrane properties of a positively charged sterol

Sterols are important constituents in cell membranes of most eukaryotic cells. The sterol structure has been well conserved through evolution and only a few sterol species are found among the diversity of eukaryotic cells (*212-214*). Cholesterol, the main mammalian sterol, is an important modulator of cellular lipid bilayers where it can eliminate densely packed gel phases as well as increase the acyl chain order of loosely packed membranes (*215;216*). The changes in membrane fluidity and thickness caused by cholesterol can also help modulate the function of membrane proteins (*217*). The β-hydroxyl group on carbon 3, a planar ring sterol skeleton with the two protruding methyl groups, a double bond at Δ^5 and an iso-octyl side chain are all important for the proper membrane functions of cholesterol, and studies were these molecular structures are altered have shown considerate effects on membrane structure and function (*123-125;218-221*). Apart from cholesterol, some cells also contain cholesterol sulfate, a negatively charged sterol with a sulfate group at the 3 carbon (*222*). Cholesterol sulfate is present only in trace amounts in most cells but has been shown to protect red blood cells against hypotonic hemolysis and to decrease the fertilization efficiency of sperm (*222*). 3β-amino-5-cholestene is a synthetic sterol with an amino group on the 3 carbon, with a partial positive charge (pH

dependent). Although cationic lipids are not found in eukaryotic cells they have been successfully used in biotechnical applications, e.g. to complex DNA with lipids to obtain transfection competent DNA/lipid lipoplexes (*223*). Cationic sterols have been shown to be promising transfection agents as well as having antimicrobial effects (*224-226*). It was therefore of interest to study how aminocholesterol behaves in bilayer membranes.

5.2.1. Ordering of phospholipid acyl chains by sterols

To study the ordering effect of cholesterol or aminocholesterol on PSM and DPPC bilayers in the liquid crystalline phase we measured the anisotropy of DPH in those bilayers (Fig. 11). As evident from paper I, the anisotropy function correlated well with the relative deuterium order profile obtained from ^{2}H-NMR measurements and DPH anisotropy can therefore be considered a valuable tool in determining acyl chain order. Our results showed that both sterols were able to increase the order of the liquid crystalline phase of PSM equally, and that the increased order was concentration dependent (Fig. 11A and B). Also for DPPC an increased order was seen albeit aminocholesterol was less effective than cholesterol (Fig. 11 C and D). These results indicate that both sterols are able to order the acyl chains of PSM and DPPC in the liquid crystalline phase, forming a liquid ordered phase.

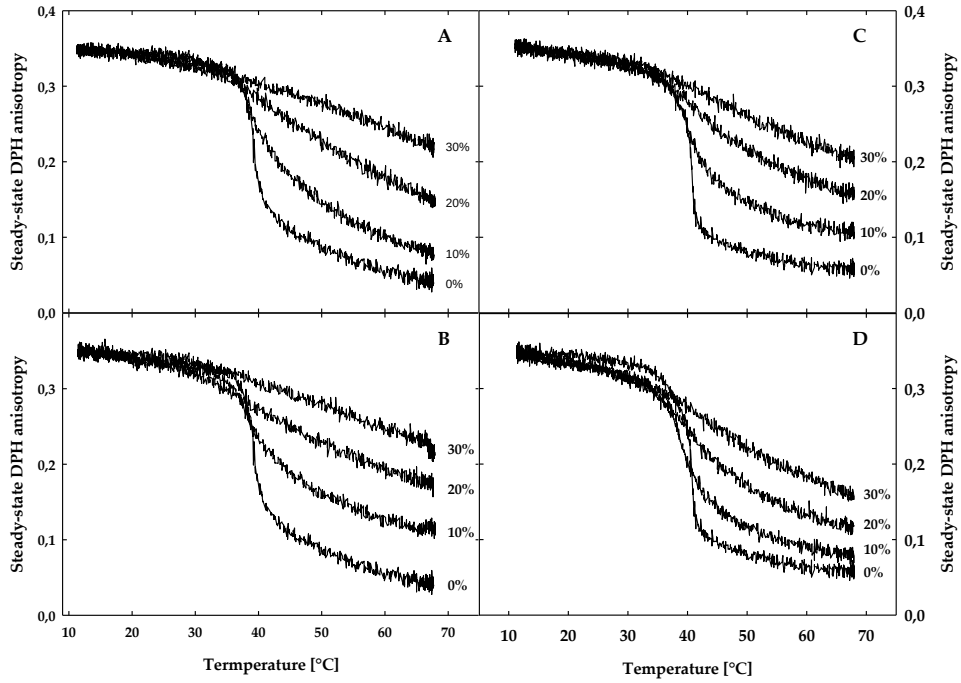

Figure 11. *Steady-state DPH anisotropy in PSM (panels A and B) and DPPC (panels C and D) with increasing amounts of cholesterol (panel A and C) or aminocholesterol (panel B and D) at the indicated concentrations. Total phospholipid concentration was 50 μM and DPH present at 1 mol%. Temperature was ramped 5 °C/min and data are representative from three experiments. The figure is adapted from paper II with permission of ACS Publications.*

5.2.2. Differential scanning calorimetry thermograms of binary phospholipid/sterol bilayers

To further investigate the effect of aminocholesterol on PSM and DPPC bilayers we employed DSC to determine the enthalpy of gel phase melting and cooperativity (Fig. 12). Cholesterol is known to reduce the enthalpy of T_m in a concentration-dependent manner (*227*) and we investigated if aminocholesterol acts analogously. Multilamellar vesicles of either PSM or DPPC with an increasing amount of aminocholesterol showed that already 5 mol% of the sterol was able to remove the pre-transition of both bilayers and reduce the gel to liquid transition enthalpy (Fig. 12). T_m was also shifted to lower values. For PSM, a two component melting was observed, a more cooperative one at 39.9 °C and a

less cooperative one at 40.4 °C (Fig. 12). With DPPC a more symmetric transition was observed at 40.5 °C (Fig. 12). Similar results were obtained when the sterol concentration was increased to 10 mol% and at 20 mol% both bilayers displayed a broad, low-enthalpy transition. The enthalpy decreased almost linearly with increasing sterol concentration, approaching zero at 30 mol% of aminocholesterol (Fig. 12).

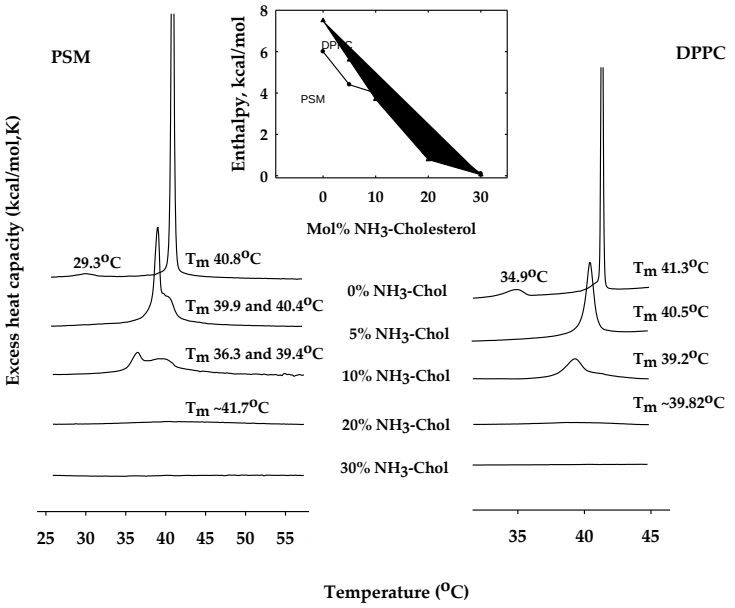

Figure 12. *DSC thermograms of PSM (left) and DPPC (right) in the presence of the indicated aminocholesterol. The phospholipid concentration was 1 mM and the scanning rate was 1 °C/min. The insert displays the calculated molar enthalpies versus aminocholesterol concentration. The figure is adapted from paper II with permission of ACS Publications.*

5.2.3Ordered domain formation in binary and ternary bilayers

DPH quenching was used to determine the presence and thermostability of the ordered domains in binary and ternary bilayers consisting of a disordered lipid, (POPC), and an ordered lipid (PSM or DPPC), with added cholesterol or aminocholesterol (Fig. 13). DPH partitions evenly between ordered and disordered domains whereas the quencher 7SLPC primarily partitions into

disordered domains (*228*). Because of this a fraction of DPH will be protected from quenching if ordered domains are present, and the thermostability of these domains can be measured. In binary POPC/PSM mixtures, ordered domain melting started already at 12 °C and melted completely at 28 °C (Fig. 13, left panel). If 10 mol% cholesterol was added the thermostability of the ordered domain was markedly increased with the end off melting at approximately 50 °C. Surprisingly, aminocholesterol failed to affect the thermostability of the PSM rich domain. When PSM was replaced by DPPC the thermostability for the binary mixture was close to that of PSM but neither of the sterols was able to induce any observable change (Fig. 13, right panel).

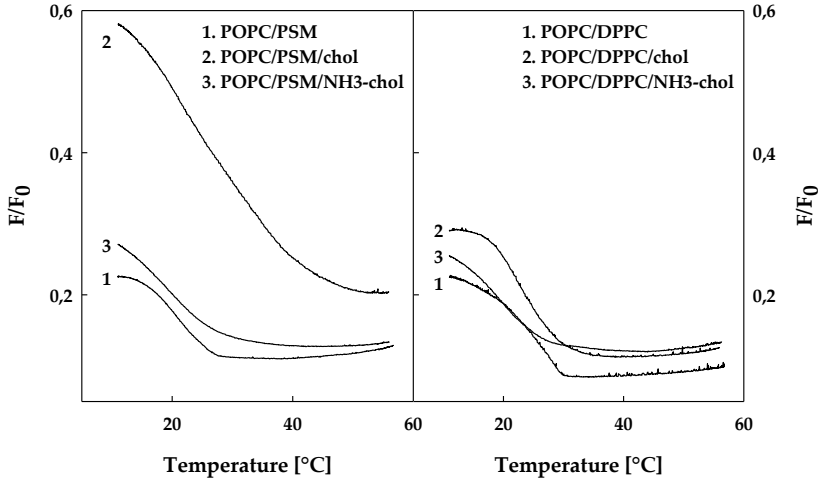

Figure 13. *Quenching of DPH fluorescence in ternary bilayers. POPC was present at 60 mol%, PSM (left panel) or DPPC (right panel) at 30 mol% and the sterol at 10 mol%. Curves are representative of at least three different experiments for each composition. The figure is adapted from paper II with permission of ACS Publications.*

5.2.4. Lifetime analysis of trans-parinaric acid

In order to further explore the ability of aminocholesterol to form a liquid ordered phase we performed lifetime measurements of the probe tPa (Fig. 14). Our samples consisted of POPC and PSM or POPC and DPPC with added cholesterol or aminocholesterol. The lifetime of tPa is relatively short in fluid bilayers and becomes significantly longer if gel phase is present while the

lifetime in the liquid ordered phase is intermediate (*229*). For the POPC/PSM mixture the lifetime was ~12.5 ns indicating that PSM was partially mixed with POPC (Fig. 14) and not in a gel state (the lifetime for pure PSM in gel phase is ~40 ns (*229*)). Addition of cholesterol or aminocholesterol increased the lifetime to 19 and 16 ns respectively. Since the lifetime of tPa in the liquid ordered phase has been determined to be between 15 and 25 ns (*229*) it is evident that both sterols do indeed induce the formation of a liquid ordered phase, aminocholesterol being somewhat less effective. When PSM was replaced with DPPC similar results were obtained and again aminocholesterol was less ordered than cholesterol (Fig. 14).In a binary POPC/DPPC system, however, the tPa lifetime was ~34 ns, indicating that POPC was less miscible with DPPC compared to PSM and that DPPC was able to form a gel phase.

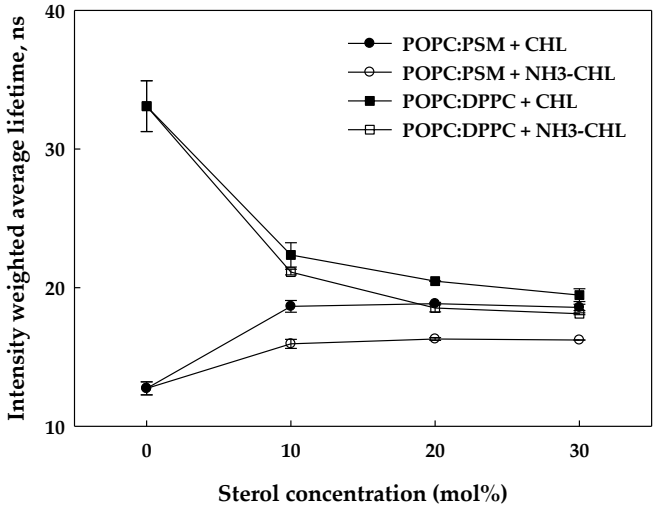

Figure 14. *The lifteime of tPa in ternary bilayers. POPC was present at 60 mol%, PSM (left panel) and DPPC (right panel) at 30 mol% and the sterol at 10 mol%. Each value is the average of three measurements ± SEM. The figure is adapted from paper II with permission of ACS Publications.*

5.3. Membrane properties of a sterol with a large head group

The phospholipid head group structure has been shown to be important for cholesterol interactions in many studies (*118;230*). Cholesterol favors lipids with large head groups such as phosphocholine(*230*). Decreasing the head group size will decrease the interactions between cholesterol and the SM or the less

favorable PC lipid. For example, phosphatidylethanolamines have a markedly lower affinity for cholesterol than PCs (*230*). Also, reducing the PC head group size by removal of methyl groups will reduce cholesterol affinity for the bilayer (*231*). The effect of a large head group could be explained by the umbrella model; cholesterol has a small head group and needs lipids with larger head groups to shield it from unfavorable interactions with water like an umbrella (*113;232-234*). Larger head group lipids also support a higher cholesterol solubility in membranes (*230*).

Ceramide is a sphingolipid with two free hydroxyl groups, one in the C1 position and one in the C3 position in the long chain base (*235*). The hydroxyl in the C1 position is an analog to the 3-hydroxyl of cholesterol. Ceramide interacts favorably with SM and has been shown, under certain conditions, to displace cholesterol from ordered domains indicating that Cer has a higher affinity for SM than to cholesterol (*26;236;237*). Ceramide was also thought to depend on the large head group of SM for protection from water and indeed the solubility of Cer in SM is very close to that of cholesterol (*238*). Recent studies have however shown that the heads group size of SM is not crucial for Cer interactions (*239*). The main reason for the poor mixing of cholesterol and ceramide has been attributed to the small head group size of both lipids and the inability to form stable bilayers on their own. However, when ceramide is given a phosphocholine head group, becoming a SM, the interactions with cholesterol becomes favored. How would the situation be if cholesterol would acquire the head group instead of Cer? Previous studies gave some clues to this question, Chol-PC was shown to form stable bilayer structures with both dimyristoyl glycerol and cholesterol (*240*). A reasonable continuation to this study was to study the interactions between Chol-PC and ceramide. In the following experiments we have studied Chol-PC behavior in binary and ternary bilayers and its interactions with ceramide and saturated as well as unsaturated phospholipids and cholesterol.

5.3.1. Formation of vesicles from cholesteryl phosphocholine and colipids

The first step in the experiments was to test if Chol-PC is indeed capable of forming bilayers together with ceramides and cholesterol (*240*). We prepared both giant unilamellar vesicles (GUVs, Fig. 15) using electroformation and large unilamellar vesicles (LUVs, Table S1 in paper III) using lipid extrusion. The GUVs and LUVs contained an initial 1/1 molar ratio of Chol-PC and either a saturated ceramide PCer, an unsaturated ceramide OCer or cholesterol. Because of the size of the GUVs we were able to visualize them in a confocal microscope

using a bilayer probe (DiI_{18}). We observed that liposomal structures were formed (Fig. 15). Many of the vesicles had complex internal structures and the GUVs containing PCer were generally smaller than the other two (however, no systematic size comparison was performed).

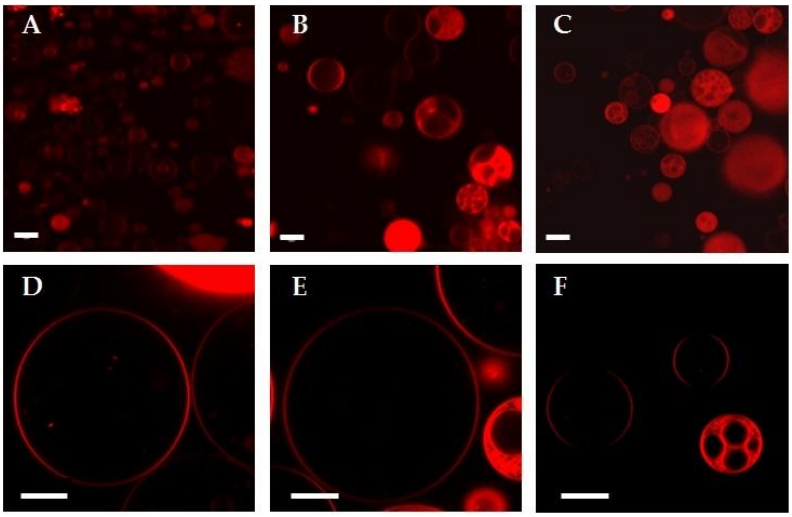

Figure 15. *Giant vesicles made of a 1/1 molar ratio of Chol-PC and PCer (panels A and B), Chol-PC and OCer (panels C and D or Chol-PC and cholesterol (panels E and F). Scale bar is 10 μm. The brightness and contrast of the images was slightly improved. The figure is adapted from paper III with permission of ACS Publications.*

LUVs are too small to be seen in a microscope, but they can be analyzed *e.g.* using light scattering. Size measurements of the different extruded (200 nm) compositions showed LUVs with a diameter of 142 nm to 195 nm (SEM ±25 nm) with no obvious dependence of size or lipid composition (Table S1 in paper III). Besides the 1/1 mixture, also 1/3 and 3/1 ratio mixtures were tested with no observable difference in size or lamellarity. Mixtures of 1/1 ratio of Chol-PC/PCer and Chol-PC/Chol were highly unilamellar and stable. To further study the packing of the bilayers c-laurdan measurements were performed (Fig. 2 in paper III). c-Laurdan is sensitive to the hydration level at the interface. Its emission will be blue shifted in densely packed domains where the interfacial hydration level is low and red-shifted in more loosely packed domains where the interfacial hydration level is higher (*191*). At 20 °C the c-laurdan spectra was observed to be blue-shifted at all compositions, in addition, OCer displayed a small red shoulder, indicating a higher hydration level (Fig. 2A in paper III). When the

temperature was increased to 40 °C the fluorescence of the mixture containing Chol-PC and PCer was moderately red-shifted. The fluorescence of the Chol-PC/OCer mixture was dramatically red shifted and the Chol-PC/Chol was unchanged (Fig. 2B in paper III). GP-values calculated from the spectra show these values decreased for both PCer and OCer when the temperature was increased, whereas that in the Chol-PC/Chol mixture was largely unaffected (Fig 2C in paper III). Lifetime measurements of the tPa probe showed no clear gel-like structures in any of the compositions (Table 1 in paper III). However it showed that Chol-PC/OCer and Chol-PC/Chol mixtures had a similar state of acyl chain order whereas Chol-PC/PCer had a higher order.

5.3.2. Properties of bilayers containing cholesteryl phosphocholine

To investigate the effect of Chol-PC on PSM and DPPC, steady-state DPH anisotropy measurements were performed (Fig. 16). The anisotropy of DPH can be used as a tool to investigate acyl chain packing since the excited state rotation of the molecule will be dependent on the degree of bilayer packing. The results showed that Chol-PC was able to destabilize the gel phase of both PSM and DPPC which was observed when Chol-PC was substituted by cholesterol (Fig. 16). This was not surprising since the large head groups of PSM, DPPC and Chol-PC will lead to packing defects because of the mismatch between the hydrophobic volume and the head group volume. A large head groups prevents a close packing of the sterol ring of Chol-PC and the acyl chains of PSM and DPPC. The ordering of the acyl chains above T_m for PSM and DPPC by Chol-PC was also much weaker compared to cholesterol (Fig. 16). To further determine how Chol-PC causes gel phase destabilization of PSM and DPPC bilayers, DSC measurements were performed (Fig. 4, paper III). Already at 5-10 mol% Chol-PC disrupted the gel to liquid crystalline phase transition, *e.g.* the DSC peaks were broadened indicating loss of cooperativity. When Chol-PC was substituted for cholesterol the peaks were much narrower, indicating a higher cooperativity between the lipid and cholesterol (Fig. 4 in paper III).

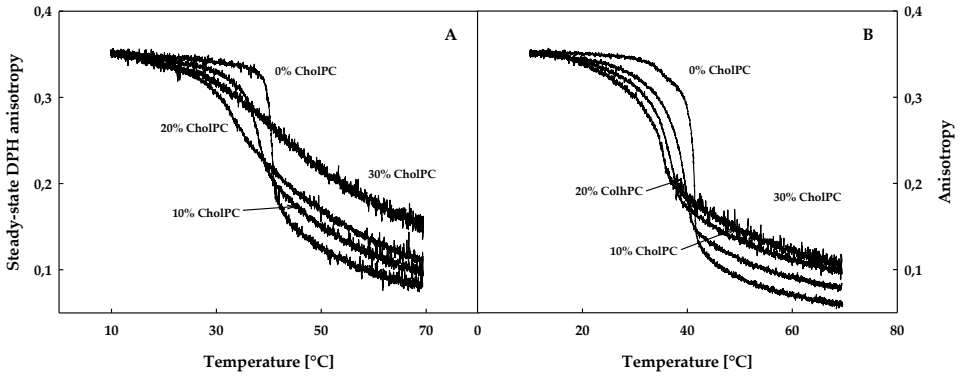

Figure 16. *Steady state anisotropy of DPH in PSM (A) and DPPC (B) bilayers with increasing amounts of Chol-PC. Lipid concentration was 50 μM and DPH present at 1 mol%. Temperature was ramped 1 °C/min. The scans are representative of at least three different experiments. The figure is adapted from paper III with permission of ACS Publications.*

Cholesterol is known to order the acyl chains of phospholipids in the liquid crystalline state. In our experiments Chol-PC failed to increase the order of PSM and DPPC bilayers (Fig. 16). To compare how the position of the head group affects the acyl chain order steady-state DPH anisotropy in equimolar mixtures of either PSM/Chol or PCer/Chol-PC was measured (Fig. 6 in paper III). The results revealed a higher order for the PSM/Chol system compared to PCer/Chol-PC indicating that placing the phosphocholine head group on the sphingolipid improved the acyl chain packing compared to having it on the sterol. The anisotropy results were confirmed with tPa lifetime measurements showing that the lateral packing density was higher for PSM/Chol than PCer/Chol-PC bilayers (Table 2 in paper III).

5.3.3. Ordered domain formation in ternary bilayers

To investigate how Chol-PC behaves in more complex systems we prepared ternary mixtures containing POPC, PCer, cholesterol and Chol-PC in different combinations and measured the ordered domain stability using tPa (Fig. 17). Since PCer is poorly miscible with POPC, it will form ceramide rich ordered domains to which also tPa will partition thereby escaping quenching by the quencher 7SLPC (that preferably partitions into disordered domains). As temperature increases the ordered domains melt and quenching becomes more efficient. For PCer rich domains this happened at 35-36 °C (Fig. 17). If cholesterol was added in equimolar amounts to PCer the domains thermostability decreased

to 28-29 °C. This drop was probably caused both by destabilization of the PCer domains by cholesterol and the ordering effect of cholesterol on the bilayer. Interestingly, Chol-PC behaved as cholesterol, *i.e.* lowering the thermostability similarly (Fig. 17). If cholesterol and Chol-PC were mixed in equimolar amounts with POPC, no ordered domains reported by tPa were detected. Lifetime measurements on tPa in identical systems were in good agreement with the quenching results, *i.e.* showed the same $\tau_{avg.}$ for PCer/Chol and PCer/Chol-PC domains and a markedly lower $\tau_{avg.}$ for Chol/Chol-PC domains (Table 3 in paper III).

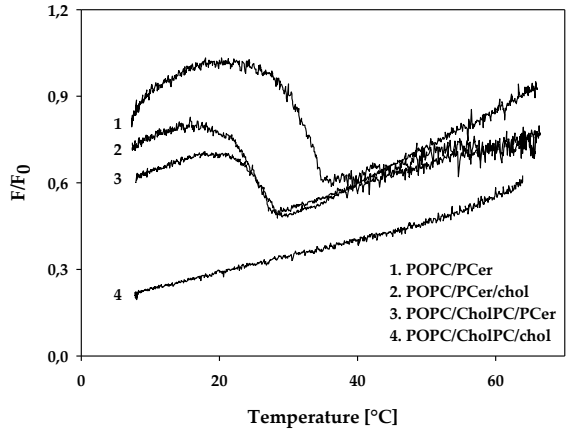

Figure 17. *Ordered domains in POPC bilayers measured as by tPa quenching. POPC was present at 85 mol% in the F_0 sample and in the F sample 30 mol% was replaced by the quencher 7SLPC. PCer and sterols were present at 15 mol% and quencher at 1 mol%. All curves are representative of at least three different experiments. The figure is adapted from paper III with permission of ACS Publications.*

5.3.4. Trans-parinaric acid lifetime in ternary bilayers as a function of sterol content

The addition of cholesterol to bilayers composed of POPC and PSM or DPPC induces a liquid ordered phase at sufficient cholesterol concentrations (*63;229*). The formation of the ordered phase can be detected using tPa lifetime measurements, since tPa preferably partitions into regions of high order where the lifetime is significantly higher than in less ordered regions. In a 6/4 molar ratio POPC/PSM or POPC/DPPC bilayer addition of 5, 10 and 15 mol% of cholesterol induced the L_o phase as evident in Fig. 18. The difference in initial lifetime for PSM and DPPC reflects the differences in the gel phase properties of

these lipids. This was primarily due to a difference in POPC solubility in the ordered domains in PSM and DPPC. When cholesterol was replaced by Chol-PC, the L_o phase no longer formed, further confirming our previous anisotropy results showing that Chol-PC is unable to induce order in PSM or DPPC bilayers.

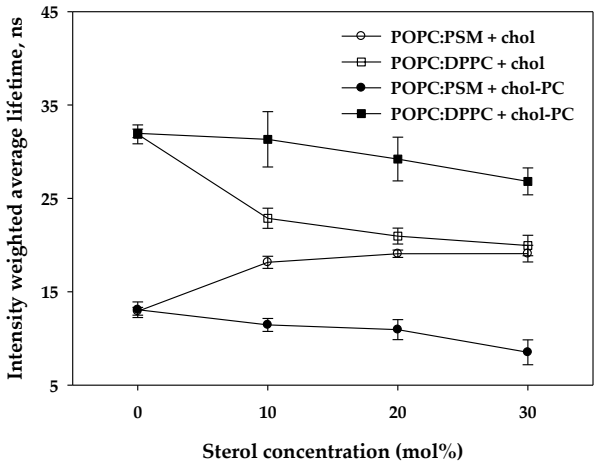

Figure 18. *Lifetime analysis of tPa in ternary bilayers. POPC was present at 60 mol% and PSM or DPPC at 40 mol%. To this the indicated amounts of either cholesterol or Chol-PC was added. tPa was present at 0.5 mol% and total lipid concentration was 0.1 mM. Each value is the average of three different experiments with ± SEM. The figure is adapted from paper III with permission of ACS Publications.*

5.4. Cholesteryl phosphocholine as a potent solvent-free ceramide deliverer

Ceramides are important in cell signaling (*241-245*) and as regulators in cell growth, proliferation and differentiation (*149;246;247*). Because of their hydrophobicity ceramides are poorly soluble in aqueous solutions and thus direct addition of naturally occurring ceramides to cultured cells is difficult. Short-chain ceramides dissolved in small amounts of organic solvent have been successfully incorporated in cell in culture but another possibility would be to use liposomal complexes. Short-chain ceramides are more potent in affecting cell viability compared to their longer counterparts, possibly due to higher cellular uptake (*248;249*). In our previous work we investigated the formation of stable bilayer vesicles composed of Chol-PC and a saturated ceramide, PCer (paper III). These complexes could serve as a new and potent solvent free way to deliver

ceramides to cultured cells. PCer is difficult to deliver to cells because of its relative hydrophobicity and it easily crystallizes in DMSO, commonly used to deliver lipids to cultured cells. We therefore chose to study delivery of short-chained ceramides in order to be able to compare the two methods. To investigate if the Chol-PC/ceramide lipid complexes can be used to deliver ceramide to cultured cells we have compared the bioactivity of N-hexanoyl ceramide (C6-Cer) when presented to HeLa or FRTL-5 cells, either in DMSO or as a complex with Chol-PC.

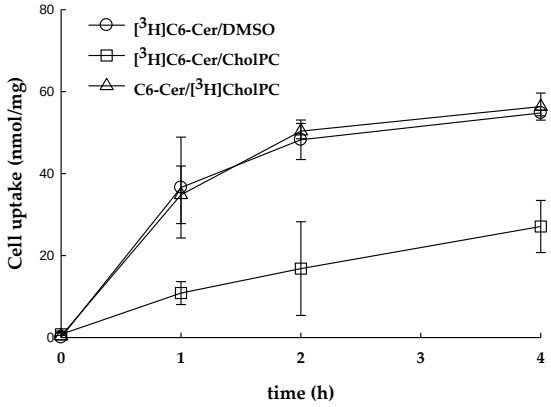

Figure 19. *Incorporation of [³H]C6-Cer or [³H]Chol-PC into FRTL-5 cells. The cells were exposed for the indicated times to 0.05 mM of [³H]C6-Cer/Chol-PC, [³H]C6-Cer/DMSO or C6-Cer/[³H]Chol-PC. The uptake was normalized to cell protein amounts and zero time labeling was subtracted. Each value is an average from 6 dishes ± SEM. The figure is adapted from paper IV with the permission of the authors.*

5.4.1. Cellular uptake of [³H]C6-ceramide from different formulations

To determine if cells are able to take up C6-Cer from the Chol-PC/C6-Cer complexes we measured the uptake of [³H]C6-Cer either in a complex with Chol-PC (at equimolar ratio) or dissolved in DMSO. FRTL-5 cells were exposed to 0.05 mM of [³H]C6-Cer and the cell associated labeling was measured over a 4 hour time period (Fig. 19). We also studied [³H]Chol-PC/C6-Cer complex to monitor the uptake of Chol-PC. The cell associated C6-Cer increased curvi-linearly with time and a more efficient cell association was achieved when C6-Cer was dissolved in DMSO then when complexed with Chol-PC (Fig. 19). Chol-PC association was also found to be time dependent and larger than that seen for C6-Cer. The fate of C6-Cer taken up by cells has already been studied in cultured

cancer cells (*250*) whereas the fate of Chol-PC was still unknown. It was therefore of value to study the fate of Chol-PC once inside the cells. We measured the extent of hydrolysis of [^{3}H]Chol-PC (to [^{3}H]Chol) and followed incorporation of the label into [^{3}H]cholesteryl esters. An increase in cholesteryl ester concentration in cells is an indicator of cholesterol influx into the cells (*251*). After 4h only 8.9±1.9% of [^{3}H]Chol-PC was hydrolyzed to [^{3}H]Chol and only 0.5±0.1% was further converted into cholesteryl esters. We therefore concluded that even though Chol-PC enters the cell it has no significant effect on the cellular mass of free cholesterol.

5.4.2. Ceramide inhibits the proliferation of FRTL-5 and HeLa cells

Since previous studies have shown that proliferation of FRTL-5 cells is inhibited by ceramide (*252;253*) we decided to compare how C6-Cer complexed with Chol-PC or C6-Cer dissolved in DMSO affect FRTL-5 cell proliferation by measuring incorporation of [^{3}H]thymidine into cellular DNA (Fig. 20A). Thymidine incorporation can be used as a proliferation marker since dividing cells will have thymidine bound to their DNA. After 48 h incubation the cells exposed to C6-Cer had incorporated significantly less thymidine than the control cells. There was a difference between C6-Cer dissolved in DMSO or in complex with Chol-PC, the latter displaying a lower incorporation of thymidine (Fig. 20). Further support for the results was obtained by counting the cell number (Fig. 20B). The experiment was also repeated with HeLa cells with similar results (Fig. 3C in paper IV). Effects ceramide were also seen when C6-Cer prepared from L-*erythro*-sphingosine or D-*erythro*-sphinganine were used (Fig. 3D in paper IV) indicating that the stereochemistry was not of importance to obtain the observed effects. To determine that it was in fact C6-Cer and not Chol-PC that caused the observed effects we performed the same experiments using chol/Chol-PC complexes, cholesterol dissolved in DMSO or Chol/mβCD complexes (Fig. 20C). All of these constructs failed to inhibit thymidine incorporation nor did they stimulate it, indicating that the effects observed were indeed due to C6-Cer.

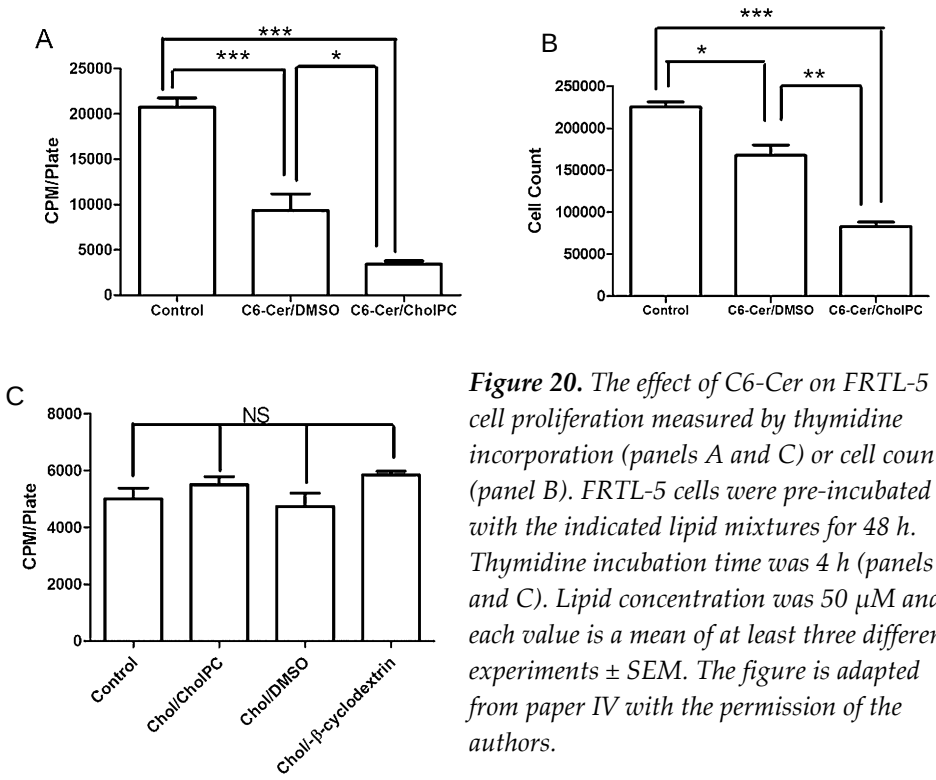

Figure 20. *The effect of C6-Cer on FRTL-5 cell proliferation measured by thymidine incorporation (panels A and C) or cell count (panel B). FRTL-5 cells were pre-incubated with the indicated lipid mixtures for 48 h. Thymidine incubation time was 4 h (panels A and C). Lipid concentration was 50 μM and each value is a mean of at least three different experiments ± SEM. The figure is adapted from paper IV with the permission of the authors.*

5.4.3. Ceramide Induces Apoptosis in the FRTL-5 Cells

Short-chain ceramides are known to induce apoptosis in FRTL-5 cells (*253*). We therefore tested how C6-Cer in complex with Chol-PC compared to C6-Cer dissolved in DMSO in inducing apoptosis (Fig. 21). FRTL-5 cells were exposed to the formula for 48 hours and then analyzed with fluorescence-activated cell sorting (FACS). Dishes not exposed to C6-Cer had less than 5% apoptotic cells after 48h whereas the cells treated with C6-Cer displayed a dramatic increase of apoptotic cells, the C6-Cer/Chol-PC complex treatment being the most effective (Fig. 21). Chol/Chol-PC complexes were used to control that the uptake of Chol-PC did not affect the apoptosis (Fig. 21). The results showed no change as compared to the untreated cells indicating that the apoptotic effect was indeed due to C6-Cer.

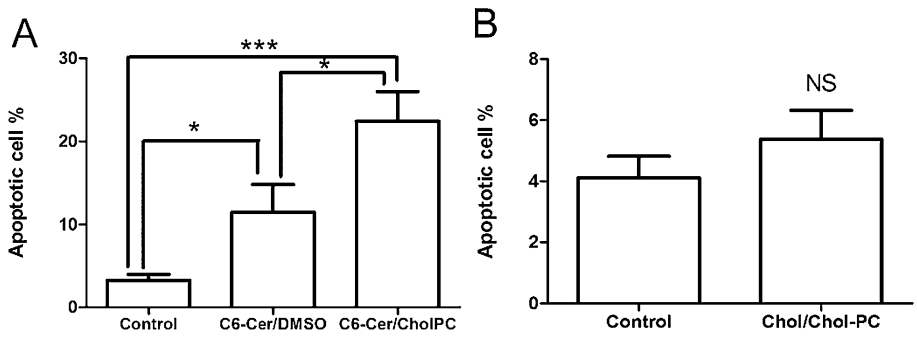

Figure 21. *Induction of apoptosis in FRTL-5 cells by C6-Cer measured by cell counting. Cells were exposed to the indicated mixtures for 48 h and the proportion of apoptotic cells was counted. Each bar value is a mean of three different experiments ±SEM. *p<0.05, ***p<0.0001, NS = no significance. The figure is adapted from paper IV with the permission of the authors.*

6. DISCUSSION

6.1. Effect of acyl chain order on cholesterol partitioning

Numerous studies have shown that several structural properties affect a sterols affinity for phospholipid bilayers (*75;200;230;254;255*). Cholesterol might either have a preference for certain phospholipids, *e.g.* those with larger head groups (*75;200;230*), saturated chains, or an aversion for others, *e.g.* with polyunsaturated acyl chains (*254;255*) forcing it to interact with other phospholipids. In paper I we used CTL as an analog for cholesterol. These two sterols are not identical; the double bond in the B and C ring of CTL yields it more polar and changes its planarity compared to cholesterol (*192;256;257*). Despite these differences, CTL partitions into the L_o phase and condenses bilayer packing as well as increases the acyl chain order (*115;258-260*). Although the absolute partitioning values are not the same for CTL and cholesterol, the relative partitioning of CTL between cyclodextrin and bilayer membranes is similar to that of cholesterol (*258;259*), thus making CTL a useful probe for measuring sterol partitioning between cyclodextrin and different lipid bilayers. The lipids used in the study are common in the cellular membranes of most cells (*206;261;262*) and were chosen because they are fluid at the temperature range where reliable CTL measurements are feasible. The PC and SM lipids studied were also fairly symmetric regarding the length of the acyl chains, eliminating the possible effect of interdigitation (*263*).

Our results revealed that at equal DPH anisotropy the affinity of CTL was highest for SM bilayers and lowest for 14:0/14:0-PC bilayers. This was true also for equal CTL anisotropy and deuterium order parameter indicating that CTL interacts more favorably with SM compared to PC when the acyl chain order parameter is equal. The difference between SM and PC also increased with increasing acyl chain order (decreasing temperature). This could be explained by the effects of temperature on the hydrogen bonding network, *i.e.* the lower the temperature the stronger the hydrogen bonds due to decreased thermal motions. Since SM can form more hydrogen bonds than PC, the net increase in hydrogen bond strength will be larger for SM than PC when the temperature is lowered. MD simulations have shown that SM can form both inter and intralipid hydrogen bonds (*17*) which decrease the lateral diffusion and rotational motion of SM and thereby indirectly strengthen the interactions with cholesterol. Hydrogen bonding between SM and cholesterol are still debated (*203*) (see above). The importance of SM hydrogen bonds is indicated in studies where the

hydrogen bonds was abolished upon methylation of the OH or NH groups of SM which led to the abolishment of SM-cholesterol interactions (*262*). Studies that have shown that the higher acyl chain order in SM compared to PC plays a role have all been done in mixed bilayers (*111;200;258*). In such mixtures SM might prefer to hydrogen bond with cholesterol instead of PC, whereas our data is focused on pure systems where the sterol can only choose to act with either PC or SM. Although SM and PC share the same head group its dynamics and orientation might differ. Several studies have shown that head group properties are of importance for Chol/PC interactions (*75;113;264*). MD simulations on PSM and DPPC point to minor differences in the head group orientation (*17*). These findings are further supported by NMR studies indicating differences in SM and PC head group orientation (*265-267*).

6.2. Effect of an amino group on the 3β carbon of cholesterol

In paper II we have explored the role of the polar moiety of cholesterol by changing it from a hydroxyl group to a (partially) positively-charged amino group. These subtle changes in the sterol structure could to discover which structures are important for maintaining the intramolecular interactions required for sterol membrane function. The hydroxyl head group of cholesterol helps to orient cholesterol in the bilayer (*268*). Replacing it with a partially charged amino group is unlikely to affect the orientation since both the hydroxyl and the amino group are of roughly the same size and both have the capability to form hydrogen bonds with adjacent lipids. However, a change in the molecule tilt angle in relation to the bilayer normal is possible. One indication that this indeed takes place is that aminocholesterol was slightly less effective in ordering DPPC bilayers compared to cholesterol. This effect is not seen for PSM/sterol bilayers, probably due to different electrostatic interactions between PSM and the sterol. The positive charge could also affect solvation and thus the depth of penetration of the sterol. This option is supported by the previously reported increase of the inter-bilayer translocation of aminocholesterol when compared to cholesterol (*269*). Our DSC measurements showed that at lower sterol concentrations (5-15 mol%) aminocholesterol and cholesterol displays very similar effect on the thermotropic behavior of PSM and DPPC. However, at higher concentrations (20 mol%) aminocholesterol almost abolished the transition enthalpy whereas with cholesterol a residual transition enthalpy. The melting profile of PSM/aminocholesterol bilayers was also more complex compared to PSM/Chol, probably because of the positive charge and how it affects the interactions with PSM. The hydrogen bonding between PSM and aminocholesterol is also likely to

be different from that of DPPC and aminocholesterol. DPH anisotropy data together with DSC and c-Laurdan measurements indicate that aminocholesterol is able to participate in a liquid ordered phase similar to cholesterol or saturated phospholipids. Previous studies have shown that in a ternary system of POPC/PSM or POPC/DPPC and cholesterol the sterol will partition into the ordered domains formed by PSM or DPPC and increases the stability of these domains probably by increasing van der Waals interactions or by excluding POPC molecules from these domains (*270*). When the sterol was replaced by aminocholesterol, the stabilization effect disappeared, apparently because of more unfavorable interactions with the ordered lipids or because a larger portion of aminocholesterol partitioned into the POPC rich phase. Experiments with tPa measurements indicated that aminocholesterol was indeed able to form liquid ordered domains together with PSM and DPPC, although those domains were less ordered compared to cholesterol containing domains.

6.3. Effect of a large head group on sterol behavior

6.3.1. Interactions with other membrane lipids

Studies by Gotoh et al. showed that a sterol phosphate can form stable bilayers together with cholesterol or saturated diacylglycerol (*240*). In paper III we explored this issue in more detail to understand how the shape of interacting molecules would affect these interactions, mainly by manipulating head group to acyl chain volume ratio. Both cholesterol and ceramide induce negative membrane curvature and the inverted cone shape of the molecules prevents them from forming stable lamellar bilayers on their own. When ceramide acquires a large head group in the form of a phosphatidylcholine, becoming a SM the acyl chain to head group volume will change. The change in lipid shape will allow the SM to form a stable lamellar bilayer on its own and also together with cholesterol and/or ceramide. We set out to investigate how cholesterol with a large head group (phosphocholine) would interact with cholesterol and ceramide or with saturated or unsaturated phospholipids in model bilayers. First, we confirmed the results by Gotho et al. showing that Chol-PC forms bilayers together with cholesterol. We also extended the experiments to ceramides which have a structure similar to diacylglycerol but with the ability to form more extensive hydrogen bonding networks (*152*). Both GUVs and unilamellar LUVs could be formed. The c-Laurdan probe is sensitive to interfacial hydration (*271*) which makes it suitable to study the interfacial packing density of lipid bilayers. All systems tested showed low interfacial

hydration at 20 °C. At this temperature PSM bilayers are in the gel state and have a GP value of 0.42 (*71*). Based on the GP value one could conclude that Chol-PC/PCer bilayers, with a GP of about 0.35, are similarly hydrated as PSM and be gel-like. However, tPa lifetime measurements displayed much lower values for Chol-PC/PCer (9-18 ns) than for pure PSM bilayers (~32 ns (*229*)). In fact, the lifetime obtained for Chol-PC/PCer is much closer to those reported for PSM/Chol bilayers in a liquid-ordered state (*229*). The reason for the fluid state of the Chol-PC/Cer bilayers is probably due to the rough surface of Chol-PC with protruding methyl groups in the sterol body which prevents efficient van der Waals interactions between the lipids, thus weakening the packing. The formation of Chol-PC/Chol bilayers can largely be explained by the umbrella model; a lipid with a large head group can solubilize a co-lipid with a small head group (*113;230*). The phosphocholine head group of Chol-PC can shield cholesterol from unfavorable water exposure, thus favoring a bilayer formation.

It is known that SM and a saturated PC do mix and that the ideality of the mixture depends on the hydrophobic interactions between the acyl chains (*272*). When Chol-PC was added to either PSM or DPPC bilayers a dramatic destabilization of the lipid gel phase took place. This destabilization is most likely due to mismatch between the head group and the hydrocarbon volume. It has been estimated that PSM has a volume per lipid of 1175 $Å^3$ (*273*), whereas the volume for cholesterol is only 630 $Å^3$ (*274*). If PSM is stripped of its head group (creating PCer) the volume drops to 990 $Å^3$ (*273*). Based on these numbers it becomes clear that the hydrophobic part of Chol-PC will not be able to fill the intermolecular void when it is mixed with PSM, resulting in the observed destabilization.

6.3.2. Cholesteryl phosphocholine/ceramide vesicles as potent ceramide vehicles

Ceramides are bioactive molecules that affect many cellular processes *e.g.* via ceramide rich domains, enzyme modulating or formation of membrane pores. Ceramides are able to induced cytochrome leakage from the mitochondria (*275*). To study the effects ceramide in cells one can generate ceramides either through the action of sphingomyelinases degrading SMs to ceramides or by transferring ceramides from an extracellular carrier in the medium. The hydrophobicity of ceramides makes it difficult to transport them via the aqueous culture media and thus organic solvents such as DMSO are often used. In paper III we showed that Chol-PC is able to form stable fluid bilayers together with C16-Cer. In paper IV we explored the possibility to use such complexes to deliver short-chain

ceramides to cells. The antiproliferation effects of short-chain ceramides are well documented (*147;276*) and in this study we chose to focus on C6-Cer, known to affect cell growth and apoptosis. Chol-PC/C6-Cer complex was indeed much more effective in prohibiting cell growth and induce apoptosis than C6-Cer dissolved in DMSO although radiolabeled C6-Cer was taken up more efficiently when in DMSO. The higher uptake is likely caused by the crystallization of the ceramide when mixed with the medium and crystals sticking to the cell surface rather than being taken up into the cell. C6-Cer was effective in inhibiting cell growth in both FRTL-5 and HeLa cells and unexpectedly the same effect was seen for dihydroceramide or the unnatural L-*erythro* form of C6-Cer. It is therefore evident that even though several studies have noted the importance of right stereochemistry (*277-279*), for cell growth both the L and the D-isomers are equally effective. Observing the apoptotic levels of treated dishes revealed that the complex-treated cells displayed the highest levels of apoptotic cells, indicating good ceramide entry into the cells. To verify that the effect was indeed due to the effect of C6-Cer, we studied Chol-PC/Chol vesicles and observed no effect on the cells, thus showing that the observed effect was indeed due to ceramide.

The cellular entry mechanism and the fate of C6-Cer inside the cell was not explored in this work. Ceramides dissolved in DMSO are likely to precipitate when added to the aqueous culture media and thus the bioavailability of crystalline ceramide is low. By mixing the ceramide with Chol-PC the crystallization can be avoided. The fluid nature of the bilayer and the fact that ceramide probably prefers to interact with phospholipids over Chol-PC would allow for transfer of monomeric ceramide from the complex to the plasma membrane. It is know that the hydrophobicity of lipids correlate negatively with the efficiency of transfer between membranes (*280;281*). The longer the acyl chains the slower the transfer rate. This was the main reason for choosing short-chain C6-Cer in this study. It is possible that some of the Chol-PC/Cer complex could fuse with the plasma membrane. Once the ceramide has entered the cell it could be re-acylated to form long-chained ceramides. The ceramide would also need to be transferred to the ER and mitochondrial membranes and the nucleus in order to induce the observed effects.

7. CONCLUSIONS

The results in paper I clearly demonstrate that acyl chain order markedly influence the sterol/phospholipid interactions; however when the acyl chain order is equal the sterol will prefer to interact with SM over PC. It seems that sterols have a larger ordering effect on the acyl chains of SM, possibly due to the stronger interactions between SM and sterol compared to PC. Both differences in head group area and hydrogen bonding properties could be the reason for this difference. SM cholesterol interactions are important for the formation of ordered domains in cell membranes. These domains may function as cell signaling platforms, aiding in receptor clustering and thereby be a vital part of proper signaling transduction and support important cellular functions.

In paper II the hydroxyl group of cholesterol was changed to an amine group, making it more polar. The results indicated that this modification did not largely affect the membrane behavior of aminocholesterol compared to cholesterol. This is promising in the aspect of using aminocholesterol as a cationic lipid in biotechnical applications where a positive charge and a competent sterol are needed for optimal function.

In paper III we investigated how a large phosphocholine head group on cholesterol (Chol-PC) affects its membrane properties. It was clear that Chol-PC preferred to interact with lipids having small head groups, *i.e.* cholesterol or ceramides and that it disrupted the packing of lipids with larger head groups, *i.e.* PSM and DPPC. The results indicated the importance of lipid shape in forming stable bilayers and the prospect of stable and fluid vesicles with a high ceramide content may open up new possibilities in drug delivery and other medical applications.

In paper IV we explored the use of Chol-PC/C6-Cer lipo-complexes as a solvent free ceramide delivery system for cultured cells. The results showed that the complex was much more efficient in causing inhibition of proliferation, apoptosis compared to C6-Cer delivered dissolved in DMSO. We have proposed that C6-Cer in complex with Chol-PC provides a better bioavailability compared to the use of solvents and can be successfully used to deliver ceramides to cultured cells.

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