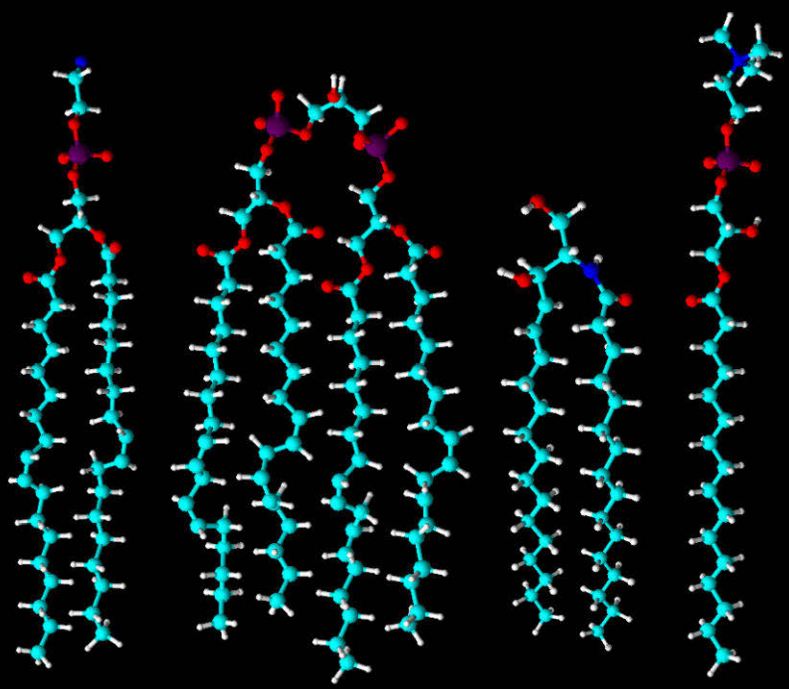
Anna Möuts

Membrane lipid frustration: Contribution of ceramide and its effects on lateral segregation

Studies on ceramide interactions with non-bilayer prone lipids



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Author contribution in the original publications:

I. Participated in study design with the supervisor. Experiments were performed together with Elina Vattulainen, while the ceramide analogues were provided by our Japanese collaborators. All authors contributed to the interpretation of the data and writing of the manuscript.

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Abstract

Ceramides are both sphingolipid precursor molecules and breakdown products with effects on membranes that are of interest both from biophysical and physiological points of view. When present in a membrane, ceramide induces the formation of highly ordered domains. Ceramide accumulation in biological membranes has in turn been suggested to be involved in committing the cell to death, possibly through effects on membrane fluidity or as a signalling molecule involved in the apoptotic cascade. Though ceramide aggregates at low concentrations in the membrane, this effect can be promoted by other major membrane lipids. Notably some of these are lipids typical of mitochondria, which also is the proposed site of ceramide mediated apoptotic induction. In the interaction of ceramide with its co-lipids the proportion of the headgroup size to the volume occupied by acyl chains appeared as an important factor to consider along with alterations of the hydrogen bonding competency of ceramide itself to other surrounding lipids. The promoted lateral segregation of ceramide by co-lipid molecular shape seems to be driven by different mechanisms. Lipids with proportionally small headgroups interact unfavourably with ceramide which as a result is pushed away, while lipids with large headgroups and small acyl chain volume provides shielding from the aqueous environment.

Abstrakt

Ceramider är både byggstenar och nedbrytningsprodukter av sfingolipider vars inverkan på membraner är intressanta ur både biofysiskt och fysiologiskt perspektiv. Vid närvaro av ceramid i ett membran bildas domäner med ökad ordningsgrad i förhållande till omgivande membran. Ansamling av ceramider i cellens membraner har påvisats ha en koppling till att förpassa cellen till att genomgå programmerad celldöd, möjligen genom att påverka membranets fluiditet eller som en signalmolekyl som utlöser en apoptotisk kaskad. Ceramider aggregeras vid låga halter i membraner, men denna effekt kan förstärkas av andra betydande lipidklasser. Anmärkningsvärt är att vissa av dessa lipider är typiska för mitokondrier, som också anses vara startpunkten för utlösandet av apoptos. I ceramiders samverkan med andra lipider i membranet visade vi att förhållandet mellan huvudgruppens storlek och acylkedjornas volym samt ceramidens vätebindningspotential är viktiga faktorer att ta i beaktande. Den förstärkta laterala segregeringen av ceramider som förmedlas av omgivande lipiders form verkar ha olika bakomliggande mekanismer. Lipider med förhållandevis små huvudgrupper samverkar ofördelaktigt med ceramid och har en frånstötande effekt, medan lipider med stora huvudgrupper och liten acylkedjevolym ger ceramiden skydd från omgivande vatten.

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1. Review of litterature

1.1 Introduction

One fundamental aspect of life is its separation from the outside environment by a membrane and sometimes a cell wall, composed of various lipids and proteins, resulting in the smallest unit of a living organism; the cell. This semi-permeable membrane allows biomolecules to reach concentrations at which biological processes can take place at a higher rate than if said molecules were not confined within a limited space. Eukaryotic organisms have also developed organelles to further compartmentalize biochemical reactions. The plasma membrane and the membranes of organelles are the site for membrane proteins involved in both cell signalling and in energy metabolism, as well as in a multitude of biosynthetic pathways [1]. The fluid mosaic model [2] by Singer and Nicolson in the early 1970's presented the lipid bilayers of the cell membrane and the membranes of organelles as fluid systems in which lipids can diffuse laterally, as can membrane proteins embedded in the bilayer. Still, the lipids in the membranes are far from a passive solvent for membrane proteins, but can in themselves function as potent signalling molecules [3] or by providing distinct structural variations in the membranes that in turn can regulate protein activity [4]. Considering this, membranes are far from homogenous in terms of fluidity and distribution of both lipids and membrane proteins [5-7].

Membrane lipids are amphipathic molecules that contain a polar headgroup and hydrophobic acyl chains. The spontaneous organization of lipids into a bilayer is a result of minimizing the unfavourable interactions of hydrophobic parts of the molecules to water, by sequestration of the hydrocarbon chains to the membrane interior while the headgroups are oriented to face the aqueous environment of the lipid molecules [8]. One important characteristic of the membrane is thus that it is formed spontaneously, without the need for input of energy in order to be assembled [9]. The bilayer consists of two lipid leaflets, with headgroups facing outwards to the aqueous environment while the acyl chains are facing the interior of the membrane [10]. Within the interior of the membrane the dominating type of interaction between the acyl chains are weak van der Waals forces, though the weakness of singular such bonds is outweighed by the amount interactions between the hydrocarbon chains [11]. Closer to the interfacial region of the membrane there is an increased amount of hydrogen bonding potential [12] and with headgroup-water interactions and headgroup-headgroup interactions including both hydrogen bonding and polar interactions [13, 14].

Biological membranes are usually organized into lamellar phases, though this is not the only possible structure lipids may organize into. Depending on properties such as molecular shape and functional groups of the lipid in question, or the degree of hydration, some lipids will spontaneously form inverted hexagonal phases [15]. Cubic phases may also form under specific

conditions [16]. Micelles or lipid crystals are other aggregates that may form. Membranes are thus highly dynamic structures with their physical state being a result of the ensemble of interactions of a multitude of lipid species, ever changing both trough biological and physical cues.

1.2 Membrane structure

1.2.1 Bilayer lipids

Cells contain thousands of lipid species of which membrane lipids have specific function as an ensemble [1, 17]. Phosphatidylcholine (PC) is the most abundant lipid class in mammalian cell membranes. Other abundant glycerophospholipids are phosphatidylethanolamine (PE) and phosphatidylserine (PS), as well as slightly lower concentrations of phosphatidylinositol (PI). Cardiolipin (CL) is mainly found in the mitochondrial inner membrane and is a diphosphatidylglycerol. Another significant lipid class is the sphingolipids, of which sphingomyelin is one of the most common ones in plasma membranes [1] while glycosphingolipids being particularly abundant in myelin producing oligodendrocytes [18]. Sterols are a third important class of lipids, of which cholesterol is the sterol found in animal cell membranes [1].

1.2.1.1 Glycerophospholipids

The glycerophospholipids have a glycerol backbone as their defining structure, which binds the fatty acids of the acyl chains through covalent bonds at the *sn*-1 and *sn*-2 positions. The headgroup is also covalently linked to the resulting diacylglycerol skeleton. Biologically relevant lipids often have mixed acyl chains, the acyl chain on the *sn*-1 position is usually saturated, while the *sn*-2 acyl chain in turn is unsaturated [19, 20]. Due to the position of the lipid in the membrane, there is a slight mismatch in the length of the acyl chains even if they would contain an identical number of carbons, as the acyl chain at the *sn-2* position has to bend in order to be perpendicular to the plane of the membrane [21, 22]. The acyl chains are usually even numbered and between 12 and 22 carbons [23] in length with linear structures, although bacteria can also contain lipids with branched acyl chains and acyl chains with an odd number of carbons [24], of which the branched acyl chain containing lipids seemingly functionally replace unsaturated lipids in maintaining membrane fluidity [25]. In addition, the acyl chains may be unsaturated to varying degrees [26], which is the presence of one or more *cis*- double bonds in either one or both chains. *Trans*-double bonds occur to a lesser degree in animal membrane glycerophospholipids [27], though they can be important components of bacterial [28] and photosynthetic membranes [29]. Presence of *cis*-double bonds increases the space that the acyl chain occupies within the membrane which results in weaker interactions with adjacent lipids which in turn translates to increased fluidity of the membrane [30].

1.2.1.2 Phosphatidylcholine

Phosphatidylcholines (PC) have a choline headgroup that together with typical acyl chain conformations comprise a molecule with a roughly cylindrical shape [31]. Due to the relatively neutral molecular shape, PC species readily form bilayers and are a major component in biological membranes [1]. These lipids have been extensively studied as model membranes. The most common acyl chain lengths in PC's in biological membranes are 16 and 18 carbons, with typically one chain being saturated and the other one being mono- or polyunsaturated [32] that can categorize the lipid as a so called hybrid lipid [33]. Nevertheless, both PCs with two fully saturated acyl chains or two unsaturated chains are also common. The majority of PC synthesis takes place at the endoplasmic reticulum where the rate-limiting step is catalysed by cholinephosphate cytidylyltransfease [34] in the CDP-choline (Kennedy) pathway [35].

1.2.1.3 Phosphatidylserine

PS in mammalian cells is asymmetrically distributed in the plasma membrane, found in the cytosolic leaflet [36]. Its translocation to the extracellular leaflet is a known apoptotic signal [37]. The serine headgroup has a negative charge that despite potential electrostatic repulsion causes PS to occupy a smaller area in a membrane than PC with corresponding acyl chain compositions, causing a more condensed state [38]. PS is largely synthesized from PE and PC in the mitochondria-associated membrane in the ER by exchanging the headgroup [39, 40].

1.2.1.4 Phosphatidylethanolamine

Phosphatidylethanolamine is an abundant lipid in the mitochondrial membranes and at the cytosolic leaflet of the plasma membrane [41], while it is also an important lipid species in bacterial membranes, as bacteria generally lack PC in their membranes [42-44]. Phosphatidylethanolamine is produced by the other branch of the Kennedy pathway [35] or by decarboxylation of PS in the mitochondria [45, 46] of which the latter seems to be the more important source in mammalian cells. Due to the small size of the PE headgroup compared to other membrane lipids, it is considered having a conical shape and thus creating curvature stress in its lipid environment. Therefore, PE promotes negative curvature in both model membranes and also in biological membranes [47], where PE is found involved in membrane fusion events [48, 49] and also in the polar ends of rod shaped bacteria [50] in membrane regions of high curvature. In model membranes PE promotes the transition into both cubic phases [16] and inverted hexagonal phases (H_{II}) while also lowering the transition temperature to the nonlamellar phases with increasing PE content [51]. Localization to the inner leaflet of membranes is energetically favoured, and can cause coupling of phase transitions in asymmetric vesicles [52]. Another effect of the comparatively small ethanolamine headgroup is that it allows PE molecules to pack more

tightly [53], thus increasing the thermostability of PE compared to PC molecules with identical acyl chain compositions. The headgroup structure binds fewer water molecules than PC, leading to an increased hydrogen bonding capability and stronger intermolecular interactions that also lead to an increased propensity to form dehydrated structures or aggregation or abandonment of lamellar phases [54, 55].

1.2.1.5 Cardiolipin

Cardiolipin is mainly found in the inner mitochondrial membrane, though it can be externalized to the mitochondrial outer membrane under specific conditions, such as to function as a signal for mitophagy of defective mitochondria [56]. CL is also found in bacterial membranes, especially in the polar ends and septa [50, 57], thus giving support to the bacterial origin with the endosymbiotic hypothesis of the mitochondria. Its structure is quite distinct from other membrane lipids in that it is essentially composed of two diacylglycerol molecules connected by glycerol [58]. CL carries two negative charges that upon binding a divalent cation such as Ca^{2+} can induce the formation of inverted hexagonal (H_{II}) phases if the acyl chains are unsaturated, while a lamellar phase is preferred in absence of divalent cations [59]. The charges have also been thought to have a function in the mitochondrial inner membrane in trapping protons [58, 60] from the oxidative phosphorylation pathway. It has been shown that CL has a function in stabilizing respiratory supercomplexes in the mitochondrial inner membrane [61, 62].

1.2.2 Sphingolipids

Sphingolipids are distinct from glycerophospholipids due to their different backbone structure, first discovered by their degradation products [63]. Among sphingolipids found in cells, the sphingosine base is the most common backbone structure, though dihydrosphingosine and other structural varieties are also commonly found [64]. The sphingosine molecule has a long hydrocarbon chain with a *trans*- double bond near the headgroup region, whereas dihydrosphingosine is fully saturated [65]. The usually saturated acyl chain is N-linked to the sphingoid base and forms the simplest sphingolipid structure, ceramide [66]. The ceramide molecule functions as a precursor for complex sphingolipids, of which galactosylceramides and glucosylceramides represent two branches of glycosphingolipids with separate synthesis pathways, and as precursor for sphingomyelins that constitute abundant plasma membrane lipids [67]. Sphingolipid synthesis occurs through three possible pathways; *de novo* synthesis [68, 69], the salvage pathway [70-72] from ceramidase action [73], and from sphingomyelin (SM) breakdown by SMases [74, 75]. Ceramide is synthesized within the ER by six different ceramide synthases, of which each produce ceramides with different acyl chain lengths with high substrate specificity

[68, 76-78]. Expression levels of the ceramide synthases is dependent on the tissue type [79-81]. Ceramide is transported away from the ER by the ceramide transport protein (CERT) to the Golgi network to be converted into sphingomyelin [82]. Glycosphingolipids are also synthesized from ceramide in the Golgi, but seem to originate from a different pool of ceramides than those delivered by CERT [82, 83].

1.2.2.1 Sphingomyelin

Sphingomyelin is enriched in the outer leaflet of the plasma membrane, and is also found along its biosynthetic pathway, with the main site of synthesis being the lumenal side of the Golgi [84] by sphingomyelin synthase 1 [85]. Sphingomyelin has a choline headgroup for which phosphatidylcholine functions as a donor [86]. Sphingomyelin is transferred to the plasma membrane by vesicular transport [87]. Upon the plasma membrane sphingomyelin can be degraded by sphingomyelinases, while free ceramides also can be converted back into SM by sphingomyelin synthase 2 [85, 88]. The large headgroup contributes to the favourable interactions that sphingomyelin has with cholesterol and ceramides. While the preferential interaction of SM and cholesterol [89-91] has been known for a long time, it has been shown that the SM headgroup adopts a conformation which by increasing the relative area of the headgroup improves the shielding effect from water in interactions with cholesterol, however, this is not a specific interaction of the headgroup and hydroxyl group of cholesterol [92]. This conformational change can be specifically sensed by Ostreolysin A [93]. Sphingomyelin has pronounced hydrogen bonding properties due to the structure of its long chain base with the amide and hydroxyl group in the membrane interfacial region [94, 95]. The intermolecular hydrogen bonding capabilities and the generally saturated acyl chains confer to higher order parameters than phosphatidylcholines with corresponding acyl chain lengths [96].

1.2.3 Nonbilayer lipids

1.2.3.1 Ceramide

Ceramide is the structurally simplest sphingolipid that is normally present at very low concentrations in the membranes of the cell, at a few mol% at the most. Due to its effective lack of headgroup, or the small hydroxyl group located at the C1- position on the ceramide molecule, it is very hydrophobic and is as a result prone to laterally segregate in membranes in which ceramide is present [97, 98]. 1-deoxy ceramide can be generated under specific conditions and has been found to be elevated in type 2 diabetes patients plasma [99] and when CerS are blocked by fumonisin [100], and of which 1-deoxy dihydro varieties especially are even more hydrophobic than ceramide [101]. The acyl chains in ceramides are generally fully saturated, except for the trans-Δ^4 double bond in the sphingosine backbone, while

dihydroceramides are fully saturated. Ceramides have extensive hydrogen bonding capability in the interfacial region, where the 1-OH and 3-OH groups are important for intra- and intermolecular hydrogen bonds [102, 103]. The 1-OH group forms intermolecular hydrogen bonds through a bound water molecule, while the 3-OH participates in intramolecular hydrogen bonding to the 1-OH group and by functioning as an acceptor of a hydrogen bond with a water molecule that interacts with the trans double bond between carbons 4 and 5 in the sphingosine base [104]. Changes in the conformation of the sphingoid base only leads to minor differences in physical behaviour in ceramide analogues as compared to palmitoyl ceramide [105], as the hydrocarbon chain length of the sphingoid base seems to be more important for the ability to induce formation of domains with increased order in membranes [106].

The lack of headgroup also facilitates ceramide translocation from one leaflet to the other with lipid flip-flop occurring at much higher rates than for phospholipids or other sphingolipids [107-109]. The following lipid scrambling has a destabilizing effect on membrane permeability and integrity [110]. Lack of headgroup sets preferences to what lipids ceramide interacts favourably with, ideally the co-lipids have larger headgroups that provide shielding from the aqueous membrane exterior, while the hydrogen bonding properties of the co-lipid are more important [111]. As a result of this, ceramides are unable to form bilayers on their own, though there are reports of stacked bilayers being possible structures in the stratum corneum of the skin or in pure ceramide systems and at low hydration levels [112-114]. Ceramide has an intrinsic negative curvature, and facilitates the lamellar to hexagonal phase transition in PE membranes [115].

Ceramide prefers high degrees of order within the membrane while also contributing itself towards the formation of highly ordered domains. In phospholipid membranes that are highly unsaturated, both ceramide and dihydroceramide will form more highly ordered ceramide rich domains than in membranes that contain saturated or partially saturated hybrid lipid species [116]. Ceramides and cholesterol share the characteristic of being very hydrophobic molecules without headgroup, and the preference of partitioning together with sphingomyelin [117]. In model membranes with this combination of lipids, ceramide competes with cholesterol for access to sphingomyelin and is able to displace cholesterol out of the liquid ordered domains [118]. Even as low ceramide concentrations as 4% in ternary lipid systems can have a profound effect on the organization of domains, in which there seems to be coalescence of SM together with palmitoyl ceramide, forming very highly ordered subdomains that exclude most fluorescent probes within liquid ordered domains [119], while also being topologically taller than the surrounding membrane, indicating higher degree of order [120].

The acyl chain length of the ceramide affects its miscibility into SM membranes and the stability of domains formed; short acyl chain ceramides

with a mismatch in chain length of the N-linked acyl chain and the sphingoid backbone perturb the stability of SM bilayers, while ceramides with longer acyl chain lengths stabilize domains [121]. Dihydroceramides, lacking the trans-Δ^4 double bond, allow for even closer interactions with SM and other dihydroceramide molecules and thus decrease void space between molecules while also resulting in larger subdomains than ceramide [122].

Ceramide generation is involved in several signalling processes in the cell [123] as well as in cellular senescence [124]. Though ceramide concentration in membranes is normally very low and is strictly regulated [125], in some instances ceramide concentration may acutely increase. This has been shown to be the case when the cell is exposed to high level of cell stress [126], ionizing radiation [127] and anticancer drugs [128]. Ceramide generation has also been linked to apoptosis [129], to which a few possible mechanisms have been proposed. Translocation of ceramide to the mitochondrial outer membrane has been shown to induce apoptosis together with the proapoptotic Bax protein [130]. The apoptotic cascade has been indicated to begin with mitochondria starting to leak cytochrome C [110], which then triggers the following steps in programmed cell death. Ceramide has a destabilizing effect on membrane structure due to its ability to flip-flop [109, 131], but it has also been reported that ceramide would form channels [132-134] in the mitochondrial outer membrane and thus triggering apoptosis. Formation of ceramide pores has been considered controversial, and as a result membrane destabilization through surface area mismatch caused by SMase action [135] on SM and/or ceramide flip-flop between leaflets [136] have been suggested as more plausible explanations.

1.2.3.2 Sterols

Sterols have a basic structure of four fused carbon rings, which grants them a planar and rigid structure. Cholesterol is the main sterol found in animals, though they also contain low quantities of oxysterols which are more hydrophilic and also function as receptor ligands [137]. Due to the very hydrophobic structure of sterols and the small headgroup, they are unable to form bilayers on their own and need co-lipids to interact with [138]. However, cholesterol is able to form complexes with lysophosphatidylcholine that when together can form vesicles [139]. Cholesterol is enriched in the plasma membrane and is believed to be enriched in the outer leaflet due to its preferential interaction with sphingomyelin [140, 141], though there are conflicting reports on the cholesterol distribution in leaflets [142], and it has also been suggested that an increase of cholesterol content in the outer leaflet would induce negative curvature stress which in turn would force cholesterol into the inner leaflet, though the interactions with inner leaflet lipids is less favourable and that themselves have negative intrinsic curvature [143]. Lateral diffusion rates of cholesterol are similar to those of phospholipids and SM in model membranes, albeit slightly faster, but significantly faster in live cells [144]. Depending on the other lipid species present in the membranes,

cholesterol has an ordering or disordering effect resulting in the formation of liquid-ordered domains [145].

1.2.3.3 Lysophospholipids

Lyso-phospholipids are a breakdown product from the degradation of phospholipids by the action of several different types of phospholipase A_2. The result is a lyso-phospholipid and a cleaved off acyl chain [146]. A cytosolic phospholipase $A_2\alpha$ preferentially cleaves DAG or PC with one of the acyl chains being arachidonic acid [147]. Arachidonic acid in turn is a precursor in the production of eicaosanoids involved in inflammatory responses [148]. Lysophosphatidylcholine has in turn been shown to be able to permeabilize mitochondria to both Ca^{2+} and cytochrome C [149]. Presence of lysophosphatidylcholine can inhibit the the transition to an inverted hexagonal phase in phosphatidylethanolamine membranes due to its molecular shape [150]. In model membranes in a liquid ordered phase, composed of either PC or SM with cholesterol, lysoPC is able to promote membrane fission [151]. Lyso-phospholipids are normally present at low membrane concentrations as they are generally reacylated in the Lands cycle [152, 153].

1.3 Order, disorder and lateral segregation

A lipid that has two saturated acyl chains has a significantly higher melting temperature than a lipid with acyl chains of an equal number of carbons but which include cis- double bonds in each. The melting temperature of a lipid with mixed acyl chains is higher than that of a di- unsaturated lipid, but is closer to its unsaturated counterpart than it is to the mean of the melting temperatures of the unsaturated and fully saturated varieties [154, 155]. Acyl chain length is another factor that affects the thermostability, due to the increased contact surface and potential for van der Waals interactions of the acyl chains within the membrane. Further, the presence and position of cis-double bonds on the acyl chains can cause a marked difference in the behaviour of the lipid as the order parameter is overall reduced compared to fully saturated lipids [156]. Double bonds near each end of the acyl chain, deep in the interior of the membrane or close to the headgroup region cause less disturbance in the space between acyl chains as the order of the membrane decreases. The order parameter in membranes is highest between the acyl chains near the middle of the acyl chain and thus cis- double bonds in this region have a much larger effect on fluidity [157, 158]. The thermostability of the lipids affect the overall order in the membrane.

Strong lateral segregation occurs in lipid mixtures of lipids with very different acyl chain compositions and results in a high line tension between the L_o and L_d domains [159]. Binary lipid mixtures that contain one lipid species with two saturated acyl chains, while the other lipid has two unsaturated acyl chains will likely phase separate and where the saturated

lipid will form an ordered phase while the unsaturated lipid surrounds the ordered domain as a liquid disordered phase. Before reaching equilibrium, the ordered phase has a flower or branched like appearance while the size is determined by the rate of cooling resulting in lipid demixing, with a rapid cooling leading to more numerous but smaller domains [160, 161]. When the proportion of unsaturated lipid is decreased or exchanged for a lipid with one saturated and one unsaturated acyl chain the line tension between the lipids is decreased, and as a result the domains decrease in size and become more irregular in shape [162]. Inclusion of cholesterol in a membrane results in a ternary system in which cholesterol can decrease the amount of PSM required for the formation of L_o domains when one of the acyl chains in a hybrid lipid is highly unsaturated [145]. Polyunsaturated phospholipids interact poorly with cholesterol [163] and will thus increase the stability of the L_o domain formed by cholesterol and a higher T_m lipid [164]. Within unsaturated membranes cholesterol has an ordering effect on the lipids, though the ordering effect is weaker when the *cis*- double bond is located in the middle part of the acyl chain [165], while the cholesterol interaction with saturated lipids has a disordering effect. In a situation where cholesterol can choose lipid environment, there is a preference for ordered environments due to thermodynamics, as increasing the entropy is more favourable. The ordering effect of cholesterol on the acyl chains with subsequent condensation effect allows for the headgroups of surrounding lipids to better accommodate cholesterol beneath them in accordance to the umbrella model that explains the requirement of large headgroup co-lipids for cholesterol [166].

Neither biological nor model membranes are particularly homogenous considering lipid distribution. It was early discovered that some lipids preferentially associate with each other [91]. Lipid rafts have been considered as the fraction of membranes that have been able to be isolated by cold detergent extraction, consisting mostly of sphingomyelin- and cholesterol-rich domains of the plasma membrane [167, 168]. It has however also been shown that the use of detergent can in itself promote lipid de-mixing and raft formation [169]. Lateral organization can lead to co-existence of multiple phases in the lipid system [170], depending on the lipids present and can be presented as phase diagrams as a means to classify lipid behaviour according to common structural denominators of the lipids in the phase diagram [171].

1.4 Lipid packing frustration

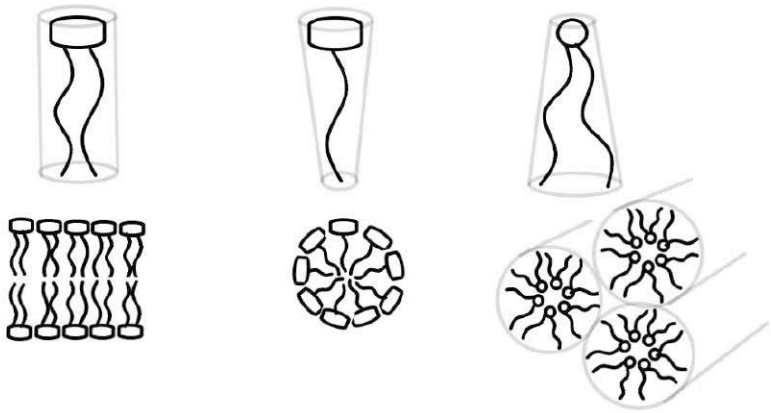


Figure 1. Lipids with a cylindrical shape prefer lamellar phases. A large headgroup area coupled with a small volume occupied by the acyl chain(s) results in a conical lipid shape and will preferentially form micelles. Small headgroup lipids with bulky acyl chains on the other hand are prone to from inverted hexagonal (H_{II}) phases. Image modified from [172].

Lipids are thought to have intrinsic curvature or a shape factor based on the ratio of the relative size of the headgroup compared to the area the acyl chains occupy. In lipid monolayers a mismatch between the relative areas of headgroup and acyl chains resulting in a strain at the lipid-water interface from which the lipids would curl. The radius of curvature, R, is given as intrinsic curvature R_0 for a lipid in a relaxed state [173]. Lipids with small headgroups in proportion to the space occupied by the acyl chains have a negative curvature and negative R_0 values while lipids which prefer lamellar phases or have positive curvature have larger R_0 values, while the intrinsic curvature of a membrane C_0 is the inverted value of R_0 [172]. When in a bilayer, presence of lipids with negative intrinsic curvature that would bend in a monolayer are prevented from doing so by the other leaflet which would prefer to curl in the opposite direction and are creating curvature stress [174]. While most cellular membranes are in a lamellar phase, regions of high curvature are present especially in the cristae of mitochondria. Membrane fusion is suggested to involve brief non-lamellar phase transitions, and is promoted by negative intrinsic curvature lipids such as PE and cholesterol [175]. In influenza virus hemagglutinin (HA) driven membrane fusion the viral envelope and the target membrane are brought into close contact upon HA binding [176], which results in a dehydration of proximal membrane surfaces and the subsequent formation of a stalk phase at the fusion site [177]. The fusion proceeds with the stalk transitioning into a hemifusion diaphragm when membranes have a high negative spontaneous curvature [178] but is also modulated by the elastic properties of the membrane [178, 179]. The

bending of membranes is a significant feature in several biological processes, such as cell division when content of negative curvature inducing lipids increase upon M phase [180] and vesiculation in signalling as well as in viral entry and budding. Many of these processes are membrane protein driven [181], while proteins can simultaneously be dependent on the lipid components in the membrane in order to function and the lateral pressure profiles they confer [182]. Binding of membrane proteins is also affected by curvature strain, among others CTP:cholinephosphate cytidylyltransferase that regulates PC synthesis and preferably binds membranes with high presence of negative curvature causing lipids and is activated to increase PC content that ultimately can alleviate curvature strain [183].

1.5 Biological membranes

Biological membranes are dynamic assemblies of lipids with considerable lateral heterogeneity and both temporally and spatially organized domains of higher order than the surrounding membrane [184]. As a result, lipids associating in domain formation have slowed diffusion rates in the membranes of live cells [185]. Distribution of lipids is uneven, and a strict asymmetry is upheld between the inner and outer leaflets of the plasma membrane as a loss of asymmetry is detrimental for membrane impermeability [186]. This asymmetry is maintained by flippases that move lipids from the outer leaflet to the cytosolic side [187, 188], and floppases, that work in the opposite direction [189] in order to prevent translocation of especially PS to the outer leaflet as it acts as an apoptotic signal [190]. Externalization of PS can also occur through the action of scramblases, that move lipids bidirectionally across the membrane [191]. Lipid compositions also vary with membranes of different organelles and reflects the function of each membrane. The endoplasmic reticulum is the main site of both lipid and protein synthesis [192]. The membrane of the organelle is much more fluid than the plasma membrane, as especially sphingolipid precursors are transported away to reach their final form in other organelles, most notably the Golgi. The gradually increasing membrane thickness due to increasing presence of saturated lipids and cholesterol is also reflected in the increasing length of transmembrane domains of integral membrane proteins [193]. These domains are thicker than the surrounding membrane and are also enriched in membrane proteins [194].

1.5.1 Mitochondrial membranes

Mitochondria have partial capacity to synthesize some of their own lipids, but also rely on lipid transfer at contact sites with the endoplasmic reticulum known as mitochondria associated membrane or MAM [195]. Since sphingolipid or ceramide concentrations at mitochondrial outer membranes are very low, it is suggested that in processes leading up to apoptosis, a pool of sphingomyelin residing in the mitochondrial outer membrane would be

degraded by sphingomyelinases to ceramide and ultimately lead to membrane permeabilization following Bax translocation to the mitochondrial membrane [196, 197]. Cytochrome C release is a hallmark of apoptosis and it has been found that ceramide enhances the membrane leakage promoted by proapoptotic Bax [198]. A recent report suggested that mitochondrial ceramide induced apoptosis would occur through ceramide binding to the voltage dependent anion-selective channel (VDAC) 2 by potentially blocking the re-translocation of Bax to the cytosol [199]. It has however also been shown that cholesterol levels in mitochondrial membranes are increased in some cancer cell lines, granting resistance towards apoptotic stimuli by inhibiting oligomerization of pro-apoptotic Bax and subsequent outer mitochondrial membrane permeabilization [200].

1.6 Fluorescent probes in lipid research

Though the plasma membrane of the cell is visible under a regular light microscope, more detailed study requires for it to be stained. As single lipids are small molecules compared to other components of a cell, it is preferable that the fluorescent dye used is a small molecule itself, in order to perturb the native membrane as little as possible to avoid introducing artefactual effects. The fluorophores need to be lipophilic in order to associate with the membrane and commonly contain aromatic rings and/or conjugated double bonds. The delocalized electrons in these functional groups are excited by light at specific wavelengths and as the electrons return from their excited state they will emit photons with lower energy, or longer wavelength. Depending on the chemical structure of the fluorescent probe, it can have preference for a specific type of membrane environment. These properties can be used to gain information about the physical state of the membrane [201].

Diphenylhexatriene, or DPH, is a widely used fluorescent probe used in characterization of membrane dynamics in model membranes [202]. The partitioning of DPH into membranes is even, with no particular preference to certain degrees of order [203]. 2-Dimethylamino-6-lauroylnaphthalene (Laurdan) is sensitive to changes in the membrane environment. The emission wavelength of Laurdan changes according to the degree of water penetration into the membrane near the interfacial region of the membrane [204]. When membrane order is increased, the emission shifts to shorter wavelengths, i.e. blue-shifts, while a decrease of membrane order leads to a red shift [205]. BODIPY is usually conjugated into a lipid analogue, rendering it strongly fluorescent. It is widely used for microscopy due to its brightness [206]. The fluorescent group is rather large and the tagged lipid analogue may not behave like the native lipid species would in a membrane, as B-cholesterol does not order the membrane as native cholesterol does, but does partition into the L_o domain [207]. Rhodamines can be used either on their own in staining mitochondrial membranes [208], or conjugated to a lipid analogue

[209]. As a lissamine-rhodamine B dioleoylphosphatidylethanolamine conjugate, it prefers liquid-disordered domains in membranes and will self-quench in ordered or gel domains [209]. 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD) can be conjugated into the acyl chains of phospholipids, though it has been found that the relatively polar fluorophore causes the fluorescent group to be localized close to the membrane-water interface [210]. Fluorescent tags conjugated to lipid analogues have different partitioning behaviour than their native lipid counterparts [211].

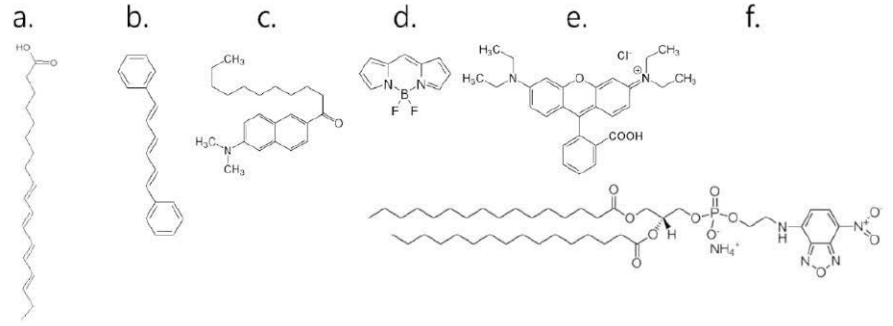


Figure 2. Fluorescent probes with use in lipid research. ***a****. trans-parinaric acid* ***b****. DPH* ***c****. Laurdan* ***d.*** *BODIPY* ***e****. Rhodamine B* ***f.*** *NBD conjugated to the headgroup of PE*

1.6.1 Polyenes

Polyenes are linear alkyl chains with conjugated double bonds and a polar, often carboxylic acid, at one end of the chain [212]. Parinaric acid derivatives are essentially fluorescent fatty acids, and thus most closely resemble the acyl chains in membranes [213]. Because of this, these fluorophores do not disturb the membrane environment as much as many other probes conjugated into the acyl chain of fluorescently tagged lipid analogues. When fed to cells, polyene fatty acids are incorporated as acyl chains into lipids as would radiolabelled varieties of equal acyl chain length and are also further metabolized as native fatty acids [214]. Parinaric acids are useful either as free fatty acids, or can be conjugated to form fluorescent varieties of either phospholipids or sphingolipids [213]. Parinaric acids have a strong affinity for membranes, and are virtually nonfluorescent in water [215]. *Cis*-parinaric acid was the first variety to be isolated from a plant (*Atuna racemosa, formerly Parinari glaberrinium or P. laurina)* and was found to have aforementioned properties. It has an even distribution into different phases in membranes. *Trans*-parinaric acid, tPA, in which all the conjugated double bonds are in trans conformation, favours partitioning into ordered domains in membranes [215]. tPA is oriented parallel to the membrane normal in POPC bilayers and retains this orientation in disordered systems. Structurally tPA behaves like a

saturated acyl chain in membranes, which likely helps the partitioning into ordered domains [216]. Fluorescence lifetime decay for polyenes is multiexponential and is represented by the presence of multiple lifetime components in recorded lifetime spectra of a given membrane [217]. Temperature and order of the solvent in which a polyene resides affects the fluorescence intensity, with high order and low temperatures resulting in the highest intensity [218]. When tPA is located in a membrane environment with a high degree of order, it stays in its excited state for a longer time before emitting the photon and returning to the ground state, hence it gets a longer fluorescence lifetime [219]. Increase in lifetime has been shown to have good correlation to the order in membranes and is indicative of the lipid phases present [220]. Both *cis*- and *trans* parinaric acid are prone to oxidation [221] and are also photobleached very fast, making them impractical for use in microscopy. However, addition of one more double bond to the four conjugated double bonds improves photostability of the probe allowing for use in microscopy, still with some limitations to photostability and low quantum yield [214]. The partitioning preferences of pentaenes shift to a more even membrane distribution among ordered and disordered domains [222].

2. Aims

The aims of this thesis was to gain an understanding on ceramide interactions with lipids presenting different shape factors and what implications these effects might have on ceramide lateral segregation. In short effects that were to be studied; modification of ceramide structure, effect of negative curvature and the effect of positive curvature strain.

I. For the first paper we explored the effects on oxidizing or removing the 1-OH group of palmitoyl ceramide, as well as removal of the 3-OH group, for the implications of ceramide-rich domain forming ability.

II. For the second paper the aim was to investigate the effects of typical mitochondrial lipids known to induce negative curvature, PE and CL, on ceramide lateral segregation to try to shed light on the possible involvement in the role of ceramide in apoptosis.

III. For the third paper the specific interaction between lyso-phospholipids and ceramide was studied due to their inherent shape factors completing each other and to evaluate the effect of a positive curvature inducing lipid on ceramide behaviour.

3. Materials and methods

3.1 Lipids

All glycerophospholipids were purchased from Avanti Polar Lipids, while cholesterol and bovine heart cardiolipin and was purchased from Merck. Palmitoyl ceramide was purchased from both Avanti Polar Lipids and from Larodan. 1-carboxy- and 1-carboxy-methyl ceramide were kindly provided by Matsufuji et al. 1-deoxy ceramide was synthesized in house from 1-deoxy-sphingosine (Avanti Polar Lipids) and palmitic acid (Larodan). Palmitoyl- and stearoyl sphingomyelin were purified in house from egg-SM and brain-SM (Avanti) using reverse phase HPLC with a C18 column and methanol as eluent.

Stock solutions of glycerophospholipids and sphingomyelins were prepared in argon-purged methanol. Stock solutions of cardiolipin and ceramides were prepared in 1:1 (v/v) chloroform:methanol, while cholesterol was dissolved in 3:2 hexane:isopropanol. Concentrations of glycerophospholipid, sphingomyelin and cardiolipin stock solutions were determined by total phosphorous assay [223], while ceramides were carefully weighed and dissolved in a known volume of organic solvent. Concentration of cholesterol was determined by monolayer collapse using a surface barostat. All lipid stock solutions were stored at -20°C, and were brought to ambient temperature 1 hour before sample preparation. tPA was synthesized in house from α-linoleic acid [224]. The product was purified by crystallization with a total purity of >96%. Identity of the product was verified by analytical HPLC and by fluorescence spectra.

Samples were prepared by pipetting the lipids into glass test tubes to which 1 mol% of fluorescent probe was added. The samples were mixed by brief vortexing and then dried under a flow of nitrogen at a 40°C water bath, until a lipid film was formed. Then, pre heated and argon-purged MQ-water or TBS buffer (10mM Tris, 140mM NaCl) was gently added and tubes were sealed with samples left to hydrate undisturbed at 65°C for 1h. After hydration, the samples were again vortexed and then bath sonicated (FinnSonic) at 65°C for 5 minutes. The samples were then left to equilibrate to ambient temperature for 1h in a dark cupboard before beginning measurements.

3.2 Fluorescence lifetimes

Fluorescence lifetime decay of tPA emission in membranes was recorded with a PicoQuant100 instrument, to which a PDL unit was coupled. The setup also contained a 400nm cut off filter and temperature control by water bath. Most lifetimes were recorded at 23°C, while some were recorded at 37°C for which samples were kept at a heating block prior to measurements. Constant stirring was used during experiments. Collected lifetime data was subsequently analysed by FluoFit (PicoQuant) software. The intensity-weighted average fluorescence lifetime of tPA emission [201] is given as:

$$\langle\tau\rangle=\Sigma i\alpha i\tau 2i/\Sigma i\alpha i\tau i$$

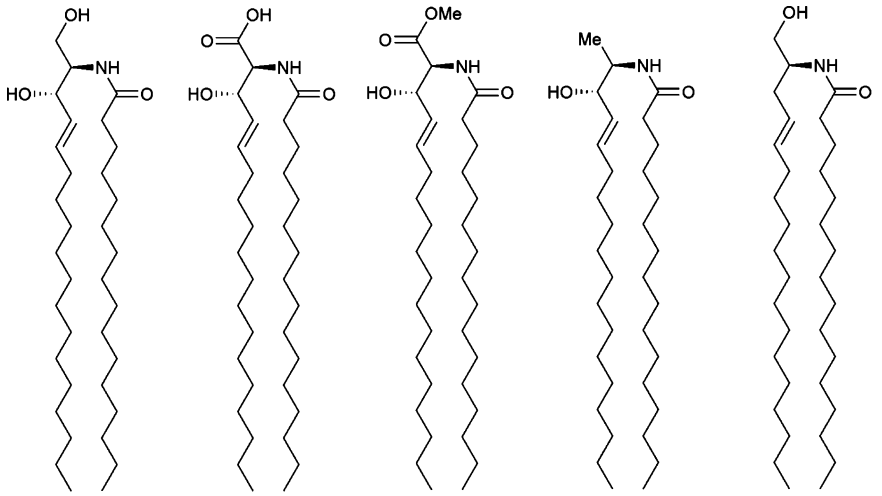
3.3 Fluorescence anisotropy

Steady state anisotropy was measured with a PTI QuantaMaster1 instrument with a Xe-arc lamp as light source. tPA anisotropy was measured with 305nm as excitation wavelength and 430nm as emission wavelength. The peak of emission lies closer to 420nm, but 430nm was chosen in order to minimize noise. Temperature was controlled by a Peltier element, with temperature being increased by 5°C/minute. Magnetic stirring was set to 300rpm. Anisotropy data was analysed with Felix32 software. Fluorescence anisotropy (r) was calculated [201] according to:

$$r=(IVV-GIVH)/(IVV+2GIHV)$$

4. Results

4.1 Role of 1- and 3-hydroxyl groups for ceramide-co-lipid interaction (Paper I)



In paper I we explored the role of the 1- and 3-hydroxyl groups for ceramide interaction with co-lipids in the membrane. The hydroxyl groups on either the 1- or 3- position were removed to yield 1-deoxy ceramide and 3-deoxy ceramide respectively. Additionally ceramide analogues with the 1-OH groups substituted for a carboxyl group or a carboxy-methyl groups, characterized in an earlier paper [225], were included. It has been found that 1-deoxy ceramide can accumulate during specific conditions under which ceramide synthases are inhibited [100]. Here we compared interactions of ceramides in a POPC based membrane, with inclusion of PSM and cholesterol as co-lipids.

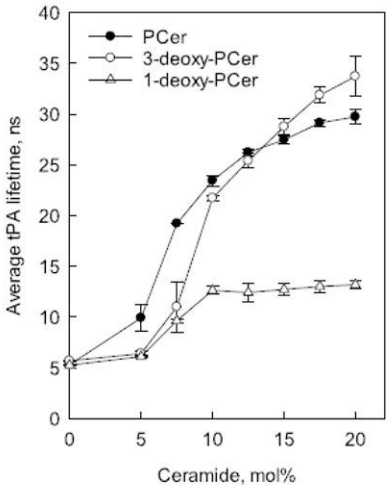


Figure 3. Comparison of tPA lifetimes in POPC membranes with, PCer, 1-deoxyPCer and 3-deoxyPCer. The increase in lifetime occurs at similar ceramide concentrations with all of the ceramides.

We found that in POPC vesicles the onset of an ordering in the membrane occurred at similar ceramide concerntrations despite differences in functional groups. Canonic PCer does however seem to have a slighty higher propensity for order in the membrane, thus creating a ceramide rich ordered domain at slighty lower membrane concentrations than 3-deoxy PCer, which at lower concentrations doesn't seem to laterally segregate as strongly. 1-deoxy PCer has an onset at the same concentration as 3-deoxy PCer, but appears to have a significantly weakened ability to form an ordered phase, which is considered to have an average tPA lifetime of over 15 ns. A long lifetime component is present at the onset concentration of 1-deoxy ceramide (data not shown) but is present at very low amplitudes compared to the other ceramides studied. At increasing ceramide concentrations, 3-deoxy ceramide causes the longest average lifetimes, indicating a higher degree of order in the membrane.

As with measurements of the thermostability of the ceramide-enriched ordered domains through tPA anisotropy, some differences between ceramides with different functioal groups were observed. Again, there is not very much difference between PCer and the 1-OH oxidized varieties and 3-deoxyPCer. 1-deoxyPCer on the other hand seems to have a lowered ability to stabilize the ceramide-rich ordered domains as seen with lowered values of anisotropy both at the start of measurements and with a lower end melting temperature. Increasing the amount of 1-deoxyPCer does not either lead to a similar increase in ordered domain stabilization as PCer and the other analogues, indicating weaker intermolecular interactions and thus a more disordered membrane.

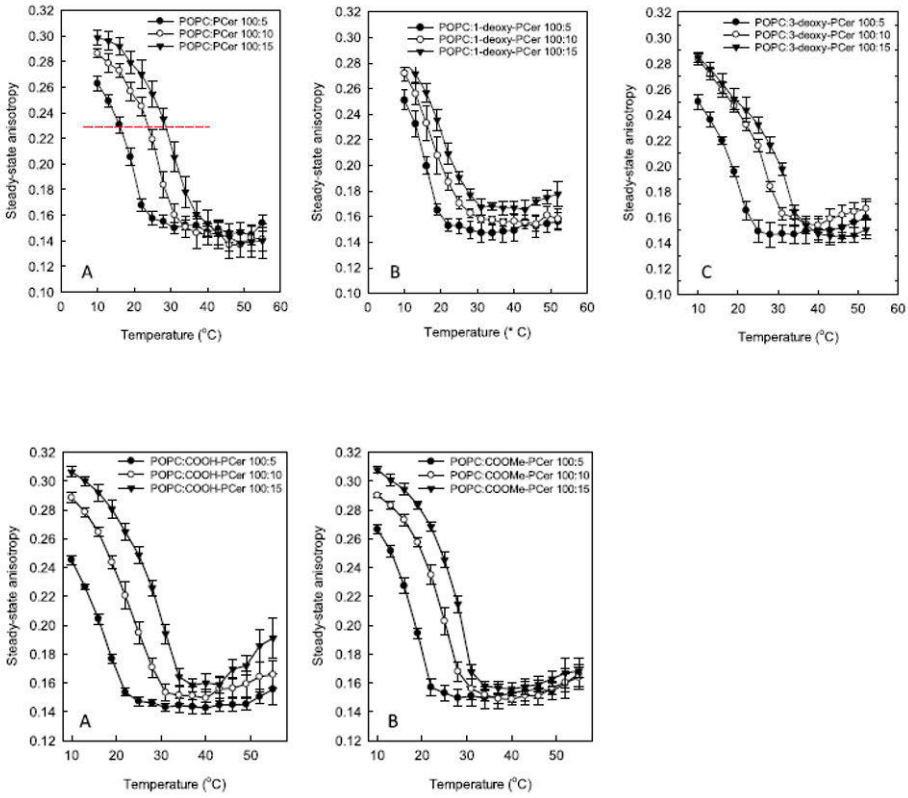


Figure 4. tPA anisotropy of ceramides in POPC membranes. All ceramides show an increased thermostability with increasing ceramide content, though 1-deoxyPCer to a lesser degree.

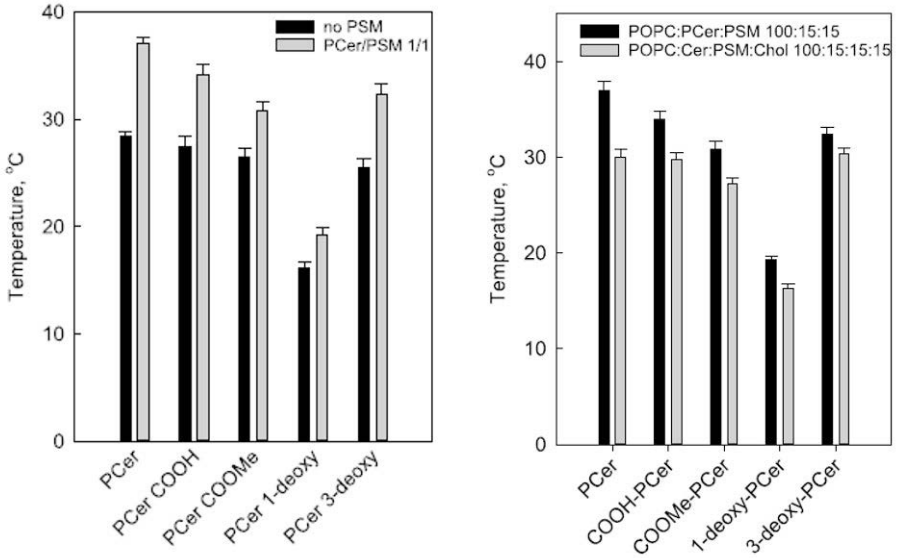


Figure 5. Panel A shows increased thermostability measured by tPA anisotropy in all lipid systems upon addition of PSM. Panel B shows a comparison of membranes with ceramide analogues and PSM and in membranes with similar compositions where cholesterol is included, resulting in decreased thermostability.

PSM interacts very favourably with PCer in both binary and more complex lipid mixtures by providing both a large headgroup and saturated acyl chains for the ceramide to associate with. We determined the effects on thermostability of PSM interaction with the PCer analogues in POPC liposomes by tPA anisotropy. The thermostability of the ceramide-rich ordered domains were increased by PSM with all ceramide analogues, though to a somewhat lower extent in the case of COOMePCer and even less for 1-deoxyPCer. The effect of adding cholesterol to the systems was also assessed by the same method, as cholesterol competes for association to PSM and in general fluidises highly ordered systems. The addition of cholesterol had a smaller effect on thermostability than PSM on its own, so temperatures were still slightly higher than in binary systems. The effect of cholesterol was however larger on PCer than it was on any of the analogues.

4.2 Ceramide interaction with lipids typical of mitochondria (Paper II)

In paper II we studied how lipids that are abundant in mitochondria can affect the lateral segregation of PCer, as increased ceramide levels in mitochondrial membranes have been implicated in the apoptotic cascade. The focus in this study was on the lipids with intrinsic negative curvature due to their propensities to induce the formation of non-lamellar phases, mainly H_{II} hexagonal phase. Further, membrane compositions were mainly made to mimic the outer mitochondrial membrane, as initiation of the apoptotic cascade is due to permeabilization of this membrane.

Fluorescence lifetimes of tPA was measured in model membranes composed of combinations of di-unsaturated and hybrid PC and PE species, with increasing PCer concentrations. As compared to the bulk lipids, POPC or DOPC, ceramide had a significantly larger effect on promoting the formation of a ceramide-rich ordered domain in DOPC based membranes with PE presence, although the onset of the ordered domain formation starts at significantly higher ceramide concentrations in pure DOPC than in POPC [226]. Inclusion of both DOPE and POPE promoted the formation of a ceramide-rich ordered domain in a linear manner when increasing the concentration of each PE species, with POPE causing the onset to occur at the lowest ceramide concentration. In POPC based membranes, neither DOPE or POPE induced strong promotion of the ordered domain onset. Only at 40% POPE in POPC was there an onset at lower PCer concentration.

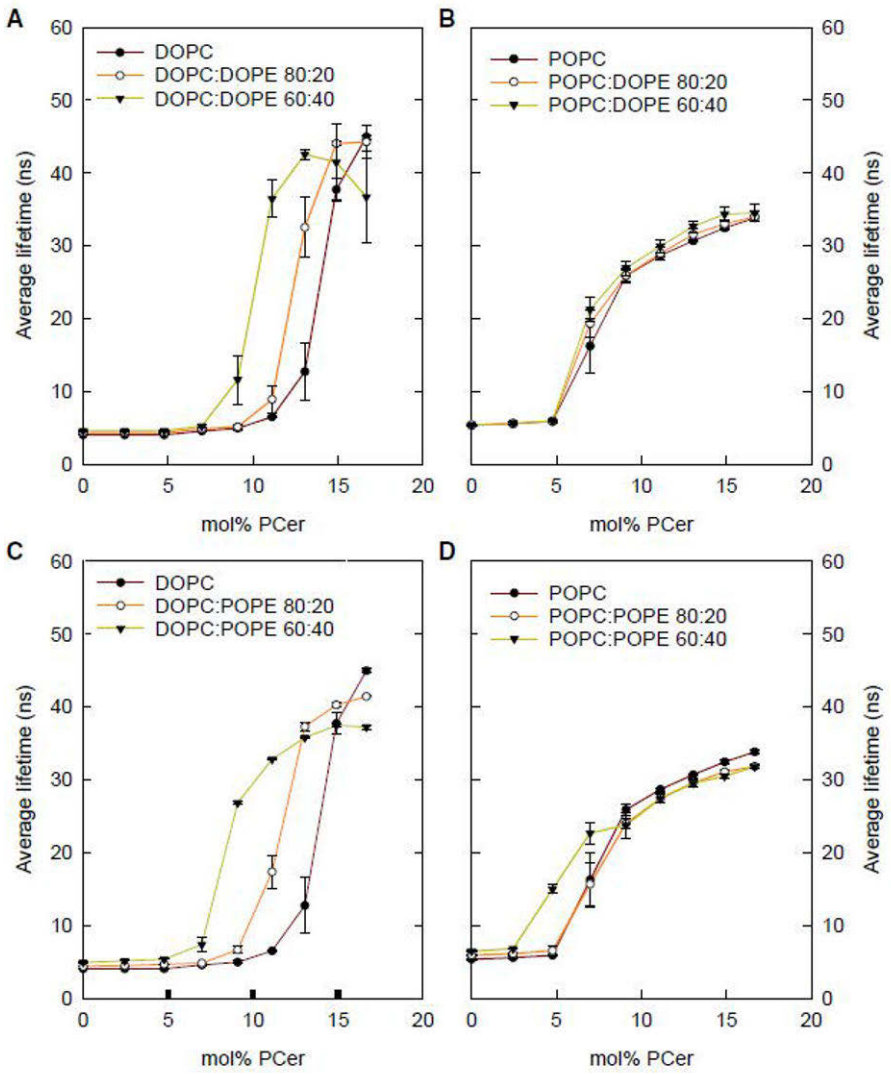


Figure 6. tPA average lifetimes in PC:PE combinations as a function of PCer concentration. Panel A shows that an increased proportion of DOPE in a DOPC based membrane reduces the amount of PCer required to form a ceramide-rich ordered domain. Panel B shows that DOPE is not able to promote further order in a POPC based membrane. In panel C it is shown that POPE in DOPC is, similarly as in panel A, able to promote the formation of a ceramide-rich ordered domain. In the POPC:POPE membrane shown in panel D, only the higher concentration of PE causes a slight shift in the onset.

To measure the thermostability of the ceramide-rich ordered domains in membranes with similar compositions as with lifetime measurements, tPA anisotropy was measured. Both DOPC-DOPE and POPC-POPE systems showed a linear increase in thermostability, however the increase was smaller in the latter system. In the mixtures of unsaturated and hybrid lipid species increase in thermostability occurred in a more step-wise manner, with the increase in thermostability again being more subtle.

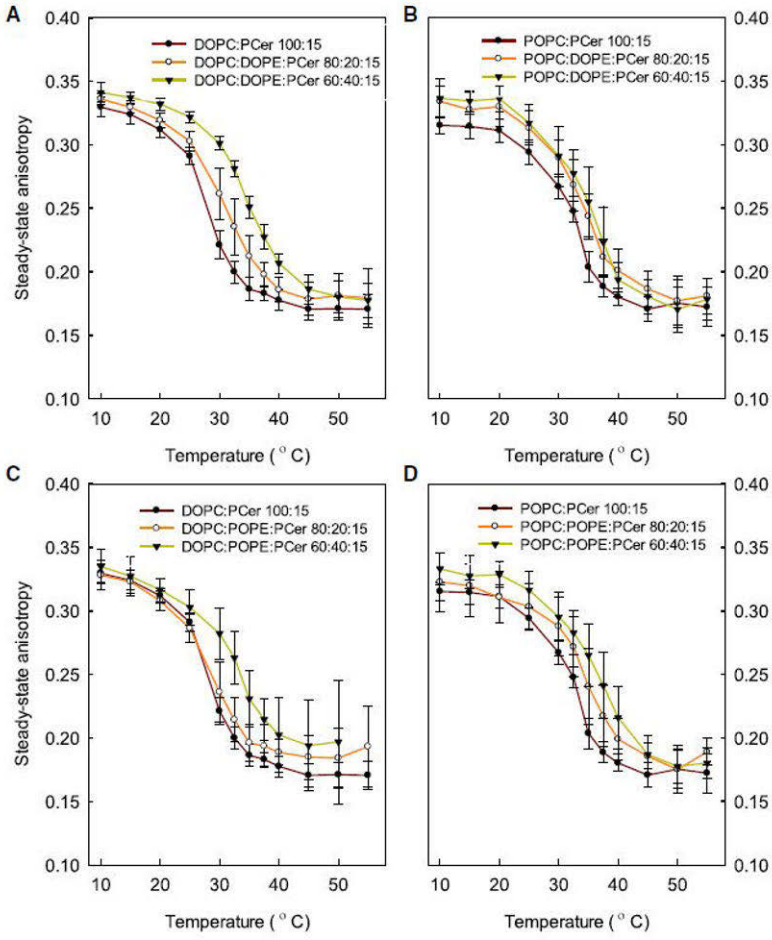


Figure 7. Panels A-D show the effect of PC:PE combinations on the thermostability of the ceramide-rich ordered domain as measured by tPA anisotropy. Panel A shows increased thermostability with increasing amounts of DOPE in DOPC. Panel B shows a modest increase of thermostability by DOPE in POPC. Panel C in turn shows a modest effect on thermostability of POPE in DOPC. Panel D shows a relatively linear increase of thermostability in POPC:POPE membranes.

In order to determine if the lack of promotion of ceramide-rich ordered domain onset in POPC based membranes is due to a small dynamic window at 23°C due to the higher Tm of POPC and POPE compared to their doubly unsaturated counterparts, tPA lifetimes were also measured at 37°C. The higher temperature should thus cause the phospholipids to be completely fluid, and should resemble the behaviour of DOPC based systems. We also included lipid compositions of pure POPE with PCer and POPC:POPE at a 20:80 proportion, and measurements were done stepwise from pure POPE to pure POPC. tPA anisotropy was also determined to assess the stability of the ceramide-rich ordered domain in all of the compositions. At 37°C the average lifetimes are overall shorter compared to measurements at 23°C due to the increased fluidity of the membranes. The ceramide-rich ordered domain onset occurs at the lowest PCer concentration when the phospholipid was entirely POPE. There is a nearly linear transition to higher ceramide concentrations required to induce the onset when the POPE amount decreases. Interestingly the average lifetimes are not the longest for POPE phospholipid membranes but are increased when POPC concentration in the membrane increases. The average lifetimes are the longest at the plateau of the 80:20 POPE:POPC phospholipid composition. The average lifetimes however start decreasing when adding more POPC. These results are reflected in the anisotropy measurements where the end melting temperatures are in the same order as the highest average lifetime at ceramide saturation, or plateau of the graphs.

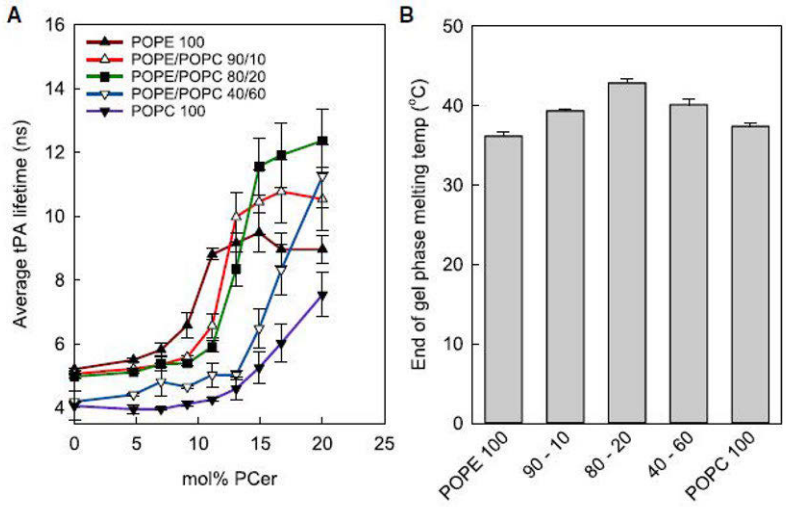


Figure 8. Panel A shows average tPA lifetimes at 37°C as a function of PCer content in POPE based membranes into which POPC is added in increasing amounts, ultimately replacing POPE. Increasing POPC content shifts the onset of the ceramide-rich ordered domain formation to higher percentage of PCer required, while still leading to an increased average lifetime in membranes in which both phospholipids are present. Panel B shows end melting temperatures of corresponding lipid mixtures by tPA anisotropy.

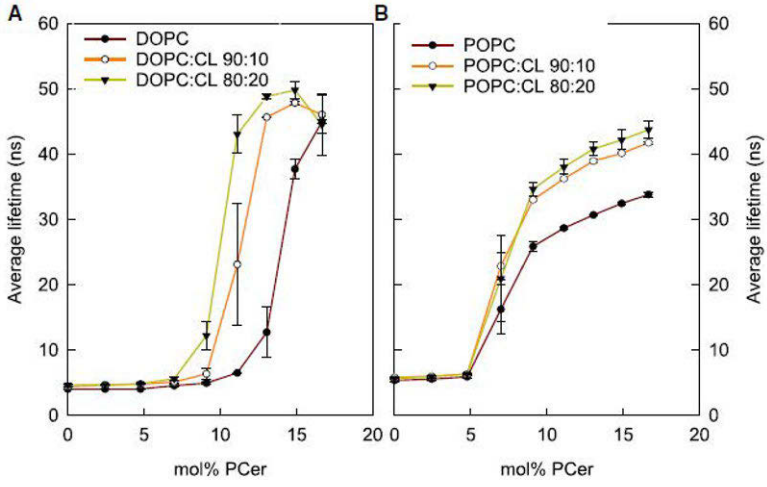


Figure 9. Panel A shows a promotion of ceramide-rich ordered domain formation onset with increased cardiolipin content in DOPC membranes. Panel B shows corresponding lipid mixtures with the phospholipid exchanged for POPC. No onset promotion is prevalent, but average lifetimes are increased when CL is included.

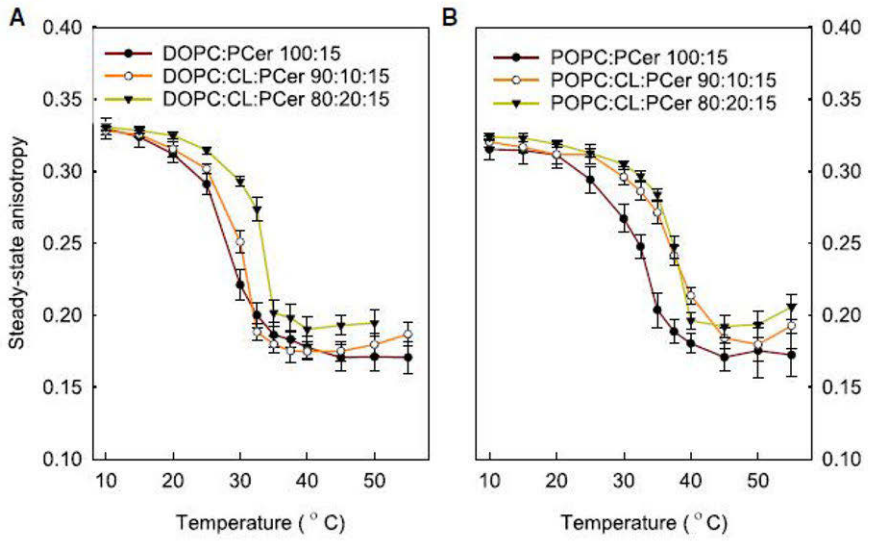


Figure 10. Panel A shows a modest increase of thermostability as measured with tPA anisotropy in the 80:20:15 DOPC:CL:PCer mixture. Panel B shows that thermostability increases in all POPC based membranes where CL is included.

Cardiolipin is another typical mitochondrial lipid that we included in the experiments. Though it is mostly associated with the inner mitochondrial membrane, it can be found in smaller quantities in the outer mitochondrial membrane as well [56]. The amount of cardiolipin included was half of that compared to PE in POPC and DOPC membranes due to cardiolipin essentially being two conjugated phospholipids, having four acyl chains. Both tPA lifetimes and anisotropy were measured in cardiolipin containing membranes based on POPC or DOPC. 1mM $CaCl_2$ was included in the buffers used for the experiments as Ca^{2+} has been shown to induce non-lamellar phases in cardiolipin membranes [227]. tPA lifetimes in DOPC:CL with increasing ceramide concentrations show an earlier onset of the ceramide-rich ordered domain formation when the cardiolipin concentration is increased. The effect is significant, but less pronounced than with either PE species previously used. In POPC based membranes there is again no promotion of an earlier ceramide-rich ordered domain onset, while there is instead a clear increase in the average lifetime after the onset, with only small differences between the cardiolipin concentrations tested. tPA anisotropy of similar lipid compositions show an increase in ceramide-rich ordered domain stability for the higher cardiolipin content in DOPC membranes, while in POPC based membranes there is an increase in thermostability for both cardiolipin concentrations used.

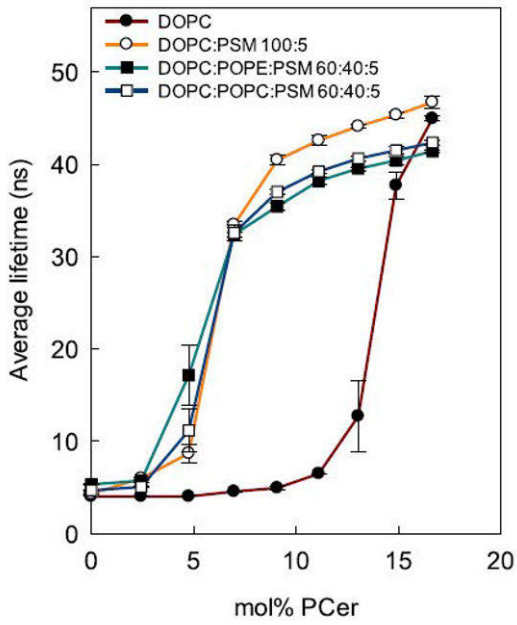
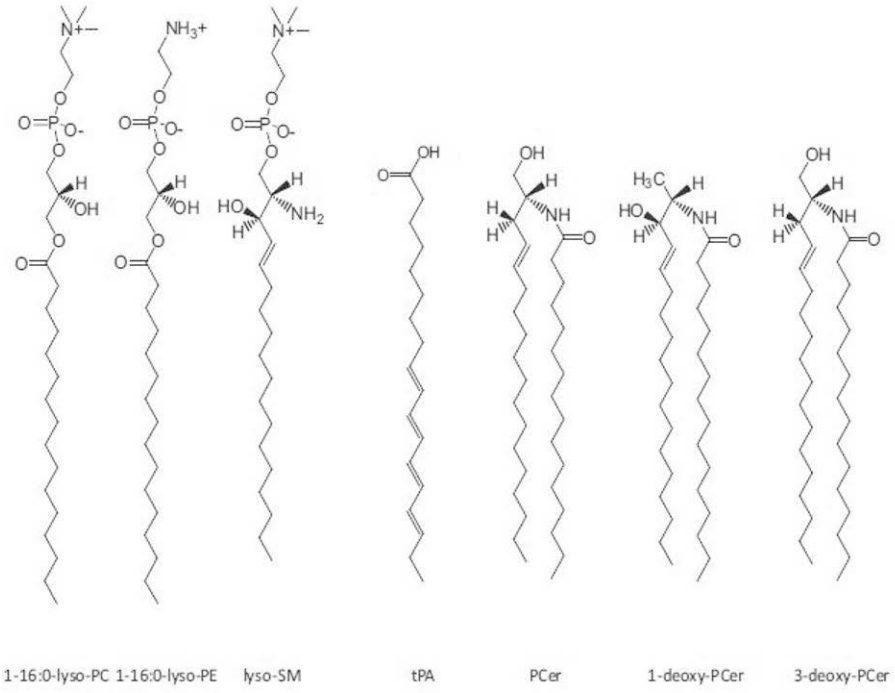


Figure 11. Inclusion of PSM into a DOPC based membrane masks all other onset promoting effects, or lack thereof, by other phospholipids present in the lipid mixture.

Finally, we compared the effect of PSM on the ceramide onset in DOPC membranes or DOPC with either POPE or POPC, to elucidate the effect of headgroup or acyl chains in the presence of a lipid with which ceramide is known to interact very favourably with. Due to the very strong preference of PSM interaction instead of any other lipid in these membranes, the small amount of PSM causes the onset to be shifted to very low PCer membrane concentrations, thus masking any effect that the other lipids would have. With POPE in the DOPC membrane there is a slight elevation of the average tPA lifetime at 5mol% PCer.

4.3 Ceramide complexes with lysophospholipids (Paper III)



For this paper we wanted to find out if lyso -phospholipids could reverse the effect of onset promotion by lipids with intrinsic negative curvature, such as PE and CL. Lyso-phospholipids should thus, due to their intrinsic positive curvature caused by the large headgroup and lone acyl chain, be able to counteract the effect of negative curvature induced lateral segregation. However, we quickly found that the effect of including lyso-PC did not have the intended effect, quite the opposite instead.

Inclusion of lyso-PC at a 80:20 DOPC:lyso-PC concentration led to a similar promotion of the formation of the ceramide-rich ordered domain as inclusion of POPE at a 60:40 DOPC:POPE ratio, as seen by tPA lifetime measurements. Similar tendencies were also seen with tPA anisotropy, with the end melting temperature being increased by about 10°C as an indication of increased thermostability of the formed ceramide-rich ordered domains.

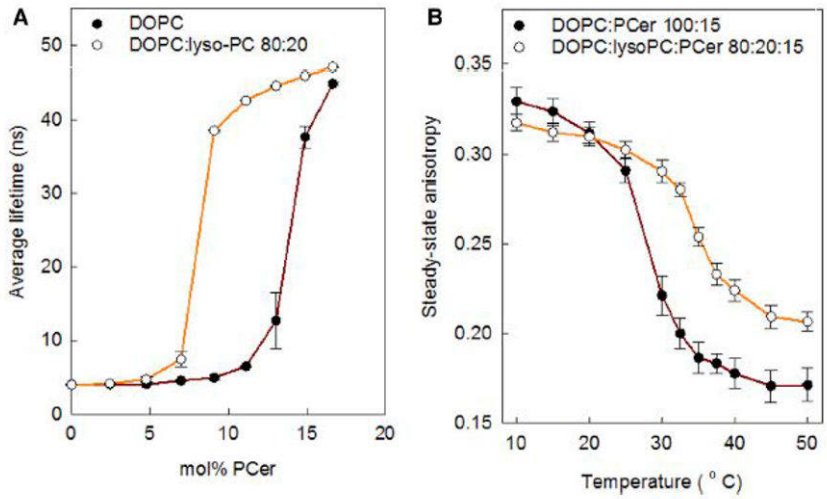


Figure 12. Figure A shows increased tPA lifetimes at lowered PCer concentrations and a promotion of ceramide-rich ordered domain formation when PE is replaced by lyso-PC in DOPC. Panel B shows tPA anisotropy of corresponding lipid mixtures and a significant increase of ceramide-rich ordered domain thermostability.

Next, to elucidate the effect of the headgroup of the lyso-lipid on ceramide interaction, we measured tPA lifetimes at 23°C while included the lyso-lipids at equimolar concentrations to PCer in DOPC based vesicles. DOPC was chosen as the main phospholipid in order to have a larger dynamic window in which the effects of different lyso-lipids could more easily be discerned. We found that there was little difference between different headgroups of the lyso-lipid component. Only with oleoyl lyso-PC was there a significant difference to the other lyso-lipid species, however, oleoyl lyso-PC also promoted the formation of a ceramide-rich ordered domain in comparison to PCer only in DOPC.

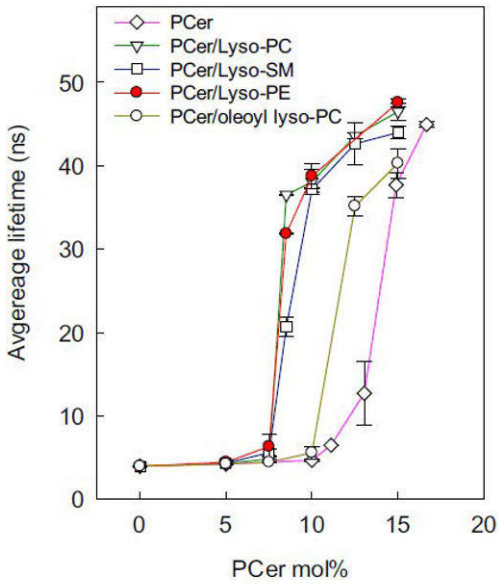


Figure 13. Average tPA lifetimes of equimolar concentrations of PCer and lyso-lipids in DOPC promote the formation of a ceramide-rich ordered domain as the onset is shifted towards lower ceramide concentrations required.

A comparison of the thermostability of the lyso-lipid/ceramide complexes was assessed by tPA anisotropy. Here, there is a significant increase in the thermostability of the ceramide-rich ordered domains in the ternary bilayers in which the lyso-lipids have a saturated acyl chain. Oleoyl lyso-PC confers a thermostability similar to, or slightly less than PCer only in DOPC.

In order to determine how structural variations in the ceramide molecule itself affects the complex forming properties together with lyso-PC, tPA lifetimes were measured. Notably, 1-deoxy ceramide which has very weak ordering effects in POPC [102] does not form ceramide-rich ordered domains at all in DOPC. However, in the presence of lyso-PC an onset occurs at 12,5mol% 1-deoxy ceramide concentration, which is a lower concentration that at which PCer forms a ceramide-rich ordered domain in DOPC. 3-deoxy ceramide on the other hand behaves more like PCer in DOPC.

As with acyl chain modified ceramides, 24:1-ceramide forms a ceramide-rich ordered domain at a higher concentration than even chained PCer derivatives, but is also subject to the strong ordering effect of lyso-PC. Oleoyl ceramide did not form ordered domains neither on its own or together with lyso-PC in DOPC.

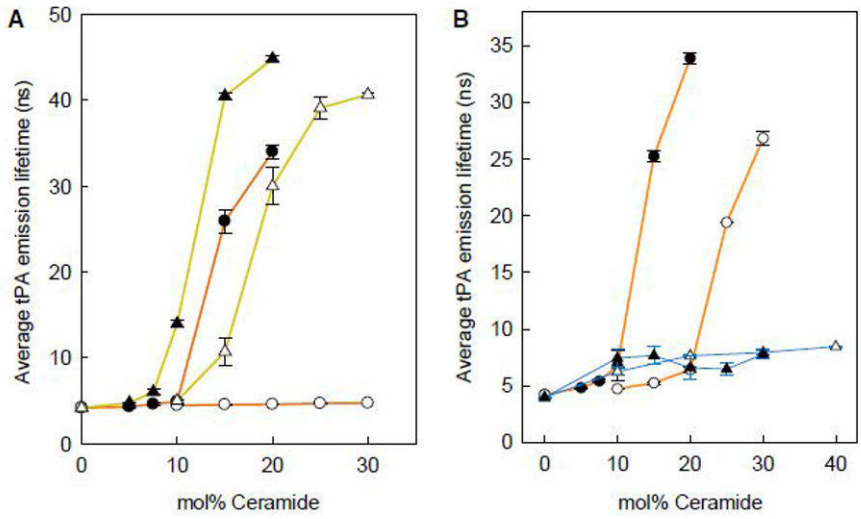


Figure 14. Figure A shows a comparison of tPA average lifetimes of 1-deoxy ceramide (orange) and 3-deoxy ceramide (yellow) with of without lyso-PC in DOPC. Panel B shows oleoyl ceramide (blue) and 24:1-ceramide (orange) with or without lyso-PC.

To determine the stoichiometry of the PCer/lyso-PC interaction in DOPC, tPA anisotropy was measured to determine the thermostability of the ternary system. The PCer concentration was kept constant at 10%, while lyso-PC was added in increasing amounts. The end melting temperature increased sharply with addition of lyso-PC, until the amount of PCer and lyso-PC reach equimolar membrane concentration. After the lyso-PC concentration exceeds that of PCer, there is still an increase in the end melting temperature of the ceramide-rich ordered domains, but at a slower rate.

Neither ceramide nor lyso-PC are able to form bilayers on their own, ceramide forms crystals and lyso-PC forms micelles. These lipids are dependent on co-lipids in order to be incorporated into a membrane. Since ceramide and lyso-lipids interact very favourably with each other, and our precious experiments with chol-PC and ceramide allows for formation of vesicles we tried this with oleoyl ceramide and lyso-PC as well. Oleoyl ceramide was chosen due to its significantly lower melting temperature compared to other ceramides. Together, oleoyl ceramide and lyso-PC formed vesicles, though with slightly irregular shapes as imaged with TEM. The vesicles appeared unilamellar, and their irregular shapes were likely due to the vesicles being in gel phase at the imaging temperature of 23°C.

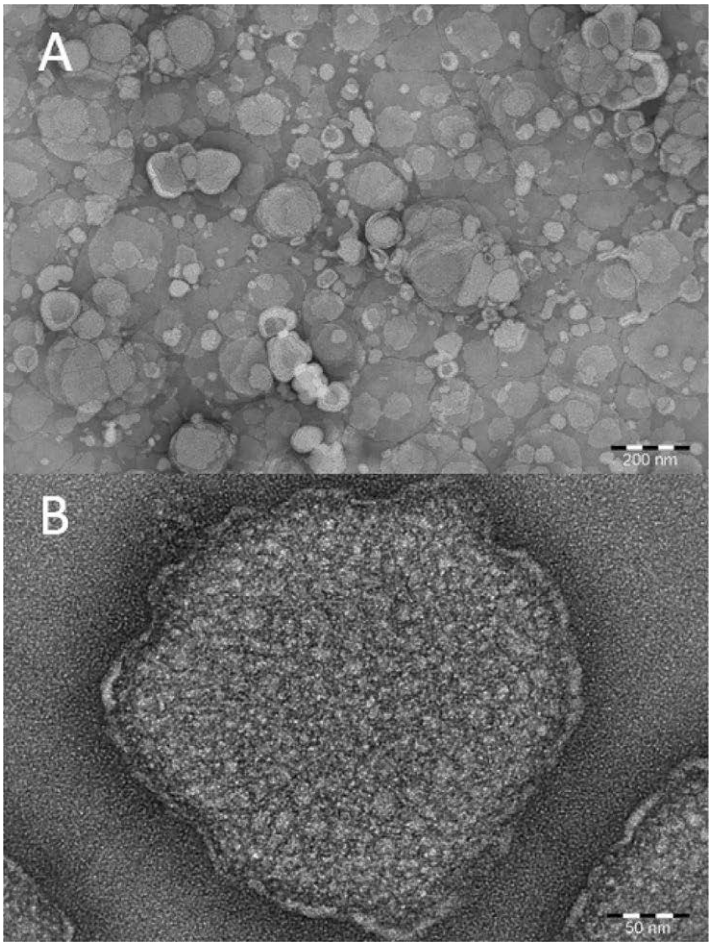


Figure 15. TEM images of a 1:1 oleoyl ceramide:lyso-PC complex with uranyl acetate lipid staining.

5. Discussion

5.1 Effect of ceramide "headgroup" structure on lateral segregation properties

Ceramide interactions with surrounding lipids in the membrane is dependent on how closely they are able to come in contact with each other. As most ceramides are very hydrophobic in comparison to other phospholipids and sphingolipids the interactions are largely affected by the availability of saturated acyl chains in order for ceramide to co-segregate[228]. In unsaturated membrane environments, ceramide is very likely to laterally segregate [229]. Cholesterol and ceramide do not however interact very favourably together despite both being very hydrophobic but are instead competing for favourable interactions, especially with sphingomyelin [119, 226]. When present at sufficiently high proportion at equimolar ceramide and cholesterol concentrations in saturated phospholipid membranes, they are able to coexist despite each other, forming a complex ternary gel-like phase [230, 231]. When the phospholipid ratio is increased, ceramide appears to be able to displace cholesterol from PSM, apparently relegating cholesterol to the edges of the domain [231]. This is likely due to competition for shielding from the aqueous environment provided by large headgroup lipids as both ceramides and cholesterol have very tiny hydroxyl groups that serve as headgroup [226].

With the structural modification of the 1-OH group on palmitoyl ceramides we were able to show the importance of its hydrogen bonding capabilities to surrounding lipids. A slight increase in the relative size of the ceramide headgroup by oxidation into the 1-COOH or to 1-COOMe did not cause much change in the average lifetimes of the ceramides in comparison to canonical PCer in MQ-water, while in a fully protonated form. In order to have a fully protonated state in the membrane, the lipids were treated with acetic acid. This indicated that either there is sufficient hydrogen bonding competence with the modified headgroup position in order to retain similar interactions with co-lipids as canonical PCer, or that the slightly increased relative headgroup size in itself is able to provide some shielding from the aqueous environment. However, following an increase in pH and consequent deprotonation of the 1-COOH ceramide, the ability to form ceramide-rich ordered domains is strongly attenuated [225]. The other ceramides are not as strongly affected by exchanging MQ-water for buffer at ph 7,4, suggesting that the negative charge at the headgroup position is detrimental for interlipid interactions, especially causing electrostatic repulsion between ceramide molecules.

The 1-deoxy ceramide is a potentially a biologically interesting lipid as the D-erythto-sphingosine analogue, spisulosine, originally extracted from the *Spisula polynyma* clam, corresponds to the 1-deoxy ceramide headgroup region structure. Spisulosine has been shown to be cytotoxic to some cancer cell lines by inducing apoptosis following ceramide synthesis upregulation

[232]. The mechanism remains unclear, but it was speculated that spisulosine could function as a substrate for ceramide synthase due to the structural similarity to D-erythro-sphingosine [232]. Clinical phase I trials on the anticancer effect by spisulosine were performed, but were cancelled due to poor efficacy and neurotoxicity [233, 234]. There has been some rational drug design based upon spisulosine, but where the 1' position has been exchanged for other functional groups. A compound with a 4-methoxyphenyl group at the 1' position had increased activity against several cancer cell lines, but mode of cell death turned into autophagy instead of apoptosis seen triggered by spisulosine [235].

As with 1-deoxy ceramide and 3-deoxy ceramide, lifetimes in POPC based membranes for these ceramide analogues again highlight the importance of the 1-OH position for co-lipid interaction. These results are in agreement with the previously reported, significantly weakened interactions with PSM [101]. Despite the slight increase in average lifetimes for 1-deoxy ceramide at similar concentrations to when PCer and 3-deoxy ceramide show an onset of ceramide-rich ordered domain formation, the average lifetime stays below 15ns which can be considered to indicate the presence of an ordered domain in a membrane, despite increasing concentrations of 1-deoxy ceramide. It is possible that this slight increase is due to an ordering effect on the POPC acyl chains by 1-deoxy ceramide [203] and due to a failure of tPA to interact with the ceramide analogue (al Sazzad et. al, unpublished results). 3-deoxy ceramide on the other hand shows an onset at a slightly higher membrane concentration than PCer, like 1-COOH and 1-COOMe ceramides. At higher concentrations it does however reach higher average lifetimes than PCer, that suggests it forms domains which are more ordered than PCer domains. This could be due to the increased hydrophobicity of 3-deoxy ceramide near its headgroup region, which would allow for the ceramide molecules to associate more closely to each other while simultaneously weakening the interactions with the phospholipid component of the membrane by decreasing the hydrogen bonding potential. This is in agreement with the 3-OH group being more involved in intramolecular hydrogen bonding with the 1-OH group[104] and is also supported by data that methylation of the 3-OH group does not cause very notable perturbations of interactions with other bilayer lipids [236].

The tPA anisotropy is in agreement with the average lifetimes of the same probe in similar lipid mixtures. With all ceramides, there is a gradual increase in thermostability with increasing ceramide content of the membranes, with the largest increase in thermostability generally occurring in the same ceramide concentration range as where the onset of long average tPA lifetimes are found in samples with similar lipid compositions, showing good correlation with methods. With 1-deoxy ceramide the increases in thermostability are small, further showing the seemingly weak interactions with the surrounding phospholipid, though this is likely a result of tPA failing to report on the ceramide rich ordered domain with its inability to interact

with the headgroup lacking ceramide species. It has been shown that monolayers of 1-deoxy ceramide are quite unstable and that miscibility in PSM is relatively low, as aggregates of 1-deoxy ceramide have been detected at 30 mol% in PSM [101], which also raises the question of the solubility in phospholipid membranes.

Addition of PSM to the membranes led to increases in thermostability for all ceramide species, but the stabilizing effect varied, apparently most affected by the structure of the 1-OH position or its modifications. The increase in thermostability was largest with canonical PCer, with a similar effect in the 3-deoxy variety. The domain stabilizing effect is slightly decreased for the 1-COOH variety, and further diminished with 1-COOMe. The thermostability of the ceramide-rich ordered domain with 1-deoxy ceramide is increased by only about 2°C, but considering the low starting value to begin with, this increase could be considered quite proportional to the decrease of thermostability in the other ceramide species. As the ceramides apart from 1-deoxy ceramide are more similar in their thermostabilities comparison of the effect of PSM is easier. As canonical PCer is stabilized the most, it suggests that the large headgroup provided by PSM both provides shielding from the aqueous environment while the sphingosine backbone participates in hydrogen bonding with the ceramide, for which the 1-OH group appears important[101]. As the 1-OH is present in the 3-deoxy ceramide, it does not cause a disruption for these interactions. With the oxidized ceramide species, the introduced carbonyl group does not seem to be very disruptive either to the hydrogen bonding network between the hydroxyl group to the choline headgroup of PSM. With COOMe-ceramide it appears that the methyl group weakens the hydrogen bonding potential, or possibly causes steric hindrances to closer interactions. The modest increase of thermostability of 1-deoxy ceramide together with PSM points towards the function of the 1-OH group in interactions with other membrane lipids in general, as the slightly increased melting temperature likely is only due to the presence of a higher amount of saturated acyl chains in the membrane.

As cholesterol competes with ceramide for SM interactions at lower concentrations [237, 238] the ability of cholesterol to compete with the ceramide analogues was measured by including it in equimolar concentrations to both PSM and the ceramide species in question. Of all the ceramide species cholesterol had the largest effect on the membranes containing PCer, decreasing the thermostability of the ceramide-rich ordered domains to values only slightly above that of binary POPC:PCer membranes. COOMe ceramide and 1-deoxy ceramide are affected in a similar way as PCer, with melting temperatures returning close to the starting values. Cholesterol has a smaller effect on COOH ceramide and 3-deoxy ceramide, with the latter being least affected. These results would suggest that COOH- and 3-deoxy ceramide have higher relative affinities to PSM than cholesterol does, since cholesterol has a low affinity to ceramide.

5.2 Lipids typical of mitochondria promote ceramide lateral segregation

Due to the proposed role of ceramide in the triggering of apoptotic events [239] and to try to gain a bit more understanding about possible mechanisms, we selected lipids typical of mitochondrial membranes. PE and CL are both lipids that are considered to induce negative curvature pressure in membranes [240], due to the small headgroup and proportionally large volume of acyl chains. PCer in itself is also considered to induce negative curvature pressure with resulting promotion of H_{II} phase formation in PE membranes, while some models suggest ceramide would form pores in the mitochondrial outer membrane [241, 242]. This model has been criticized [136] due to the high energetic cost for such a structure to be formed.

Both tPA lifetimes and anisotropy showed that the presence of PE in the membranes in which DOPC was the main lipid led to a promotion of the formation of a ceramide-rich ordered domain. Clearly increased order could be detected in all lipid compositions except for the POPC:DOPE phospholipid mixtures. This is likely due to two separate reasons, both ceramide avoidance of highly unsaturated lipid environments and negative curvature stress caused by small headgroup lipids. Though curvature stress has been difficult to show experimentally, simulations have opened some insights into the lateral pressure profiles of some lipids and concomitant behaviour in the membrane [243]. DOPE is usually assigned a wedge like shape with its small phosphatidylethanolamine headgroup mismatching the relative area of its two unsaturated acyl chains. POPE is also attributed with some negative curvature inducing slight area mismatch between its headgroup and acyl chains, but to a lesser degree. The promotion of the formation of ceramide-rich ordered domains in the DOPC based membranes with addition of either DOPE or POPE can possibly be explained by different mechanisms. First, in the systems containing DOPC and DOPE, PCer will not have very favourable interactions with either phospholipid due to the unsaturated acyl chains in both species. Here the increase in average lifetime due to the promotion of the ceramide-rich ordered domain formation could arise from PCer interacting less favourably with DOPE, as its small headgroup provides insufficient shielding from the aqueous environment. It is possible that DOPE could locally create areas with negative curvature where the presence of PCer would add further negative curvature stress. As a result, DOPE would thus effectively be pushing ceramide away from itself, while PCer also would be more likely to co-localize with DOPC and with the locally increased ceramide content the ceramide-rich ordered domain is formed.

In the DOPC:POPE phospholipid system there is a similarly clear effect on the onset of the ceramide-rich ordered domain formation while the cause may be different from the situation with DOPE. With its saturated acyl chain, POPE would be a more favourable co-lipid than DOPC despite its small headgroup. The average lifetimes of the ternary lipid mixtures of DOPC:POPE with the highest PCer concentration hint that ceramide-rich ordered domain may be

more heterogenous than in DOPC:PCer. With increasing POPE concentration the average lifetime is somewhat decreased which could indicate that POPE is incorporated to a higher degree into the ceramide-rich ordered domain, while the longer average lifetimes in DOPC:PCer could be caused by stronger lateral segregation and hence "purer" gel-like domains, as less DOPC would be incorporated into the domain in the binary system, similar to earlier results by our group [116].

With the POPC:DOPE lipid compositions, addition of PCer does not further shift the onset of the ceramide-rich ordered domain towards lower ceramide concentrations. This can be explained by the preference for saturated acyl chains by ceramide. Similarly, in POPC:POPE there is only a slight shift to a lower amount of required ceramide in order to form a ceramide-rich ordered domain. In both of the POPC based lipid mixtures, the average lifetime with the highest PCer concentration are shorter than in corresponding DOPC based systems, especially binary DOPC:PCer. Though the onset of the ceramide-rich ordered domain occurs at much lower PCer concentrations in POPC based membranes, the shorter average lifetimes when the lipid system has reached saturation of ceramide, or when lifetimes are not further increased with higher ceramide concentrations, is due to the higher miscibility of POPC and PCer.

The exact composition of the ceramide-rich ordered domains cannot be known for certain due to limitations of our method. It has been shown by DSC and NMR that in binary POPE:PCer systems fluid lamellar phases can coexist with ordered phases at physiological temperatures, while at higher temperatures a three phase coexistence including a H_{II} phase is possible. Inclusion of ceramide lowers the temperature at which the H_{II} phase is formed in POPE membranes [244]. In order to gain more insight on the POPC and POPE interactions of PCer and whether there is a preference for either phospholipid, lifetime measurements were also carried out at 37°C. This temperature was chosen as to gain a larger dynamic window to detect differences with POPC:POPE ratios as both lipids are in a more fluid state at this temperature, and to have data at a biologically relevant temperature. Higher POPE concentrations were also included. Average lifetimes were significantly shorter than in measurements at 37°C, which is both a result from the increased dynamics of the membrane at this temperature, but also a feature of tPA [215]. Though the onset of the ceramide-rich ordered domain occurred at the lowest PCer concentration in binary POPE:PCer, it is not necessary a sign of preference for interaction with the phospholipid. As POPC concentration is increased, the onset is slightly shifted towards higher PCer concentrations required while also the average lifetime is increased. The highest average lifetimes are reached at a POPC:POPE ratio of 20:80, after which the onset is more markedly shifted towards higher PCer concentrations required for the ceramide-rich ordered domains to form, while the average lifetimes start decreasing again. Domain stability is reflected in tPA anisotropy measurements of corresponding lipid mixtures, with 20:80

POPC:POPE resulting in the highest thermostability. It is possible, that when POPC is present in smaller amounts in a POPE-rich membrane, ceramide will seek to associate with POPC to alleviate potential curvature stress imposed by POPE and also to take part of shielding from the aqueous environment by the choline headgroup of POPC. On the other hand, POPE would provide denser packing which should be a more favourable membrane environment for PCer. It should however be considered, that when only POPE is available to PCer, the interaction is forced resulting in more loosely packed domains. It is likely that the ordering effect on POPE by ceramide is lower than in POPC due to POPE being innately more ordered than POPC because of the smaller headgroup and hydrogen bonding properties of the ethanolamine, and thus interaction with POPC would be favoured due to entropic effects [244].The longer average lifetimes with POPC present could thus be a result of ceramide crowding due to the limited amount of POPC. The consequent decrease of the average lifetimes with increasing proportion of POPC in the membrane would then be a result from PCer being able to mix more freely.

Cardiolipin is a more special type of lipid, being essentially a double phospholipid, which is why the proportions of CL used in experiments were half the molar ratio of PE used in similar experiments, to try to keep the proportions of acyl chains from each lipid species at more similar proportions. CL was able to promote the formation of a ceramide-rich ordered domain in DOPC although to slightly lower degree than either PE species used in previous experiments, while its effect on POPC based membranes was an increase in the average lifetime. tPA anisotropy also showed a clearer effect in the POPC based systems. These results are interesting, since CL in general is highly unsaturated which would suggest that ceramide should not have very favourable interactions with it. CL is also attributed with negative curvature stress and promotion of hexagonal phase formation and has been shown to segregate into membrane regions of negative curvature [245, 246]. Still, the mechanism of the interactions within both DOPC and POPC based membranes could be the same as for corresponding experiments with PE, namely ceramide seeking more favourable interactions with co-lipids. The more modest promotion of the formation of a ceramide-rich ordered domain in DOPC based membranes by CL as compared to the effects of either PE species might be due to weaker forces repelling or attracting PCer. As both phospholipids have unsaturated acyl chains both should be equally favourable/unfavourable interactions. However, since CL is a conjugated lipid, DOPC might provide a higher degree of flexibility to better accommodate the more rigid, saturated acyl chains of PCer. Again, with POPC, there is not much potential for a lipid to further promote an onset of ceramide-rich ordered domain formation. CL does however significantly increase the average lifetimes after the onset which is indicative of increased order within the membrane. The effect is also seen in tPA anisotropy as both CL concentrations used cause a similar increase in thermostability of the membranes. This is in contrast of POPC based membranes in which DOPE is

included, where no increase in average lifetime or higher thermostability can be seen. A possible explanation could be that interfaces of POPC:CL interaction provide local environments that ceramide may further order.

Finally, inclusion of a small amount of PSM to DOPC based membranes has a strong ordering effect on all lipid compositions, effectively overriding all other order increasing effect that may be present due to the high affinity of PCer to PSM. The effect was equally strong in bilayers where POPE had been exchanged for POPC, as in DOPC with PCer. A minute difference at one PCer concentration in the middle of the onset of DOPC:POPE:PSM might give a hint of two simultaneous order increasing effects, but due to the very narrow dynamic window caused by the very potent effect of PSM it is not possible to draw any conclusions.

The implications of the lipid component in the mitochondrial outer membrane permeabilization (MOMP) of apoptosis have been a subject of interest and speculation, with results being far from conclusive. The ceramide component of apoptosis especially raises a few questions regarding it that need addressing. Are pure ceramide channels [241] or membrane destabilization induced by ceramide [136] sufficient for MOMP and subsequent leakage of large solutes, triggering apoptosis? While it has been shown that diverting ceramide to the mitochondrial outer membrane by CERT mediated transport leads to apoptosis that involves the apoptotic Bax protein [130] the question remains how sufficient amounts of ceramide to trigger apoptosis are transferred to mitochondrial membranes naturally. Mechanisms are likely either degradation of mitochondrial sphingomyelin present at low levels [196, 197] or through contact sites to the mitochondria-associated membrane of the ER [195]. It has been suggested that ceramide would enhance the pore forming capability of Bax [247] or that Bax would preferentially insert into mitochondrial ceramide-rich domains [248]. A more recent study suggests a role of ceramide regulating Bax membrane association/disassociation indirectly through regulation of the voltage-dependent anion channel 2 (VDAC2) [199]. On the other hand, other mitochondrial lipid components have also been shown to be essential for MOMP to occur. Bax mediated permeabilization enhanced by Bid has been shown to require CL [249], though the permeabilization is more dependent on CL to occur than to require activation from Bid, as the lipidic pores fail to form in PC only membranes [250]. Additionally, presence of oxidized PC species further seems to facilitate Bax membrane insertion by perturbing the acyl chain region of the membrane [251, 252]. Apparently, positive membrane curvature favours membrane permeabilization by Bax, while lipids with intrinsic negative curvature inhibit this [253] which also supports the mechanism of increased permeabilization by oxidized phospholipids mentioned previously. Recently, it has been found that the asymmetry of the mitochondrial outer membrane actually has an inhibiting effect on permeabilization by Bid activated Bax, with higher activity in symmetrical vesicles and by extension, in mitochondrial outer membranes in which

asymmetry is lost [254]. In this model system of PC, PE and CL, PE was mainly located in the inner leaflet of the asymmetric vesicles, alleviating curvature strain by the lipid and thus allowing for tighter lipid packing in each leaflet [254].

Taken together, our results cause more questions in the light of results of other groups on the membrane interaction of pro-apoptotic Bax. What is the actual role of ceramide in membrane permeabilization? While formation of ceramide-rich ordered domains is promoted in membranes containing mitochondrial lipids it appears that Bax is preferentially inserted into membranes with curvature stress or membrane defects [254, 255]. Ordering of the membrane by ceramide does thus not seem to be a likely cause of events leading to membrane permeabilization, which is maybe also hinted at by ceramide potentially preferring forming ceramide rich domains together with PC rather than PE according to our results. In any case, content of PE should be lower in the outer leaflet of the mitochondrial outer membrane prior to apoptosis. CL appears to be the preferred lipid for membrane binding of pro-apoptotic proteins. Ceramide appears to have little direct effect in the initiation of apoptosis, even though ceramide levels in mitochondria are elevated. It is therefore more likely that it has a more indirect role, for which interaction with VDAC2 and regulation of Bax membrane binding is a potential explanation [199]. Could ceramide also promote the loss of membrane asymmetry with its ability to relatively easily flip-flop from one leaflet to another?

5.3 Lyso-PLs form complexes together with several ceramide species

The reasoning in exploring the interactions between ceramide and lyso-PLs was originally intended as a means of trying to undo potential negative curvature effects by PE by the positive curvature supposedly induced by lyso-PLs. The positive intrinsic curvature of lyso-PLs is due to the area mismatch of their single acyl chain and the headgroup, granting a shape more similar to an inverted cone. The ability of lyso-PC to inhibit the transition to H_{II} phase in PE [150] and ceramide containing systems has previously been shown [110]. Instead, when lyso-PC was included in a DOPC:DOPE membrane in an attempt to push back the onset to higher PCer concentrations needed for ceramide-rich ordered domain formation, the effect was the opposite, with an even stronger onset promotion as a result. The increase of order persisted when PE was removed and replaced with lyso-PC, indicating a very strong affinity of PCer for lyso-PC. Also, in this case the effect is reflected by tPA anisotropy, revealing an increase in end melting temperature of ceramide-rich ordered domains of approximately 10°C. The very favourable interaction of PCer and lyso-PC is not really surprising, considering the large headgroup providing shielding from the aqueous environment while there is extra space beneath it due to the lack of one acyl chain. The single, saturated acyl chain in itself is

also a very attractive partner to PCer that favours interactions with saturated lipids in general.

To investigate whether PCer has a preference towards specific headgroups in the context of lyso-lipid interactions as well as the role of the acyl chain component, palmitoyl lyso-PE was also included, along with palmitoyl lyso-SM and oleoyl lyso-PC. Of these lyso-lipids, all except oleoyl lyso-PC exhibited very similar effects in inducing similar amounts of order in the membrane as measured by tPA lifetimes. Oleoyl lyso-PC did increase the order as compared to DOPC:PCer only, but significantly less than the other lyso-lipids, highlighting the importance of the saturated acyl chain for lyso-lipid/PCer interactions. Because of the unsaturated acyl chain, and consequently increased occupation of the area below the headgroup, ceramide cannot form as close interactions as with the saturated lyso-lipids. This is reflected by the end melting temperatures of equimolar mixtures of PCer with the different lyso-lipids in DOPC, where the oleoyl acyl chain gives an end melting temperature equal to, or slightly less, than in DOPC:PCer. Still, the amount of PCer is half of that in the system without a lyso-lipid. The highest end melting of PCer/lyso-SM is likely possible due to the additional hydrogen bonding capability of the backbone structure, as compared to both lyso-PC and lyso-PE.

In order to take into account the role of ceramide structure for interactions with lyso-PC, 1-deoxy ceramide was selected for its lack of "headgroup" while 3-deoxy ceramide lacks the hydroxyl group on the 3' position which according to the first paper discussed in this thesis is not as important for ceramide/co-lipid interactions as the primary hydroxyl group. The acyl chain compositions of the ceramide were considered with the inclusion of oleoyl ceramide and 24:1-ceramide to take into account acyl chain mismatch. Rather unsurprisingly, the behaviour of 3-deoxy ceramide and its interaction with lyso-PC was very similar to that of PCer, further highlighting the notion that this supposed hydroxyl group is not essential for ceramide interaction to co-lipids in the membrane. Interestingly, 1-deoxy ceramide did show a significant stabilization in the membrane due to the influence of lyso-PC, considering that 1-deoxy ceramide fails to induce any order in a DOPC based membrane. According to unpublished DSC data of POPE and 1-deoxy ceramide membranes (al Sazzad et al., unpublished results) the ceramide appears to separate completely from the phospholipid. This could be an explanation for lack of onset of a ceramide-rich ordered domain by this ceramide species, leading 1-deoxy ceramide to form aggregates that cannot be detected by tPA lifetime measurements. It is also possible that the in order for tPA to properly associate with the ceramide rich domain, and by extension report it, the hydrogen bonding competence of the 1' position is required. Still a further possibility of 1-deoxy ceramide formed domains is that their membrane depth is affected and out of reach of tPA for that reason. On the other hand, presence of aggregates can also be a sign of miscibility problems in the process of sample preparation. Regardless of the aggregation state of

1-deoxy ceramide in DOPC, when lyso-PC was included the ceramide was able to form ceramide-rich ordered domains together with lyso-PC. Merely presence of lyso-PC is not sufficient to increase the order in a membrane, as is evident from the first tPA lifetime figure of membranes including lyso-PC (figure 12), which suggests that lyso-PC is able to recruit 1-deoxy ceramide to the membrane in case it has not been present but as free aggregates previously. More data of 1-deoxy ceramide membrane behaviour would be preferable. As for the ceramides with unsaturated acyl chains, though 24:1 ceramide requires high membrane concentrations to form a ceramide-rich ordered domain in DOPC membranes, this amount is significantly lowered by added lyso-PC. It appears that the low position of the unsaturation upon the acyl chain of 24:1 ceramide is not interfering with interaction with lyso-PC. The shorter average lifetimes than those in saturated ceramides suggest that the membrane is still more disordered. Oleoyl ceramide on the other hand was the only ceramide species that we tested that was not forming a ceramide-rich ordered domain neither on its own nor together with lyso-PC, remaining in a fluid, disordered state.

To gain more understanding of the preferred ratio of ceramide and lyso-PC interactions, tPA anisotropy was measured. Increasing the amount of lyso-PC in relation to PCer led to a gradual increase in the end melting temperature until one point when the increase ceases to be linear, but starts plateauing instead. This point through which two linear lines could be drawn is at a 50/50 mole ratio of PCer and lyso-PC, which suggests that a 1:1 interaction is the most favourable for these lipid species as the stabilizing effect becomes saturated. It is also possible that the domains formed by lyso-PC and PCer could contain a higher ratio of lyso-PC, but for this our methods do not yield direct evidence.

Finally, since ceramide is unable to form bilayers on its own, except reported stacked bilayers with low hydration levels [112, 113], and as lyso-PC neither can form bilayers on its own but will form micelles, we tried preparing liposomes from ceramide and lyso-PC. The interactions of the two lipid species are very favourable as is evident with our results and have shape factors that complete each other. It has also been reported that lyso-PC can form bilayers with cholesterol [139], and since ceramide is similarly hydrophobic it would thus give more insight on some of ceramides similarities to cholesterol. Oleoyl ceramide was selected so that liposomes would be in a fluid phase for EM imaging, which revealed unilamellar liposomes formed by only oleoyl ceramide and lyso-PC. The irregular shapes of the liposomes were likely due to the staining method.

6. Conclusions

For this thesis I have explored the functions of the hydroxyl groups on the ceramide molecule at the 1- and 3 positions and what implications the abolishing of these groups have, and with replacing the 1-OH group with oxidized functional groups. Based on the results it appears that in order to retain strong intermolecular interactions the ability of the functional group at 1-OH is able to function as a donor or acceptor of hydrogen bonds, whereas abolishing this group results decreases the ceramide's ability to interact with other membrane lipids as has been shown previously. We have however also become aware of limitations of our method to be able to detect possible ceramide rich ordered domains formed by the 1-deoxy ceramide, and thus further work is needed to clarify the state of this ceramide species in membranes, is it solely an issue of detection, or is it an issue of ceramide membrane solubility which also can be a partial explanation.

Despite the apparent failure to detect the ceramide rich ordered domain itself, the results would appear to show the increased order of the POPC molecules in the membrane whereas a similar ordering of DOPC is absent. This reflects the importance of the acyl chain order in the surrounding membrane as a factor in driving ceramide lateral segregation. Combining a decreased headgroup area by negative curvature inducing lipids with acyl chain interaction preferences of ceramide manifests as promoted lateral segregation. For unsaturated DOPC membranes is seems most likely that the segregation by a repulsive effect where either PE species introduce curvature stress in the membrane, and possibly membrane defects. It could be argued that while ceramide in itself also has membrane destabilizing properties and inherent negative curvature, it is a small molecule that could become localized into these areas. A preference for the PC headgroup lipid interaction can also be discerned from the lifetimes recorded at 37 °C, as differences in lipid preference cannot be distinguished at 23 °C. The effect underlying the increase in average lifetime indicative of increased membrane order by the introduction of CL seems a bit contradictory to the apparent lack of membrane ordering effect by DOPE in POPC. Though both DOPE and CL have unsaturated acyl chains and negative intrinsic curvature, and ratios of acyl chains taken into consideration in experiments, there is a clear difference in effects. Could this be caused by miscibility differences? If that is the case the CL results could indicate localized membrane environments where ceramide forms more highly ordered domains. CL also has been shown to have stronger membrane sorting properties than PE.

Preferences for different phospholipid headgroups are not as obvious when ceramides are introduced to lyso-phospholipids, as there is increased space under the headgroup, providing shielding from the aqueous exterior of the membrane. While lyso-PLs have been studied before as a means of alleviating negative curvature stress, the complex forming properties together with ceramides seems to have been neglected, as the effect is rather membrane integrity improving.

Taken together, these papers highlight the preferences ceramide have toward different phospholipids when considering their structures as a whole in determining the properties of the given membrane the ceramide species is present in. While the effects of membrane curvature remain difficult to experimentally show, lipids typical of mitochondria have significant effect on ceramide lateral segregation properties. This could have implications for apoptotic events, as mitochondrial ceramide has been shown to be able to initiate apoptosis, while a mechanism for this remains elusive. The potentiating effect on ceramide-rich ordered domain formation by lyso-PLs could be interesting to explore further, with both lipids being active signalling molecules whose generation are increased during cell stress conditions.

7. References

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