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Review

Cholesterol interactions with phospholipids in membranes

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Abstract

Mammalian cell membranes are composed of a complex array of glycerophospholipids and sphingolipids that vary in head-group and acyl-chain composition. In a given cell type, membrane phospholipids may amount to more than a thousand molecular species. The complexity of phospholipid and sphingolipid structures is most likely a consequence of their diverse roles in membrane dynamics, protein regulation, signal transduction and secretion. This review is mainly focused on two of the major classes of membrane phospholipids in eukaryotic organisms, sphingomyelins and phosphatidylcholines. These phospholipid classes constitute more than 50% of membrane phospholipids. Cholesterol is most likely to associate with these lipids in the membranes of the cells. We discuss the synthesis and distribution in the cell of these lipids, how they are believed to interact with each other, and what cellular consequences such interactions may have. We also include a discussion about findings in the recent literature regarding cholesterol/phospholipid interactions in model membrane systems. Finally, we look at the recent trends in computer and molecular dynamics simulations regarding phospholipid and cholesterol/phospholipid behavior in bilayer membranes.

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Nomenclature

DPPC,	dipalmitoyl-phosphatidylcholine;
DRM,	detergent-resistant membranes;
DSC,	differential scanning calorimetry;
N-O-SM,	*N*-oleoyl-sphingomyelin;
POPC,	palmitoyl-oleoyl-phosphatidylcholine;
POPE,	palmitoyl-oleoyl-phosphatidylethanolamine;
POPS	palmitoyl-oleoyl-phosphatidylserine;
T_m,	gel to liquid–crystalline phase transition temperature

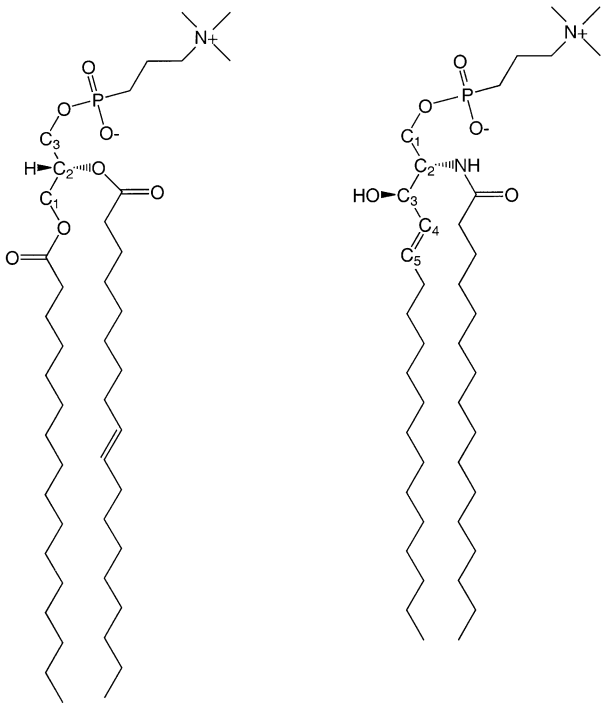
1. Phospholipids

1.1. Molecular structure of choline-containing phospholipids

The structures of both phosphatidylcholine and sphingomyelin include the same hydrophilic phosphorylcholine head group, which is zwitterionic at neutral pH. Both lipids also contain two long hydrocarbon chains, which form the hydrophobic domain of these amphiphiles (Fig. 1). The

conformation and the charge distribution are quite similar in these phospholipids, but they are certainly chemically different [1]. The differences arise from the fact that phosphatidylcholine has a glycerol backbone to which two fatty acids are linked via ester bonds, while sphingomyelin has a long-chain sphingoid base with an amide-linked acyl chain. Therefore the interfacial region is substantially more polar in sphingolipids than the complementary region in phosphatidylcholine. Another important difference in the structure of the two choline-phospholipids is that the sphingomyelin interfacial region contains both hydrogen bond donating and accepting groups, while the corresponding region in phosphatidylcholine contains only hydrogen bond accepting groups [1]. A recent study from this laboratory showed that the enthalpy of Triton X-100 partitioning into large unilamellar vesicles prepared from either dipalmitoyl phosphatidylcholine or D-erythro *N*-palmitoyl sphingomyelin was much higher for sphingomyelin membranes (+37.3 kJ/mol) compared to the chain-matched phosphatidylcholine membranes (+6.2 kJ/mol) [2]. This finding strongly suggest that the sphingomyelin membrane interface was much more extensively hydrogen bonded as compared to the phosphatidylcholine interface.

In biological membranes, the length and saturation of the hydrocarbon chains also contribute to the different properties of sphingomyelins and phosphatidylcholines. Naturally occurring phosphatidylcholines typically contain two hydrocarbon chains of nearly similar length. The acyl



1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine D-*erythro*-*N*-palmitoyl sphingomyelin

Fig. 1. The chemical structure of two typical choline-containing phospholipids, the glycerophospholipid 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine and the sphingolipid D-*erythro*-*N*-palmitoyl-sphingomyelin. The shown configurations are for the naturally occurring species, other configurations can be found in semisynthetic commercial preparations.

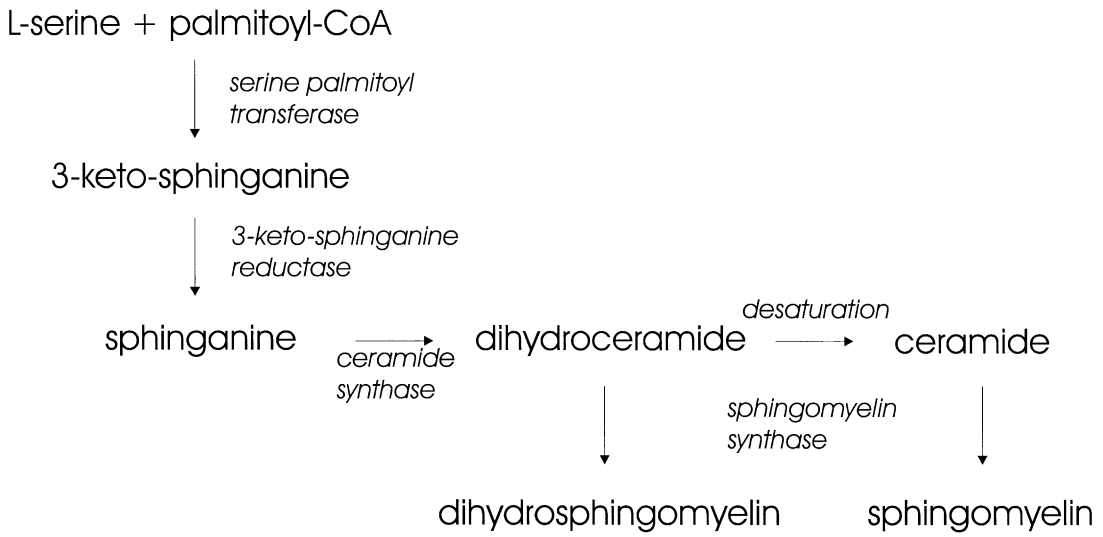


Fig. 2. The major steps in the de novo biosynthesis of sphingomyelin or dihydrosphingomyelin from L-serine and palmitic acid in cells.

chain attached to the C_1 glycerol carbon is usually saturated, while the acyl chain esterified to the second carbon is normally unsaturated [3,4]. The unsaturated acyl chain typically contains between one and six double bonds of the *cis* configuration [3,4]. The sphingomyelins in biological membranes are much more saturated than naturally occurring phosphatidylcholines [5]. Furthermore, natural sphingomyelins often have a very long amide-linked acyl chain (20–24 carbons in length), while the sphingoid base is most often sphingosine (D-*erythro*-2-amino-*trans*-4-octadecene-1,3-diol) [3,6,7]. This will lead to a mismatch in chain length for most natural sphingomyelins, and such a chain disparity has been shown to affect membrane curvature [3,8]. The chain disparity also enables sphingomyelins to participate in transbilayer hydrocarbon interdigitation [1,9,10].

1.2. Molecular structure of other common glycerophospholipids

In addition to phosphatidylcholine biological membranes also contain other glycerol-based phospholipids. As in phosphatidylcholine, the glycerol hydroxyl in the *sn*-3 position is linked to a polar phosphate-containing group and the other two hydroxyls are linked to acyl chains. Phosphatidylserine and phosphatidylethanolamine are aminophospholipids, i.e. contain a primary amine in the head group. Other common glycerophospholipids are phosphatidylinositol, cardiolipin, phosphatidic acid and phosphatidylglycerol [11]. Each of these lipid classes exhibits a characteristic acyl-chain composition. There is usually a saturated acyl chain esterified at the *sn*-1-position of the glycerol backbone, whereas the acyl-chain at the *sn*-2-position usually is unsaturated. In eukaryotic membranes, phosphatidylethanolamine and phosphatidylserine are more unsaturated than other phospholipids.

1.3. The synthesis and distribution of sphingomyelin in biological membranes

Sphingolipids are indispensable for eukaryotic life [12]. In addition to their primary role as structural components of biological membranes they have important functions in organizing the lateral domain structure of membranes and as second messengers. Sphingomyelins are also found as components of serum lipoproteins. Lately, it has been suggested that sphingomyelin also could be a "functional" constituent of food protecting against disease. At present, best evidence exists for the beneficial effects of dietary sphingolipids on atherosclerosis and colon cancer [13,14].

Sphingomyelin is synthesized by direct transfer of the phosphorylcholine moiety of phosphatidylcholine to ceramide by the enzyme sphingomyelin synthase [15–18]. The reaction also yields diacylglycerol, which can be recycled back to phosphatidylcholine [19,20]. The ceramide needed for sphingomyelin generation is mainly synthesized from palmitoyl-coenzyme A and L-serine in a series of reactions in the endoplasmic reticulum (Fig. 2) [17,18]. The major intracellular site of de novo sphingomyelin synthesis has not yet been established, since synthetic activities have been found in several compartments, including the *cis* and *medial* Golgi apparatus, the plasma membrane and the endocytic pathway [21–23]. It appears that sphingomyelin synthesis can take place in different compartments in the cell and that the major site of sphingomyelin synthesis can vary depending on the cell type. Inside the cell sphingomyelin is transported in carrier vesicles [19,24]. When needed, sphingomyelin is cleared from the cell by acid sphingomyelinase in lysosomes [19,25].

The distribution of sphingomyelin among cellular membranes is irregular, and more than half of the cellular sphingomyelins are found in the plasma membrane [19,24,26]. Within the plasma membrane sphingomyelin is, like phosphatidylcholine, mostly concentrated to the exoleaflet [27,28].

Sphingolipids are particularly relevant for the cellular physiology because of their property to cluster with cholesterol in the plane of the membrane. These sphingolipid/cholesterol clusters make up the basis for rafts in the plasma membrane. Furthermore, sphingomyelin has regulatory roles in the cell and participates in cellular signaling systems [25,29]. The sphingomyelin residing in the plasma membrane endoleaflet is the donator of second messengers in the sphingomyelin cycle [30, 31]. The cycle starts with degradation of sphingomyelin by neutral sphingomyelinase to ceramide. Depending on the activating signal for sphingomyelin breakdown ceramide can be further metabolized to sphingosine or sphingosine 1-phosphate, or the cycle can be completed by the resynthesis of sphingomyelin by sphingomyelin synthase [32–34].

1.4. Phosphatidylcholine is the major phospholipid in cell membranes

The main function of phospholipids, particularly phosphatidylcholine, is to maintain the structure of cellular membranes. Besides this function phosphatidylcholine also has regulatory roles in cells and it participates in cellular signaling. Like sphingomyelin, also phosphatidylcholine is found in serum lipoproteins [35].

Phosphatidylcholine is mainly synthesized from diacylglycerol in the endoplasmic reticulum. The last reaction in this synthesis pathway is catalyzed by CDP-choline: 1,2-diacylglycerol cholinephosphotransferase, which transfers the choline group from CDP-choline to diacylglycerol [20,36–38]. The diacylglycerol needed for phosphatidylcholine synthesis is ultimately generated

from glycerol-3-phosphate and fatty acyl-coenzyme A in a series of reactions [20,37]. In the liver a significant amount of phosphatidylcholine is generated by methylation of phosphatidylethanolamine in the endoplasmic reticulum, but this pathway is of minor significance in other cell types [20,36–38]. Inside the cell phosphatidylcholines can be transported by phosphatidylcholine transfer proteins or by vesicles [39,40]. Cellular phosphatidylcholine can be degraded by several phospholipases, namely classes A_1, A_2, B, C, and D [41]. These phospholipases reside in different cellular compartments and some of them also function in signaling pathways. Phosphatidylcholine serves as a reservoir for several lipid messengers: it is the source of the bioactive lipids diacylglycerol, phosphatidic acid, lysophosphatidic acid, platelet-activating factor and arachidonic acid [42–45].

The distribution of phosphatidylcholine among cellular membranes is more even than the distribution of sphingomyelin. Therefore there is no prominent difference in the mass fraction of sphingomyelin and phosphatidylcholine residing in the plasma membrane [3,46,47]. Like sphingomyelin, also phosphatidylcholine is concentrated to the outer leaflet of the plasma membrane [11,48].

1.5. Membrane function and distribution of aminophospholipids

Plasma membranes of cells are known to show asymmetry when the distribution of the major phospholipid classes is considered. Phosphatidylcholine and sphingomyelin have been localized to the external leaflet of the plasma membrane whereas the aminophospholipids phosphatidylserine and phosphatidylethanolamine are predominantly found on the internal leaflet of the plasma membrane. Earlier it was thought that such asymmetry rises from interactions between the phospholipids and cytoskeletal elements [49] but now it is clear that active energy requiring mechanisms are essential for the formation of this asymmetry (recently reviewed in Refs. [50,51]).

Aminophospholipids have been shown to take part in the regulation of several processes, such as blood coagulation [52], cell adhesion [53–56] and endocytosis [48,57]. The mechanisms have not yet been fully elucidated but the biophysical characteristics of these lipids have to be taken into account. Aminophospholipids are cone-shaped lipids, which means that the head group takes up less space than the acyl chains. These lipids therefore have a tendency to form non-bilayer structures such as inverted hexagonal phases or cubic phases. Non-bilayer structures have been suggested to be involved in membrane fusion [58,59]. This phenomenon seems to be of importance for example for the formation of endocytic vesicles. When non-bilayer lipids are forced into a planar structure, like a bilayer, the intrinsic pressure of the hydrophobic part of the membrane will increase. Changes in the tension of the hydrophobic portion of the plasma membrane have been suggested to regulate protein function and folding. Different aspects of the biological relevance of non-bilayer structures have been discussed thoroughly in recent reviews [60,61].

2. Cholesterol

Even if phospholipids and sphingolipids build up the matrix of cellular membranes, sterols are essential components of these membranes. In contrast to the amazing diversity of phospholipid

species, mammalian cells contain one major sterol, cholesterol, which is absolutely required for viability and cell proliferation [62,63]. Sterols differ from the other membrane lipid classes, and in principal consist of pure hydrocarbon in the form of a steroid ring structure. The maximal solubility of cholesterol in bilayers of glycerophospholipids is limited to between 50 and 66 mol%, depending on the host lipid species in the bilayer [3,64,65], but they can not alone form lamellar structures [66]. Sterols appear to have evolved to fill the flickering spaces among the acyl chains in membrane bilayers [67].

2.1. Molecular structure and cellular functions of cholesterol

The molecular structure of cholesterol includes a tetracyclic fused ring skeleton, with a single hydroxyl group at carbon 3, a double bond between carbons 5 and 6, and an iso-octyl hydrocarbon side chain at carbon 17 (Fig. 3) [68,69]. The rings of cholesterol are fused in the *trans* configuration, which makes the molecule planar and rigid, except for the flexible iso-octyl side chain [70–72]. An important notion on the three-dimensional structure of cholesterol is that the 3β-OH group, the two methyl groups and the side chain are all located on the same side of the ring skeleton (β-configuration) [68,73,74]. The hydroxyl group in cholesterol is very important, because it gives the otherwise hydrophobic compound its amphiphilic character [66,71] and therefore orients the molecule in membranes. Further, the hydroxyl group can also mediate the hydrogen bonding of cholesterol with water and possibly with other lipid components of cellular membranes [74–76].

The most important function of cholesterol is perhaps its ability to modulate the physicochemical properties of cellular membranes [72,77–79]. Cholesterol orients in a phospholipid bilayer with its polar hydroxyl group encountering the aqueous phase and the hydrophobic steroid ring oriented parallel to, and buried in the hydrocarbon chains of the phospholipids. In the membrane, cholesterol interacts with membrane phospholipids and sphingolipids and influences their behavior. Incorporation of increasing levels of cholesterol broadens and eventually eliminates altogether the cooperative gel/liquid-crystalline phase transition of the host lipid bilayer [80,81]. Cholesterol induces an ''intermediate state'' in phospholipid molecules with which it interacts, increasing the fluidity of the hydrocarbon chains below and decreasing the fluidity above the T_m (gel to liquid-crystalline phase transition temperature [80,82]). In the biologically relevant liquid-crystalline state, cholesterol increases the degree of orientational order and reduces the rate of motion of the phospholipid hydrocarbon chains [72,83]. A higher order in the

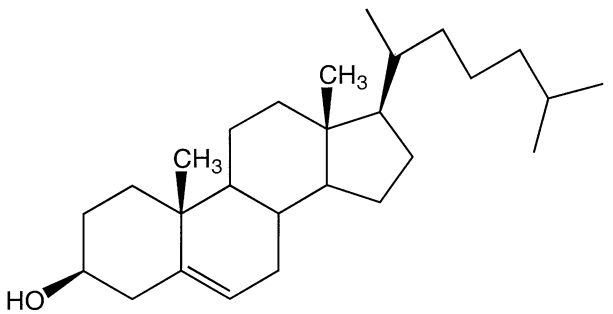


Fig. 3. The chemical structure of 5-cholesten-3β-ol or cholesterol.

membrane will lead to a laterally more condensed membrane, with increased packing density of the phospholipids [84–86]. This will increase the mechanical strength and decrease the permeability of the membrane [72,87]. Still, the relatively high rates of lateral and rotational diffusion, characteristic for fluid phospholipid bilayers are maintained [77,88,89].

Because cholesterol affects membrane properties, it can also manipulate the behavior and functions of proteins residing in the membrane [77,90]. A variety of integral membrane proteins, including ion channels, membrane receptors and enzymes, are sensitive to physical changes in the surrounding lipid bilayer [62,77,91]. Some proteins also bind cholesterol directly to become either activated or inactivated [90,92,93]. The activity of cholesterol-regulated proteins is probably mediated via sterol-sensing domains [94,95]. The proteins that partition into membrane rafts are also dependent on cholesterol for their normal function, since cholesterol is needed for the formation of the rafts [96].

Cholesterol has, besides its function in membranes, also an important role in eukaryotes as the precursor of steroid hormones in steroidogenic cells and of bile acids in hepatocytes [97,98]. Further, cholesterol acts as a precursor of the active form of vitamin D [97]. Moreover, cholesterol can participate in cellular signaling both indirectly by modulating the physical properties of the plasma membrane thereby affecting the activity of receptors and enzymes residing in it, or directly as a regulator of enzymes in the cholesterol metabolic pathways [62,97,99]. Furthermore, plasma membrane rafts and caveolae represent yet another means of cholesterol-dependent signaling since they provide platforms for efficient initiation of signaling cascades by bringing together receptors with their second messenger effectors [96,100].

2.2. Cellular distribution, metabolism and transport of cholesterol

Cholesterol is heterogeneously distributed among cellular membranes. Most of the unesterified cholesterol can be found in the plasma membrane compartment. Depending on the cell type used, and the assay method, plasma membranes have been reported to contain between 40 and 90% of the total cellular unesterified cholesterol [26,47,101,102]. Mitochondria and the endoplasmic reticulum have a very low cholesterol content, whereas the Golgi compartment contains intermediate amounts of cholesterol [72,103–105]. The distribution of cholesterol and sphingomyelin in the cell follow the same pattern, and it has been suggested that the capacity of plasma membranes to solubilize cholesterol is a function of its sphingomyelin content [106–109].

Mammalian cells require cholesterol for normal function and can obtain it in two different ways, by de novo biosynthesis or via uptake from plasma lipoproteins. All eukaryotic cells, except for mature red blood cells, synthesize cholesterol from acetate by a complicated cascade of reactions involving more than 30 enzymatic steps [71,110,111]. Cholesterol biosynthesis takes place mainly in the endoplasmic reticulum, but synthetic activities have also been found in the peroxisomes [112,113]. Alternatively, cholesterol can be taken up by the cell from lipoproteins in the circulation by receptor-mediated uptake or by direct desorption of cholesterol from the lipoprotein to the plasma membrane [114]. The synthesis and uptake of cholesterol are strictly controlled by feed-back regulation, which is achieved by transcriptional, translational and post-translational mechanisms [115–118].

The transport pathways for both de novo synthesized cholesterol and cholesterol derived via the LDL-pathway are directed towards the cell surface to maintain the high concentration of

cholesterol in the plasma membrane [119–122]. Cholesterol trafficking to and from the plasma membrane can be mediated by various pathways depending on the cell type. Both vesicular and non-vesicular mechanisms seem to be involved, but are not yet well defined [102,123–125]. It has been suggested that both de novo synthesized and LDL-derived cholesterol is transported to the plasma membrane via caveolae [102,126]. Thus caveolae could play an important role in the distribution of cholesterol in the cell [100,123].

If the amount of free cholesterol in the plasma membrane gets too high, cholesterol is transported to the endoplasmic reticulum for esterification and storage [113,127]. Esterification is mediated by the enzyme acyl-coenzyme A: cholesterol acyl-transferase (ACAT). Cholesterol moves to the endoplasmic reticulum for esterification only after the cellular free cholesterol level has reached a critical threshold level, and it has been shown that this threshold level is regulated by the sphingomyelin content of the cells [106,108,109]. The transfer of plasma membrane cholesterol to lipoprotein particles is the only way for cells in peripheral tissues to remove excess cholesterol. This is the first step in reverse cholesterol transport, which transfers cholesterol from peripheral tissues to the liver for excretion [102,114,128].

3. Is there coordinate regulation of cholesterol and choline-containing phospholipids?

The early work by Patton in 1970 revealed a positive correlation between cellular cholesterol and sphingomyelin levels in rat liver hepatocytes [129], and since then a considerable amount of studies have concentrated on unravelling the effects of sphingomyelin on cellular cholesterol homeostasis, and also the effects of cholesterol on sphingomyelin homeostasis (for recent reviews see Refs. [130–133]). The coordinated effects of phosphatidylcholine and cholesterol on each other's metabolism have received less attention, since phosphatidylcholine and cholesterol are not as widely co-localized in cells, and because phosphatidylcholines are not considered such important constituents of rafts as cholesterol and sphingomyelins are.

Altogether the gathered information suggests that the maintenance of a constant ratio of sphingomyelin and cholesterol in membranes is important to support the critical functions carried out in rafts and related membrane structures. Potential candidates to mediate the coordinated regulation of cholesterol and sphingomyelin metabolisms are oxysterols, which bind to specific oxysterol binding proteins [131,133]. Neutral sphingomyelinase in the plasma membrane has also been suggested to participate in cellular cholesterol homeostasis, because its action can mobilize plasma membrane cholesterol and also up-regulate the amount of cellular LDL receptors [134].

3.1. Effects of choline-phospholipids on cell cholesterol homeostasis

In the first studies in this research field Gatt and his coworkers noticed that an increase in the plasma membrane sphingomyelin content of fibroblasts resulted in a reduced esterification and an increased biosynthesis of cholesterol [106,135]. These results also showed that an increase in the plasma membrane sphingomyelin level led to a flow of cholesterol from intracellular sites to the plasma membrane. Later it was shown that degradation of plasma membrane sphingomyelin resulted in a dramatic activation of the endogenous esterification of cholesterol [108,136–138]. Recently, Tabas and coworkers found out that hydrolysis of plasma membrane sphingomyelin

causes an inward curvature in the membrane that eventually will lead to the formation of sealed vesicles [139]. These vesicles were suggested to transport cholesterol to the endoplasmic reticulum for esterification and also ceramide to the Golgi apparatus for conversion back to sphingomyelin [139,140]. In addition, degradation of plasma membrane sphingomyelin has been shown to result in a decreased level of cholesterol biosynthesis [108,136], and in a stimulation of cholesterol conversion to steroid hormones in steroidogenic cells [141]. Recently it was shown that cholesterol biosynthesis in sphingomyelin depleted cells is down-regulated by sterol-mediated suppression of sterol regulatory element binding protein processing [142].

A resynthesis of the degraded sphingomyelin will redistribute cholesterol in the cell, and also normalize cellular cholesterol ester levels [143–145]. However, recent studies have shown that cholesterol translocation to intracellular membranes after degradation of plasma membrane sphingomyelin is not as massive as was first thought, and most of the cellular cholesterol is still found in the plasma membrane compartment after treatment with sphingomyelinase [146]. Instead, plasma membrane sphingomyelin degradation leads to a redistribution of cholesterol in the plasma membrane so that cholesterol is more readily extracted by extracellular acceptors, if they are present [147–150]. Recent results on sphingomyelin deficient cells by Fukasawa and coworkers also imply that cholesterol remains in the plasma membrane [151]. Their studies showed that cholesterol was localized mainly to the plasma membrane even in sphingomyelin deficient mutant cells, but that it was more accessible for efflux to extracellular acceptors than cholesterol in the plasma membrane of wild-type cells [151]. Based on their results it appears that the enhanced efflux after sphingomyelinase treatment was not due to accumulation of ceramide in the membrane. The presence of extracellular acceptors has further been shown to inhibit the induction of cholesterol esterification and the down-regulation of cholesterol biosynthesis exerted by exogenous sphingomyelinase [136].

The effects of plasma membrane phospholipid degradation on cholesterol homeostasis seem to be specific for sphingomyelin, since the degradation of plasma membrane phosphatidylcholine did not affect the translocation of cholesterol to intracellular membranes [152] or increase the efflux of cholesterol from the plasma membrane to extracellular acceptors [148]. Based on this data, cholesterol seems to associate preferentially with sphingomyelin in biological membranes.

3.2. Effects of cholesterol on the homeostasis of choline-phospholipids in cells

It is fairly well established that the level of cellular cholesterol affects the rate of phosphatidylcholine synthesis. Twenty years ago, we showed that transfer of cholesterol from cholesterol-rich liposomes to cultured cells increased acetate-incorporation into phosphatidylcholines [153]. Later it was shown that enrichment of J774 macrophages with free cholesterol upregulated the CTP: phosphocholine cytidylyltransferase enzyme, leading to an increased phosphatidylcholine mass in the treated cells [154]. Recently Tabas and coworkers could show that the upregulation of CTP: phosphocholine cytidylyltransferase alpha in response to cholesterol loading is an adaptive response that attempts to prevent the cytotoxicity of free cholesterol in the cells [155]. The upregulation of CTP: phosphocholine cytidylyltransferase in response to cholesterol loading in at least human skin fibroblastas appears to be very sensitive to the structure of the sterol, since lathosterol (Δ^7) enrichment fails to upregulate the enzyme [156]. It is at present not known why lathosterol does not upregulate CTP: phosphocholine cytidylyltransferase in fibroblasts, but since

lathosterol is much less effective than cholesterol in stabilizing hexagonal phases in membranes [156], this finding may be relevant for understanding the difference between cholesterol and lathosterol.

While the modulation of cellular sphingomyelin content consistently influenced cholesterol homeostasis, alteration of cellular cholesterol synthesis and mass by a variety of methods often had varying and contradictory effects on sphingomyelin synthesis (reviewed in Ref. [133]). The increase of cellular cholesterol levels by means of cholesterol-rich lipoproteins have been shown to increase the rate of sphingomyelin synthesis in macrophages [109,157], whereas cholesterol enrichment of fibroblasts using cholesterol/cyclodextrin inclusion complexes have been shown to decrease the activity of serine palmitoyl transferase, leading to lower cellular levels of sphingomyelin [158]. Since studies in our laboratory have employed cyclodextrins which efficiently deliver cholesterol to the cells, instead of LDL particles (which also deliver neutral lipids and phospholipids to the cells), in addition to using a different cell type, it is clear that our results can differ from those reported for macrophages [109,157].

On the other hand, when cellular cholesterol levels were reduced by treatment of cells with cyclodextrin, the activity of serine palmitoyl transferase was increased and eventually led to increased sphingomyelin mass in the treated cells [158]. The treatment of fibroblasts or baby hamster kidney cells with 5 mM 2-hydroxypropyl-β-cyclodextrin for up to 60 min removed less than 2% of cell sphingomyelin or phosphatidylcholine (P. Leppimäki and J.P. Slotte, unpublished data), suggesting that the effect of cyclodextrin on the activity of serine palmitoyl transferase in the treated cells was solely due to cholesterol extraction from the cells.

3.3. The formation of cholesterol/sphingolipid-rich rafts in cell membranes

During the last years many research groups have intensively studied the function of lipid rafts and microdomains in biological membranes. The concept of rafts or glycosphingolipid/cholesterol domains, that are involved in protein and lipid transport and in several signaling cascades, was first presented in 1997 by Simons and Ikonen [159]. The raft hypothesis suggests that lipids can form domains or aggregates in the plane of the membrane and that these domains form due to interactions between the lipid molecules. The existence of such rafts has been disputed, but more and more evidence has been gathered showing the important functions of such lipid domains in the cellular membranes.

The formation of rafts seems to be driven mainly by lipid/lipid interactions that are a consequence of the physical properties of the different lipids. The properties of pure phospholipids can be described by their melting temperature (T_m), which is the temperature where the lipids change phase state from an ordered solid-like gel state to a less ordered fluid liquid crystalline state that usually is present in biological membranes. Lipids that pack tightly together have a high T_m whereas lipids that favor a fluid phase have a low T_m. The T_m of a lipid is highly dependent on acyl-chain structure. T_m increases with increasing acyl-chain length and saturation whereas T_m is decreased when the degree of unsaturation increases, because the acyl-chains *cis* double bonds interfere with lateral packing. Another factor affecting T_m is the structure of the polar head-group. For example glycosphingolipids tend to have a higher T_m than sphingolipids [160]. This could be due to hydrogen bonding between the head groups of glycosphingolipids. In mixtures of two different phospholipids, co-existing gel and fluid phases have been observed [161].

Interaction of lipids with membrane proteins has also been found to induce domain formation [162].

Sphingolipids are rich in long and saturated fatty acyl chains. This allows them to pack tightly and consequently they have a relatively high T_m. Therefore sphingolipids have been suggested to be important domain formers in biological membranes. In contrast, naturally occurring glycerophospholipids tend to have mono- or polyunsatured acyl chains and thus they have lower T_m. Cholesterol has been shown to interact preferentially with sphingolipids [132]. The interaction between cholesterol and sphingolipids is favorable since the fully saturated acyl chains of sphingolipids can interact by their complete length with the steroid ring. Furthermore, there is possible hydrogen bonding between the 3-hydroxyl group of cholesterol and the ceramide of sphingolipids [76].

Since cholesterol is present in high amounts in the plasma membrane the effects of cholesterol on the packing of phospholipids has been intensively studied. Cholesterol can enhance lateral separation of lipids in bilayers consisting of a single lipid species but also in bilayers of more complex composition [163–166]. The liquid ordered state (L_o), that seems to be of biological significance, requires cholesterol for formation. The L_o state is characterized by tight acyl-chain packing and relatively extended acyl-chains and is mainly detected in mixtures of cholesterol and phospholipids with highly saturated acyl-chains [88,167–170]. The L_o phase differs from the gel phase in the sense that the acyl-chains in the L_o phase have high lateral mobility in the bilayer [171]. In a recent study by Dietrich and coworkers [172], it was shown that lateral membrane domains could be formed in model membranes prepared from brush-border membrane lipids, and that the domains had most of the properties expected for lipid rafts in vivo. These results emphasize that the formation of glycosphingolipid- and cholesterol-rich lipid domains is driven solely by the physical properties of the lipids involved. In conclusion, it appears that sphingolipid/cholesterol rafts form liquid ordered phases in the plane of the membrane bilayer, which are surrounded by a liquid disordered phase formed by unsaturated glycerophospholipids [173, 174].

The tight packing of lipids in rafts makes them resistant to the non-ionic detergent Triton X-100. This makes Triton X-100 a powerful tool to examine different characteristics of rafts or detergent resistant membranes (DRM). Lipids in the fluid state are generally solubilised by addition of excess Triton X-100 [175,176] whereas tightly packed gel phase and L_o phase lipids are usually resistant to detergent binding and solubilisation [177,178]. The detergent resistance is mainly due to van der Waals interactions in the hydrophobic part of the membrane, but interactions between the head-groups of phospholipids also has to be considered. After extraction from cells DRM's can be isolated by density gradient centrifugation [179]. By this method one can determine the lipid and protein composition of rafts but it is not in this way possible to determine the size or subcellular localization of these rafts.

Caveolin is found in a special group of rafts, namely caveolae. Caveolae are invaginated rafts that have been shown to be involved in several signaling pathways in cells. For long the detergent resistance of lipids in cell membranes was thought to rise from the interaction of the lipids with the cytoskeleton or proteins such as caveolin. Studies on cells lacking caveolin then showed that DRMs can exist without caveolin [180,181] and that everything that can be found in the DRM fraction after DRM isolation is not necessarily localized to the caveolae in cells. Several proteins besides caveolin have been found in the DRMs. Since DRM lipids are found in a L_o-like phase one could speculate if this could be a rout for targeting proteins to DRMs. Simons and van Meer

proposed that rafts form in the Golgi and function as sorting domains for membrane proteins directed to the cell surface [182,183]. Proteins with high affinity for an ordered lipid environment could spontaneously partition into such a domain. DRM proteins often contain a saturated chain lipid group that packs nicely into an ordered lipid environment. Such proteins can have glycosylphosphatidylinositol (GPI) anchors or myristate or palmitate chains [184–188]. In contrast, one could estimate that DRM's could be scarce in transmembrane proteins and proteins containing bulky, branched prenyl groups. In fact, it has recently been shown that proteins with prenyl groups are excluded from DRMs [187]. Cholesterol depletion which should disrupt the organization of rafts has been shown to make raft-associated proteins detergent soluble [183,189,190].

There has been a lot of speculation whether DRMs are artifacts that are induced by extraction of cells. The fact that the insolubility of cellular membranes can be induced in model membranes composed of phospholipids and cholesterol [177] and that lipid domains with raft-like properties spontaneously form in model membranes composed of appropriate lipids [172] strongly suggest that rafts do exist in biological membranes. Different aspects considering the existence of DRMs and rafts in biological membranes have recently been reviewed by London and Brown [191].

3.4. Efflux of membrane cholesterol from raft and non-raft domains

The organization of cholesterol in the plasma membrane has direct effects on cholesterol efflux from the cell. When cells are exposed to an efficient cholesterol acceptor such as cyclodextrin two distinct kinetic pools of cholesterol can be found [192]. The cellular location of these cholesterol pools have recently been investigated by Haynes and co-workers [149]. In that study, it was shown that the fast pool and the majority of the slow pool of cholesterol are localized to the plasma membrane. Why there is a slow pool has not been answered. It is clear that removal of phospholipids from the plasma membrane enhances the efflux of cholesterol to an extracellular acceptor [148] and moves cholesterol from the slow pool to the fast pool [149]. When added to cells, an acceptor such as cyclodextrin would first remove cholesterol from fluid areas and from the external leaflet of the plasma membrane (fast pool). The $t_{1/2}$ of the slow pool would be determined by the rate of movement of cholesterol from tightly packed areas such as rafts or from the internal leaflet of the plasma membrane of from internal membranes to the outer leaflet of the plasma membrane. Fukasawa and co-workers [151] have recently published a study showing that the steady state level of cholesterol at the plasma membrane in Chinese hamster ovary cells is almost unaffected by sphingomyelin levels. Yet sphingomyelin levels are important for the retention of cholesterol in the plasma membrane. The effect of sphingomyelin on cholesterol efflux rates seems to be due to the specific interaction between cholesterol and sphingomyelin in the DRMs. Summarizing these studies suggests that the slow pool of cholesterol could be cholesterol associated with rafts or cholesterol that has to move from the inner leaflet to the outer leaflet of the plasma membrane before it can escape from the cell. Some of the slow pool could also originate from other cellular compartments.

3.5. The lateral distribution of phospholipids and cholesterol in the two leaflets of the red blood cell membrane—the superlattice model

The classical fluid-mosaic model of Singer and Nicholson from the early seventies suggested that lipids in a membrane formed a fluid bilayer structure in which lipids and proteins were free

to diffuse. Today we know that the lateral structure of a membrane is more complex and refined, and that lipids and membrane proteins do not have full freedom to diffuse laterally and also that the lateral distribution of lipids and proteins is heterogeneous [193]. It has already been suggested that lipid rafts exist in the membrane, but how does non-raft lipids distribute in the membrane, and what factors may govern their behavior? A model for the non-random distribution of membrane lipids have recently been presented, based mainly on results derived from model membrane studies (see the review in Ref. [194]). This superlattice model (or regular distribution model) proposes that the lateral distribution lipids tend to follow a regular pattern. The driving forces for the regular lateral distribution of phospholipids comes from both attractive (favorable van der Waals interactions) lipid/lipid and lipid/protein interactions, as well as and more importantly from repulsive interactions [both steric effects (e.g. head-group size) and coloumbic repulsion (among similarly charged lipids)]. Whereas the experimental details that led to the development of the superlattice model mostly came from model membrane studies using fluorescently labeled lipids [195–197] in addition to some recent studies with native lipids [198–201], the applicability of the superlattice model to biological membranes was first tested on the erythrocyte membrane [202]. If the phospholipid classes were grouped according to the size of the head-group (choline versus ethanolamine) or its charge (the acidic phospholipids), the superlattice model predicted a lateral distribution of outer and inner leaflet phospholipids that agreed extremely well with published compositions based on chemical analysis [202]. Whereas the superlattice model per se can predict the lateral distribution of cholesterol [197,199–201], this was not done in this erythrocyte membrane study mainly because very little is known experimentally even about the transbilayer distribution of cholesterol in the red blood cell. There is, however, one study in which the transmembrane distribution of a fluorescent dehydroergosterol was determined in red blood cells [203]. According to this study, about 75% of the membrane sterol was localized to the inner leaflet, but it is uncertain whether dehydroergosterol mimics the transbilayer distribution of cholesterol, since its structure differ from that of cholesterol [281].

4. Cholesterol/phospholipid interactions in artificial model membranes

Biological membranes contain a heterogeneous mixture of phospholipids differing from each other with respect to their head-group structure, hydrocarbon chain length, degree of unsaturation of the acyl chains, and mode of attachment of the hydrocarbon chains. Due to this complexity, it is difficult to ascertain the physical properties and functional roles of individual lipids and their mode of interaction with other lipids in natural membranes. Therefore model systems consisting of phospholipids and sterols have been a valuable tool in obtaining basic information on membrane lipid interactions. Several different methods have been used to study sterol/phospholipid interactions both in model bilayers and monolayers, and together these results contribute to our current knowledge of the lipid interactions.

In a membrane bilayer cholesterol inserts normal to the plane of the bilayer, with its hydroxyl group in close vicinity to the ester carbonyl of glycerophospholipids and the amide bond of sphingolipids, and its alkyl side-chain extending towards the bilayer center (Fig. 4) [74,77,89,204,205]. Cholesterol is intercalated in the membrane parallel to the phospholipid hydrocarbon chains, and the phospholipid carbons in positions 2–10 have been estimated to lie in

close proximity to the sterol tetracyclic ring structure [10,83,206,207]. The close proximity of the planar sterol ring system will order the hydrocarbon chains and diminish *trans–gauche* isomerization about their carbon–carbon bonds. The effective length of cholesterol has been estimated to correspond to a 17-carbon all-*trans* hydrocarbon chain [208]. Because the sterol side-chain is somewhat flexible the regions in the methyl end of the phospholipid are not as markedly ordered by the cholesterol molecule as the upper part [72,74]. In pure phosphatidylcholine and sphingomyelin bilayers the phospholipid head group is oriented approximately parallel to the plane of the bilayer [86,209]. The insertion of cholesterol into the membrane does not change the conformation of the head group [72,207,210,211].

The largest contribution to cholesterol/phospholipid interactions appears to be from van der Waals forces and hydrophobic forces [64,212]. It has been suggested that the interactions of cholesterol with sphingolipids and anionic lipids can further be strengthened by hydrogen bonding of the cholesterol hydroxyl group to the polar head group and interfacial regions in the phospholipids [132,212]. Also, the ionic phosphocholine head groups of nearby membrane sphingomyelins and phosphatidylcholines can interact electrostatically [64,72]. These intermolecular interactions are disrupted if cholesterol is inserted between the phospholipids, and as a consequence the mobility of the head group is enhanced [209].

4.1. Cholesterol's interaction with choline-phospholipids

So far, a considerable amount of data indicate that cholesterol interacts more favorably with sphingomyelin than with other membrane phospholipids, such as phosphatidylcholine, in both bilayer and monolayer membranes. The preferential interaction between cholesterol and sphingomyelin has been demonstrated with a variety of experimental approaches (for reviews see Refs. [74,79,130,132,212]). The rate of cholesterol desorption from sphingomyelin-containing bilayer membranes is known to be much slower than the desorption from membranes with acyl chain-matched phosphatidylcholines, indicating a preferential interaction of cholesterol with sphingomyelin [84,213–215]. We have obtained analogous results on the effects of the phospholipid structure on cholesterol desorption from monolayers [216]. Studies on the effect of cholesterol on the phase transitions in binary phospholipid/sphingomyelin mixtures have revealed that cholesterol preferentially abolishes the phase transition of sphingomyelin domains, indicating a preferential interaction with the sphingomyelin species [80,217]. It has further been shown that water permeability is lower in cholesterol/sphingomyelin bilayer membranes than in cholesterol/phosphatidylcholine membranes, indicative of a more dense lateral packing density and a stronger interaction in the former system [3]. In monolayers, the oxidation of cholesterol was reduced if sphingomyelin was included in the membrane, compared with the inclusion of phosphatidylcholine [218,219], which indicates a stronger interaction between cholesterol and sphingomyelin in the monolayer. Cholesterol has further been shown to interact preferentially with sphingomyelin in ternary mixed monolayers containing cholesterol, phosphatidylcholine and sphingomyelin [220].

4.2. Is cholesterol/sphingomyelin interaction stabilized by intermolecular hydrogen-bonding?

What feature(s) of the sphingomyelin molecule lead to the preferential interaction of cholesterol with this lipid? There is no exact answer to this question yet. The most striking difference between

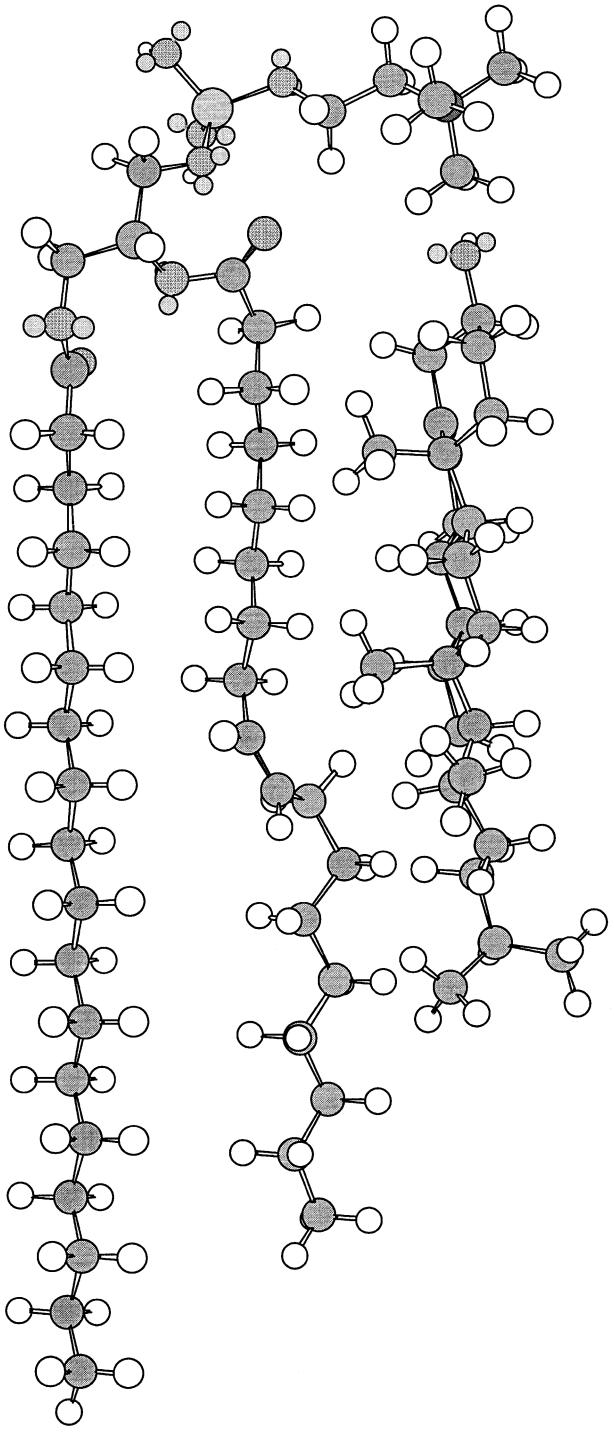


Fig. 4. A "ball and stick" representation of 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine and cholesterol, indicating their approximate orientation next to each other in a membrane leaflet. Both molecules experience rapid thermal motion, but on average the cholesterol ring structure is believed to associate with the first 8–10 carbons of the phospholipid acyl chains.

sphingomyelin and phosphatidylcholine is that sphingomyelin possesses both hydrogen bond-accepting and -donating groups at the polar/non-polar interface, while phosphatidylcholine only has hydrogen bond-accepting capacity. Still, both phospholipids could theoretically form a hydrogen bond with cholesterol's hydroxyl group [75]. There have been many suggestions on hydrogen bonding between cholesterol and certain atoms in phosphatidylcholine (for example, Refs. [221–223]), but so far there are limited experimental data supporting these suggestions (discussed in Refs. [74,207]. Sphingomyelin/cholesterol interactions, on the other hand, seem to be strengthened by hydrogen bonding [169]. Systematic alterations of the functional groups in the sphingomyelin molecule in the laboratories of Bittman and Slotte [76,224,225] have shown that the amide linkage in the sphingomyelin molecule is important for the interaction with cholesterol, while altering the hydroxyl group in the sphingosine base does not affect the interaction to a similar extent. The authors concluded that the amide function in the sphingomyelin molecule is important for the interaction with cholesterol, presumably due to the formation of a hydrogen bond between the amide and the cholesterol hydroxyl group [76]. The replacement of one or both of the acyl groups of phosphatidylcholine with alkyl groups (ether linkage instead of ester linkage) also enhanced the interaction with cholesterol, further indicating the importance of the interfacial region for the interaction [226]. In a very recent study by Veiga and coworkers [227] it was shown for egg sphingomyelin/cholesterol bilayer membranes by infra-red spectroscopy that the amide I-band maximum shifted to lower wavenumbers in the presence of cholesterol, suggesting that there was a change in the hydrogen-bonding pattern of the sphingomyelins amide group, probably involving direct hydrogen bonding to the hydroxyl group of cholesterol. In the same study, cholesterol had only small effects on the carbonyl stretching bands of egg phosphatidylcholine.

The interaction of cholesterol with sphingomyelins in monolayer membranes is known to decrease the in-plane elasticity of the membrane in a cholesterol concentration-dependent manner [178]. When the in-plane elasticities were compared for acyl-chain matched sphingomyelins and phosphatidylcholines, an equimolar concentration of cholesterol in the monolayer led to 25% lower in plane elasticity-values in sphingomyelin monolayers as compared to phosphatidylcholine monolayers. Since the phospholipids were acyl-chain matched, the difference in in-plane elasticity implies that intermolecular interactions between the acyl chains and the ring structure of cholesterol only partially account for the low in-plane elasticity. The authors speculate that an added contribution to the low in-plane elasticity in cholesterol/sphingomyelin monolayers was likely to arise from intermolecular hydrogen bonding between the hydroxyl group of cholesterol and the amide group of sphingomyelin [178].

4.3. Effects of phospholipid acyl chain composition on the interaction with cholesterol

The interaction of cholesterol with lipids depends to a large extent by the interaction between the planar steroid ring of cholesterol with acyl chains of the lipids. This favors the interaction of a fully saturated acyl chain with cholesterol. The presence of *cis* unsaturation in the phospholipid acyl chain introduces kinks that limit the formation of attractive van der Waals forces with cholesterol. Cholesterol has been shown to interact to a more favorably with both saturated sphingomyelins and phosphatidylcholines, compared to their unsaturated counterparts [84,213, 218, 219,228–231]. Still, cholesterol interacts with mono- and diunsaturated phosphatidylcholines to

some extent, while multiple unsaturation significantly decreases the interaction with cholesterol in both bilayers and monolayers [87,230,232–235]. At this point, it is important to keep in mind that natural sphingomyelins often have a long saturated acyl chain, while phosphatidylcholines usually contain one saturated chain and the other is unsaturated. This fact and the additional hydrogen bond probably explain the preferential interaction of cholesterol with sphingomyelins in natural membranes.

Studies by McMullen and McElhaney have shown that hydrophobic mismatch (i.e. the difference in hydrophobic length of the interacting molecules) affects sterol/phospholipid interactions to a great extent [236]. Their differential scanning calorimetry (DSC) studies on cholesterol/phosphatidylcholine mixtures showed that cholesterol progressively decreased the T_m of phosphatidylcholine bilayers with saturated acyl chains of 18 or more carbon atoms. If the chain length was equal or less than 16 carbons cholesterol increased the T_m. Therefore, McMullen and McElhaney concluded that the hydrophobic length of cholesterol equals the length of a phosphatidylcholine with 17 carbon saturated acyl chains, and that cholesterol interacts to a greater extent with phosphatidylcholines of the same hydrophobic length than with shorter or longer chain phosphatidylcholines [236]. A monolayer study, in which the oxidizability of cholesterol by cholesterol oxidase in mixed monolayers containing phosphatidylcholines of varying acyl chain length was compared, showed that cholesterol interacts best with phosphatidylcholines having acyl chain lengths of 14–17 carbons [237]. These results are in agreement with those by McMullen and McElhaney [236]. Another way to study the effect of hydrophobic mismatch on sterol/phosphatidylcholine interactions is to vary the hydrophobic length of the sterol, while keeping the length of the phosphatidylcholine molecule constant. Monolayer and bilayer studies, in which the length of the sterol side chain was varied have confirmed that hydrophobic mismatch greatly affects sterol/phosphatidylcholine interactions [238,239]. Hydrophobic mismatch also affects sterol interactions with unsaturated phosphatidylcholines, since the sterol side-chain length has been shown to influence their interaction with 1-stearoyl-2-oleoyl-phophatidylcholine in both monolayers and bilayers [240,241]. In all these studies, the interaction with phosphatidylcholine was strongest if the sterol side chain was as long as or somewhat longer than that of cholesterol. The effect of hydrophobic mismatch on sterol/phosphatidylcholine interactions in model membranes has recently been reviewed [74,212]. It is interesting to find, however, that the interaction of cholesterol with sphingomyelins is not affected by differences in hydrophobic length of the interacting molecules (Fig. 5) [231,242]. Ramstedt and Slotte suggested that there must be something else in addition to van der Waals forces and hydrophobic forces stabilizing the interaction between cholesterol and sphingomyelin, and therefore the interactions were not affected by hydrophobic mismatch [231]. They concluded that this stabilizing force might be a hydrogen bond between cholesterol and sphingomyelin.

There are very few studies on how the composition of the sphingoid base of sphingomyelin affects the interaction with cholesterol. Although sphingosine is the prevalent backbone in most mammalian sphingolipids, there are many other sphingoid bases as well [18]. Recent studies in our laboratory have shown that dihydrosphingomyelin (which lacks the double bond in the sphingoid base) can interact more strongly with cholesterol than sphingomyelin in monolayer membranes as indicated by measurement of the desorption rate of cholesterol [243]. A further indication of the strong interaction is that much more condensed domains were formed with cholesterol in dihydrosphingomyelin vesicles as compared to sphingomyelin vesicles. In addition,

the formed domains were stable at much higher temperatures (Fig. 6) [243]. The preferential interaction between cholesterol and dihydrosphingomyelin may result from stronger van der Waals interactions, which were apparent since the T_m of the *N*-palmitoyl-dihydrosphingomyelin used was 6.5 °C higher than for the corresponding sphingomyelin.

4.4. Evidence for complex formation between cholesterol and phospholipids in model membrane systems

To study phenomena related to the lateral distribution of cholesterol in model membrane systems, many groups have used epifluorescence microscopy to examine the formation of laterally condensed (and cholesterol-rich) phases in binary cholesterol/phospholipid monolayers at the air/water interface [244–246]. From such studies it has been observed that cholesterol (or dihydrocholesterol) form immiscible liquids in phospholipid monolayers under specific conditions of temperature, composition and two-dimensional pressure [247,248]. In binary mixed monolayers of cholesterol (or dihydrocholesterol) and a saturated phosphatidylcholine or sphingomyelin, it has been demonstrated that the mixed monolayers exhibit two upper miscibility critical points [247–249]. Such critical points have been shown to indicate the cooperative formation of molecular complexes between the sterol and the phospholipid present in the monolayer [247]. These

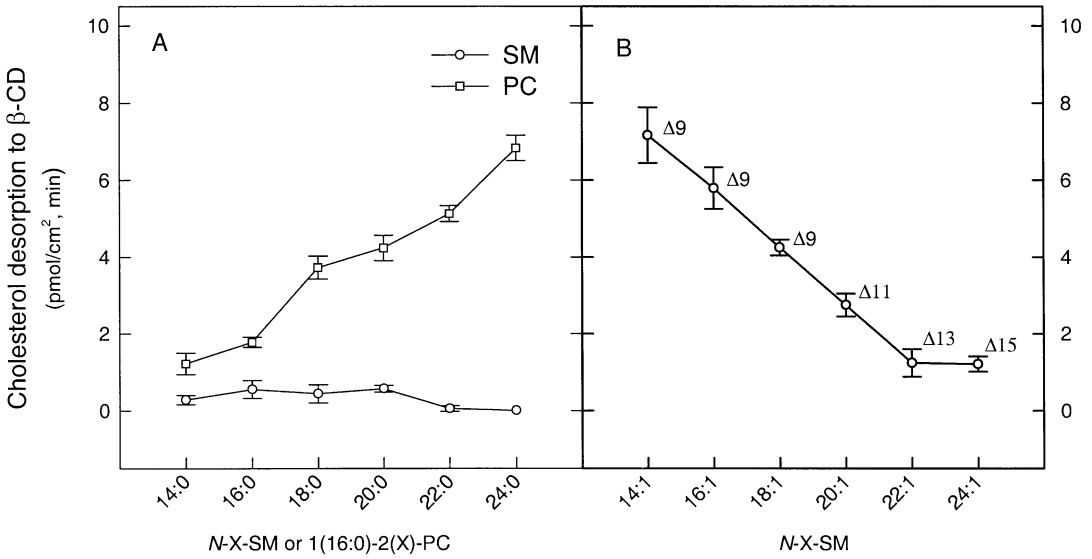


Fig. 5. Desorption of cholesterol from mixed cholesterol/phospholipid monolayers to β-cyclodextrin in the subphase. Mixed monolayers containing 50 mol% cholesterol and either phosphatidylcholines (PCs) or sphingomyelins (SMs) were prepared at the air/water interface. The monolayers were compressed to 20 mN/m and maintained at a constant surface pressure (at ambient temperature). Cholesterol desorption to β-cyclodextrin (1.7 mM) in the subphase was determined as a time function. Calculated values are plotted as a function of the acyl chain length of *N*-acyl-SM (o) or 1(16:0)-2(X)-PC (□) (panel A) or as a function of the unsaturated acyl-chain length of *N*-acyl-SM (panel B). The positions of the *cis*-double bonds are indicated in the figure. The values are given as the mean ± S.E.M. from three different monolayer experiments. Adapted from Ref. [231] with permission from the Biophysical Society.

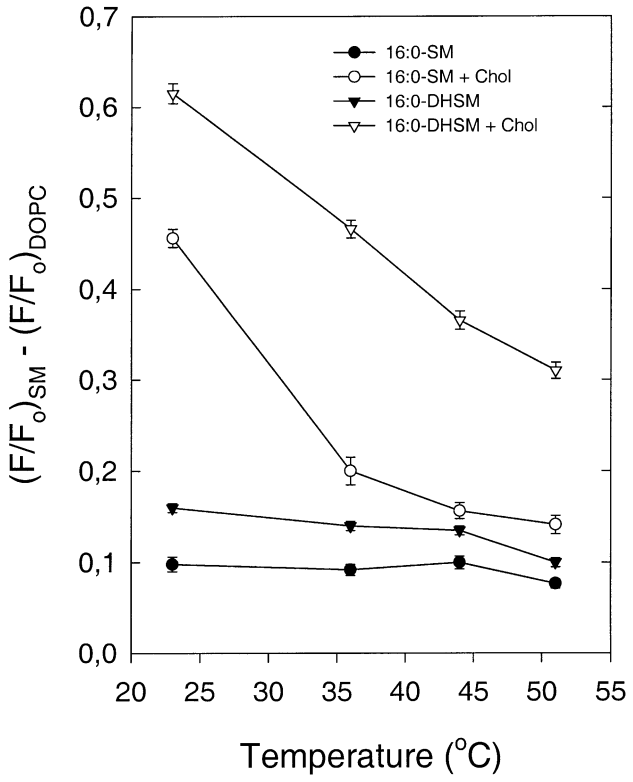


Fig. 6. Formation of laterally condensed domains in D-*erythro*-*N*-16:0-sphingomyelin (16:0-SM) and D-*erythro*-*N*-16:0-dihydrosphingomyelin (16:0-DHSM) liposomes in the presence of cholesterol, as determined by quenching of diphenylhexatriene fluorescence by palmitoyl-(12-doxyl)-stearoyl-phosphatidylcholine by the method of Refs. [164,280]. The liposomes contained the indicated sphingomyelin with or without 15 mol% cholesterol. The $(F/F_o)_{SM}-(F/F_o)_{DOPC}$ values were calculated, and plotted as a function of temperature. Each value is the average from three similar experiments ±S.E.M. Adapted from [243].

cooperative complexes form at specific stoichiometries (usually at 33 mol% sterol, but may also be found in the range 25–43% sterol [247,249]), as evidenced from cusps in the plots of critical miscibility versus sterol mole fraction, or from analysis of the mean molecular area (of the binary mixture) versus the sterol mole fraction plots. The number of molecules in the cooperative unit has been estimated to be in the order of 9–15, and for the cholesterol/sphingomyelin system the cooperative unit size does not appear to be influenced by the length of the amide-linked acyl chain [249].

The formation of cholesterol/phospholipid complexes is also inferred from measurements of the desorption rate of cholesterol from a binary monolayer at constant surface pressure [250]. It is known that the rate of sterol desorption from a monolayer is affected by the chemical composition of the monolayer [216]. McConnell and Radhakrishnan made the plausible assumption that the rate constant of cholesterol release from a monolayer was proportional to the chemical activity of the sterol in the membrane [250]. They could experimentally show that both the chemical activity of cholesterol, and the rate of cholesterol release from the monolayer (to β-cyclodextrin in the

subphase) increased by a factor of 3, when the mole fraction of cholesterol in the membrane was increased by a small increment in the vicinity of the stoichiometric composition. These findings were interpreted to demonstrate the formation of a stoichiometric complex between cholesterol and the phospholipid used in the mixed monolayer.

4.5. Does cholesterol interact with aminophospholipids

The interaction of cholesterol with phospholipids is of course also affected by the phospholipid head groups. An early DSC study of cholesterol interaction with phospholipids showed a decrease in cholesterol affinity in the following order: sphingomyelin > phosphatidylserine, phosphatidylglycerol > phosphatidylcholine > phosphatidylethanolamine [251]. The studies on the interaction of cholesterol with different classes of phospholipids usually agree on the preferential interaction of cholesterol with sphingomyelin over that with other phospholipids. Cholesterol has been reported to interact more favorably with phosphatidylcholine than with phosphatidylethanolamine in monolayers [217]. The preferential interaction of cholesterol with phosphatidylcholine over that with phosphatidylethanolamine has been confirmed in bilayers by steady-state fluorescence polarization measurements and by studies on cholesterol transfer between phospholipid vesicles [228,252]. It has also been shown that 20 mol% phosphatidylethanolamine or phosphatidylserine has no effect on the cholesterol efflux from vesicles containing 50 mol% cholesterol, whereas sphingomyelin decreases the cholesterol efflux significantly [253]. Egg phosphatidylglycerol and egg phosphatidylethanolamine have been suggested to have approximately the same affinities for cholesterol [213].

The behavior of cholesterol with the different phospholipids in bilayers has recently been studied by DSC. The results include the effect of cholesterol on the phase transition temperatures, enthalpies and cooperativity in phospholipid bilayers. The different phospholipids all seem to be affected by cholesterol in their own unique ways. Some clear differences to the behavior of phosphatidylcholine can be seen with phosphatidylethanolamine. In cholesterol/ phosphatidylethanolamine bilayers the strong inter-head group electrostatic and hydrogen bonding interactions between neighboring phosphatidylethanolamine molecules favor phospholipid/phospholipid interactions over cholesterol/phospholipid interactions. This leads to limited miscibility of cholesterol especially in the gel-state bilayer [242]. Incorporation of increasing quantities of cholesterol in phosphatidylethanolamine bilayers progressively reduces the temperature, enthalpy and cooperativity of the gel to liquid crystalline phase transition. The results vary greatly but it seems that a phase transition can persist even above 50 mol% cholesterol [242]. Cholesterol is also more miscible in palmitoyl-oleoyl-phosphatidylethanolamine (POPE) compared to dipalmitoyl-phosphatidylethanolamine and in dielaidoyl-phosphatidylethanolamine compared to distearoyl-phosphatidylethanolamine indicating a role of fatty acid unsaturation [242,254]. At low cholesterol concentrations below 20 mol% the incorporation of cholesterol reduces the lamellar to hexagonal phase transition temperature and at high cholesterol concentrations over 30 mol% the transition temperature increases by addition of cholesterol [254–257].

The reduced miscibility of cholesterol in the gel relative to the liquid crystalline phase is also seen in bilayers with phosphatidylserine although not as clearly as with acyl-chain matched phosphatidylethanolamine [229]. The reduction in phase transition temperature by cholesterol is greater in phosphatidylethanolamine as compared to phosphatidylserine bilayers [242]. For

phosphatidylserine the phase transition is almost completely abolished at 50 mol% cholesterol [258]. The magnitude of this reduction is independent of the hydrocarbon chain lengths of the phosphatidylserine, which is a clear difference to phosphatidylcholine were the transition temperature varies in a chain-length-dependent manner [236]. Results regarding the limited miscibility of cholesterol with phosphatidylserine vary greatly, but the studies seem to agree on that cholesterol is less soluble in phosphatidylserine than in phosphatidylcholine [258–261]. DSC results should however be interpreted with caution since the thermal history of the sample recently has been shown to have profound effects on the appearance of the polymorphic phase transition of cholesterol [261].

Studies of phosphatidylglycerol and phosphatidylserine have suggested that the presence of a negative charge in the head group of a phospholipid reduces the lateral miscibility of cholesterol in phospholipid bilayers [212,262]. Although the miscibility is greater with corresponding phosphatidylcholines, cholesterol is however also highly miscible in both gel and liquid-crystalline phases of phosphatidylglycerol, with decreasing miscibility when the length of the acyl chain in phosphatidylglycerol is increased [212,262].

We have recently measured the degree of interaction between cholesterol and a set of acyl chain-matched phospholipids; namely sphingomyelin, phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine. The extent of cholesterol's interaction with the phospholipids was determined in tertiary cholesterol/phospholipid (1:1) monolayers from the desorption rate of cholesterol to cyclodextrin in the subphase. The results are shown in Fig. 7 and suggest that the interaction of cholesterol with the phospholipids decreased in the following order: sphingomyelin > phosphatidylcholine > phosphatidylserine > phosphatidylethanolamine. There was not, however, a big difference in the degree of cholesterol's interaction with phosphatidylserine and phosphatidylethanolamine (Fig. 7, panel D), which is in agreement with earlier studies [229,253]. According to the results incorporation of *N*-oleoyl-sphingomyelin (*N*-O-SM) into palmitoyl-oleoyl-phosphatidylcholine (POPC)/cholesterol mixed monolayers decreased the desorption rate of cholesterol more than what would be expected if the desorption rate was a linear function of the phospholipid composition (Fig. 7, panel A). The same effect on cholesterol desorption was not observed when *N*-O-SM was incorporated into palmitoyl-oleoyl-phosphatidylserine (POPS) (panel B) or POPE (Fig. 7, panel C) monolayer membranes, rather the desorption rate was increased to some extent from the theoretical linear desorption behavior. These results suggest that phosphatidylethanolamine and phosphatidylserine are able to interfere with the formation of a hydrogen bond between cholesterol and sphingomyelin, and thereby to abolish the preferential interaction between cholesterol and sphingomyelin that consequently would lead to an increased desorption rate. The sterol desorption rate from cholesterol/POPC/POPE monolayers (Fig. 7, panel E) proved to be a linear function of the phospholipid composition. The desorption from cholesterol/POPC/POPS monolayers (Fig. 7, panel F), on the other hand, deviated from the theoretical linear desorption behavior since an addition of POPC to cholesterol/POPS monolayers led to a decrease in the desorption rate.

4.6. What can be learned from computer simulations of cholesterol/phospholipid interactions

As a complement to the experimental data on the subject there have been some recent studies on the effect of cholesterol on phospholipid bilayers by computer simulations. Computer simu-

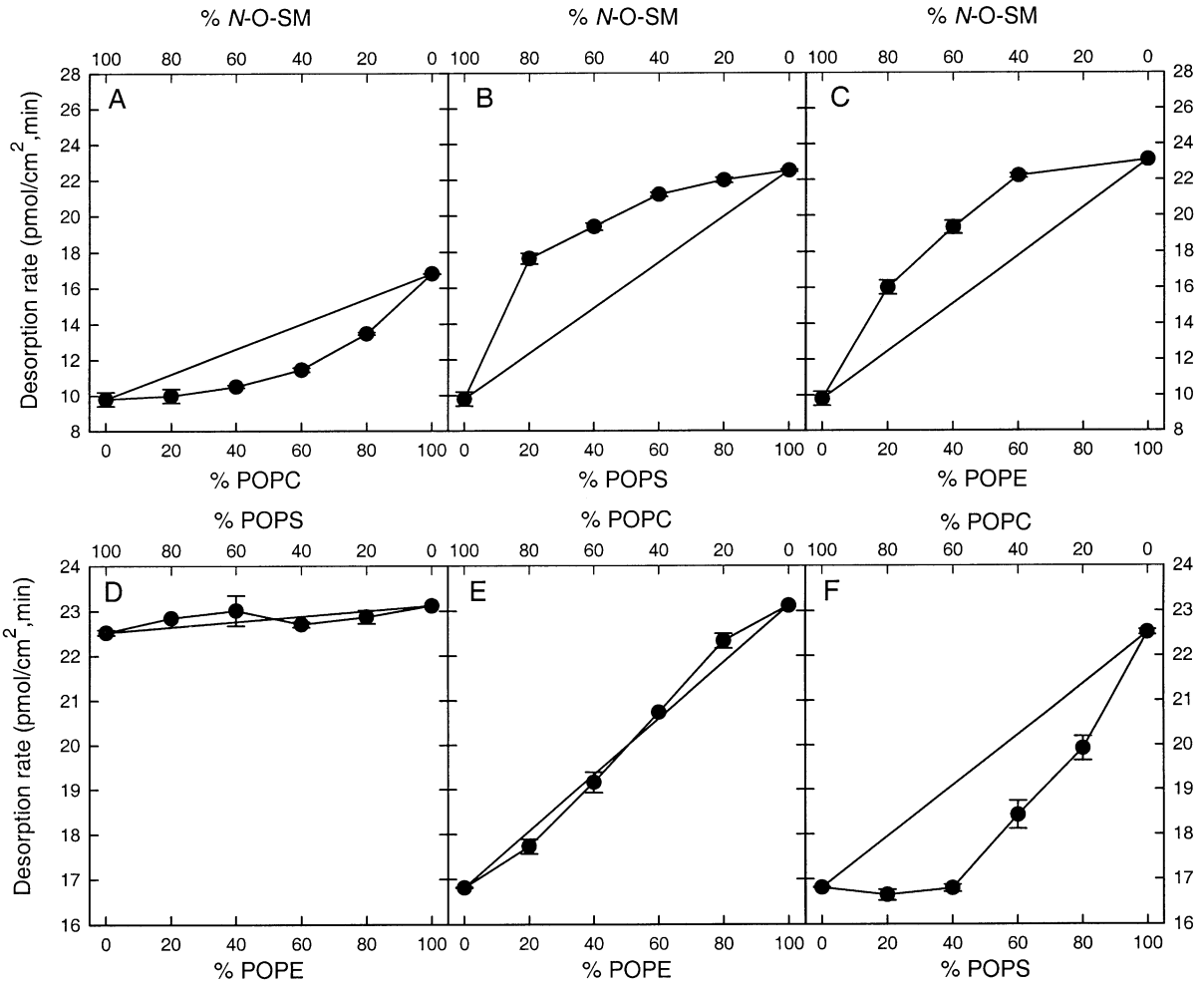


Fig. 7. Desorption of cholesterol from mixed tertiary cholesterol/phospholipid monolayers. Mixed monolayers containing cholesterol and one or two of the phospholipids (at a cholesterol/phospholipid molar ratio of 1:1) were prepared at the air/water interface. The monolayer was compressed to 20 mN/m and maintained at a constant surface pressure at 22 °C. Cholesterol desorption from (A) cholesterol/*N*-O-SM/POPC, (B) cholesterol/*N*-O-SM/POPS, (C) cholesterol/*N*-O-SM/POPE, (D) cholesterol/POPS/POPE, (E) cholesterol/POPC/POPE, (F) cholesterol/POPC/POPS mixed monolayers to 1.4 mM β-cyclodextrin was measured as a time function. Values are averages ±S.E.M. from three different experiments with each different cholesterol/phospholipid composition.

lations have been used successfully to study single-component lipid bilayers and several reviews are available on this subject (for example, Refs. [263–265]). In the last decade atomic level simulations of improving quality have been performed on mixed membranes with phospholipids and cholesterol using Monte Carlo and molecular dynamics methods [223,266–275].

In one of the first of these studies Monte Carlo calculations were used to study the effect of cholesterol on molecular order parameter profiles for acyl chains [266]. The results showed a reduction in the ability of acyl chains adjacent to cholesterol to undergo *trans–gauche* isomerization. The study also revealed that a lipid chain adjacent to the flat alpha surface of the cholesterol

ring system more easily remains in an all-*trans* configuration than an acyl chain on the bulkier side. However, the effects of the phospholipid head groups were not included in this study.

More recent simulations have been able to examine the interactions between phospholipids (mainly saturated phosphatidylcholine), cholesterol and water in more detail, due to improvements in the models and in computer power. Many of these studies have shown that the cholesterol hydroxyl group can interact strongly with both water and the phosphatidylcholine carbonyl and phosphate oxygens [223,269,270,272]. About half the cholesterols will probably interact with water and the other half with phosphate and carbonyl groups [269]. According to the study of Pasenkiewicz-Gierula and co-workers, 16% of the cholesterol molecules would be directly hydrogen bonded to oxygen atoms in phosphatidylcholine [272]. Other interactions would include water bridges between the cholesterol hydroxyl group and the oxygens in phosphatidylcholine and charge pairs between the cholesterol hydroxyl oxygen and methyl groups in the choline moiety of the phosphatidylcholine [272]. The predominant group in phosphatidylcholine involved in hydrogen bonding with cholesterol would according to the same studies be the carbonyl oxygen at the *sn*-1 position [223,269,272]. In contrast to these results an earlier energy minimization simulation suggested a minimal energy structure for dimyristoyl-phosphatidylcholine/cholesterol (1:1, mol/mol) involving a hydrogen bond between cholesterol and the dimyristoyl-phosphatidylcholine *sn*-2 carbonyl [276].

The molecular dynamics study of Tu and co-workers showed that cholesterol (12.5 mol%) sits low in the dipalmitoyl-phosphatidylcholine (DPPC) membrane leaving holes in the bilayer surface which are generally filled by choline ammonium groups from neighboring DPPC molecules which makes the bilayer surface flatter [269]. This leads to a rearrangement of water molecules and affects the electrical properties of the bilayer/water interface, so as to increase the overall membrane dipole potential. Essentially the same effect was induced by 22 mol% cholesterol in a dimyristoyl-phosphatidylcholine bilayer, giving a 7% increase in the average number of water molecules hydrogen bonded to phosphatidylcholine [272]. Another molecular dynamics study also registered an effect of cholesterol on the membrane dipole potential by 50 mol% cholesterol in a DPPC bilayer [270]. These results correlate well with the experimental results on egg phosphatidylcholine monolayers which show an increase in dipole potential with increasing cholesterol concentration in the monolayer [277].

The physical effects of cholesterol on the hydrocarbon region of the membrane have also been studied by molecular dynamics methods [223,270]. These studies show an increase in motional order of those lipid atoms adjacent to cholesterol as measured by the order parameter. There was a decrease in the probability of *gauche* conformations in the acyl-chains next to the rigid sterol ring system which gave a decrease in the population of kinks in the upper part of the hydrocarbon chains. Acyl chains adjacent to cholesterol could also not tilt as far as other lipid chains with respect to the bilayer normal. These effects would in nature affect the permeability of the membrane [223]. The simulations in the molecular dynamics study of Smondyrev et al. showed that the addition of cholesterol at both high and low (50 and 11 mol%, respectively) concentrations led to a decrease in total membrane area [270]. This was due to ordering of the hydrocarbon chains and was more pronounced in bilayers with 50 mol% cholesterol. At 11% cholesterol, the membrane thickness was comparable to that in a pure DPPC bilayer. However, at 50% cholesterol the acyl chains were more ordered and therefore longer and the membrane thickness was increased. This also gave a smaller tilt angle of the cholesterol molecules with respect to the

bilayer normal. Also cholesterol lateral motion became more restricted with increasing cholesterol concentration. Two simulations with high cholesterol content were performed, one with cholesterol rich striped domains and one with the cholesterol evenly distributed [270]. The results showed differences in these membranes, drift in membrane area and hydrocarbon chain order when cholesterol was evenly distributed, which may suggest that structures with cholesterol rich domains are favorable at high cholesterol concentrations. Membranes with a uniform cholesterol distribution seemed to be in a metastable phase [270].

Two of the major problems with computer simulations, especially when it comes to heterogeneous systems, are reaching equilibrium in finite computational time and sampling a sufficiently large region of the configuration space of the system [274]. To meet with these problems a technique combining molecular dynamics and Monte Carlo methods has recently been developed for atomic level simulation of a hydrated lipid bilayer [278,279]. The bilayer models can contain about 100–130 lipid molecules and the technique effectively takes into account hydrogen bonding between lipids and between lipids and water. This technique was recently used for simulation of fully hydrated cholesterol containing bilayers [274]. By including a fairly low amount of cholesterol (about 6% of total lipid) direct cholesterol/cholesterol interactions were avoided and the interactions of every cholesterol molecule with neighboring phosphatidylcholines were examined. At the atomic level significant differences were seen in the interaction of cholesterol with DPPC and POPC. The *cis* double bond between C9 and C10 of the *sn*-2 chain in POPC forced a kink into this chain, which hindered close packing with a neighboring cholesterol molecule. This also affected the ability of the *sn*-1 chain to pack around the cholesterol molecule, which led to order parameters for POPC not significantly different from those in a pure POPC bilayer. The saturated DPPC chains on the other hand were able to ''wet'' the surface of the cholesterol molecule. Even 6% cholesterol in the bilayer effectively altered the order parameter profile for DPPC. Through this wetting cholesterol disrupted the phase transition of DPPC due to steric hindrance of the isomeric chain disordering. The lateral diffusion of cholesterol was about the same in DPPC and POPC bilayers, which suggests that the hindrance of lateral diffusion mainly lies in the polar region of the membrane. Since these studies to our knowledge are the latest in this field, we are curious to see what results future simulation studies will reveal, especially concerning hydrophobic mismatch (since the phosphatidylcholine studied so far have contained fairly short acyl-chains) and sphingomyelin/cholesterol interactions.

5. What lies in the future?

The enormous complexity of the molecular structure of biological membranes is becoming increasingly apparent, but the ultimate goal to fully understand how membrane components interact and how different events affect membrane dynamics will be hard to achieve. Currently there is an intense effort to study rafts in membranes because of their biological interest, and focused experiments during the next few years will probably substantially increase our understanding of how lipids in membranes form rafts and how the raft structures are sustained in the dynamic membranes. Future studies on the mechanisms of the lateral distribution (i.e. heterogeneity) of lipids in membranes will probably also help to unravel some of the regulatory mechanisms which in part can explain how, e.g. lipid synthesizing enzymes are activated (or turned off) in response

to changes in membrane lipid compositions. This is already theoretically predicted by the superlattice model of the lateral distribution of lipids in membranes, but needs solid experimental confirmation. Whereas we already have some basic information about the lateral heterogeneity of the distribution of cholesterol in model and biological membranes, we still lack information about the transmembrane distribution of cholesterol. Maybe the answer to this enigma will be revealed by some novel experimental approach within the next few years.

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