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Review

Ordering effects of cholesterol and its analogues

Tomasz Róg [a,*], Marta Pasenkiewicz-Gierula [b], Ilpo Vattulainen [a,c,d], Mikko Karttunen [e]

[a] Department of Physics, Tampere University of Technology, PO Box 692, FIN-33101, Tampere, Finland
[b] Department of Computational Biophysics and Bioinformatics, Jagiellonian University, Kraków, Poland
[c] Laboratory of Physics, Helsinki University of Technology, Finland
[d] MEMPHYS — Center for Biomembrane Physics, University of Southern Denmark, Denmark
[e] Department of Applied Mathematics, The University of Western Ontario, London, ON, Canada

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ABSTRACT

Without any exaggeration, cholesterol is one of the most important lipid species in eukaryotic cells. Its effects on cellular membranes and functions range from purely mechanistic to complex metabolic ones, besides which it is also a precursor of the sex hormones (steroids) and several vitamins. In this review, we discuss the biophysical effects of cholesterol on the lipid bilayer, in particular the ordering and condensing effects, concentrating on the molecular level or inter-atomic interactions perspective, starting from two-component systems and proceeding to many-component ones e.g., modeling lipid rafts. Particular attention is paid to the roles of the methyl groups in the cholesterol ring system, and their possible biological function. Although our main research methodology is computer modeling, in this review we make extensive comparisons between experiments and different modeling approaches.

Contents

* Corresponding author. Tel.: +358 3 3115 2682; fax: +358 3 3115 2600.
E-mail address: tomasz.rog@gmail.com (T. Róg).

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1. Introduction

Cholesterol is one of the most intriguing lipid species in nature. In contrast to other lipids, it is found in high concentrations in animal cell membranes, typical concentrations being around 20–30 mol% and ranging up to 50 mol% in red blood cells [1] and as high as 70 mol% in the ocular lens membranes [2]. Due to its abundance, it is not surprising that cholesterol has numerous functions in membranes ranging from metabolism to being a precursor to hormones and vitamins, and also to providing mechanical strength and controlling the phase behavior of membranes. Here, we focus on aspects related to the two latter ones.

On the mechanistic side, cholesterol's most important effects on membranes are its ability to increase mechanical strength [3–6], to reduce passive permeability of water as well as small molecules and gases [7–13], and its capability to regulate membrane fluidity and the phase behavior of membranes [14–18]. At the molecular level, the most pronounced and easily identified effects of cholesterol are the so-called ordering [19,20] and condensing effects [21]; cholesterol has a dual nature — in the physiologically important fluid state it promotes ordering and rigidity, while in the gel state its effects are the opposite [16]. The advancing understanding of cholesterol's effects on lipid bilayers has been reviewed over the years by several authors [22–29].

In this review, we discuss in detail how cholesterol's structure and its modifications influence the interactions in lipid bilayers. Although cholesterol was discovered already in the 19th century, its structure was determined as late as in 1932 by Heinrich Wieland [30]. Structurally, cholesterol consists of three main functional elements that are important for membrane functions: the rigid steroid ring, the small hydrophilic 3β-hydroxyl group, and a short hydrocarbon chain attached to the steroid ring at the position 17 (see Fig. 1). As it was established already in the 1970s, any modification of these structural elements decreases effects of cholesterol on lipid bilayers [31]. The cholesterol steroid ring system is composed of four rings of which three have six carbons and one has five. The rings are *trans* connected and create a flat and rigid structure, which characterizes cholesterol and most of its analogues. Two methyl substituents, C18 and C19, are attached at positions 10 and 13, in relative *cis* orientations. Due to the above, the cholesterol ring system is asymmetric — one side is flat without any substituents, while the other is rough characterized by the presence of the two methyl substituents. The flat face of cholesterol is called the α-face, and all substituents located on this face (in *trans* conformation relative to C19) are called α, while the substituents located on the rough β-face (in *cis* conformation relative to C19) are called β. In the cholesterol molecule the only additional group is the polar 3β-hydroxyl group, while in other sterols there are many possible α and β substituents. Furthermore, while in cholesterol there is only one double bond between C5 and C6 in ring B (Fig. 1), in other sterols the number and positions of the double bonds varies to some extent. The chemical and the three-dimensional structures of cholesterol are shown in Fig. 1. Fig. 2 shows the cholesterol analogues, which will be discussed in this review. Technically speaking, due to the presence of the hydroxyl group, cholesterol is actually a steroid alcohol.

The effect of cholesterol on a particular matrix (typically phospho-) lipid depends on the lipid's structure. The main factors determining cholesterol's effects on a lipid are the lengths of the lipid's hydrocarbon chains [29,32,33], headgroup structure (phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), or phosphatidylglycerol (PG)) [34–40], the structure of the backbone (glycerol or sphingosine) [41], and the number and location of possible unsaturations along the hydrocarbon chains [29,32,42–44]. In this review, we will discuss the detailed atomic level interactions of cholesterol with saturated and unsaturated phosphatidylcholines and sphingomyelin. The characteristic structures of the lipid classes and the main lipids to be discussed in this paper are shown in Fig. 3.

The interactions of cholesterol with phosphatidylcholines (predominately unsaturated) and sphingomyelins (predominately saturated), the major components of the outer membranes of animal cells, have become a topic of high interest due to the raft hypothesis [45]. Lipid rafts are (believed to be) small dynamic domains composed of cholesterol, saturated phospholipids and sphingolipids, and participating in numerous cellular processes and signaling, see e.g., Simons and Toomre for a review [46]. Cholesterol seems to be vital for the formation of these highly ordered membrane domains and none of its analogues or precursors, not even the closest ones, possess the same ability to promote rafts, as will be discussed in detail later [47]. It should be noted, however, that rafts are not in the gel phase; raft systems comprise a fluid-fluid co-existence. Although lipid rafts are among the most studied individual topics in membrane biophysics and chemistry, a lot of controversy yet remains [48–53].

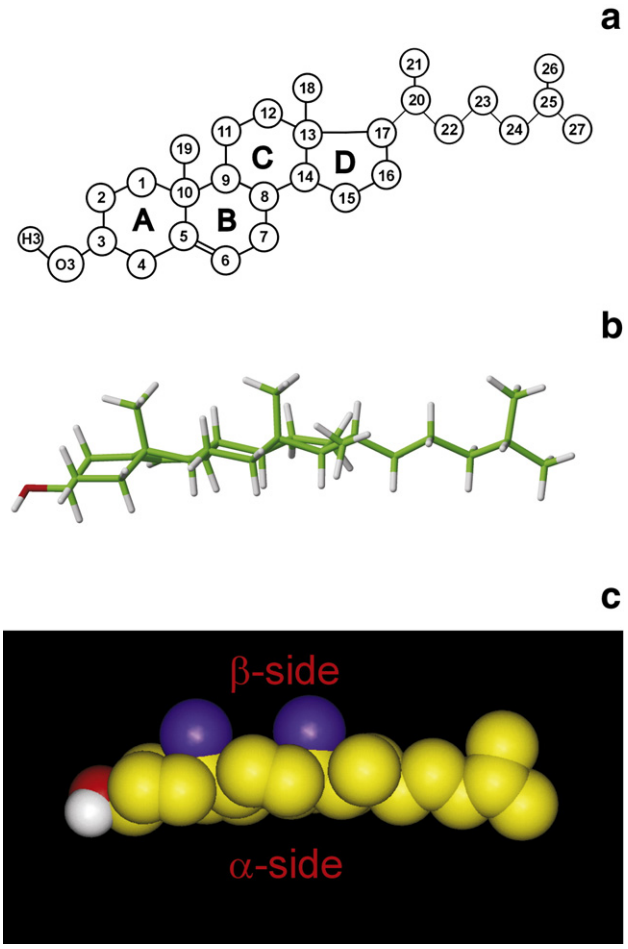

Fig. 1. Structures of cholesterol molecule: chemical structure with numbering of carbon atoms and rings (labeled A, B, C, and D) (a), three-dimensional structure in the stick representation (b) and in the CPK representation, the smooth α-face and rough β-face of cholesterol are labeled (c).

Atomistic molecular dynamics (MD) simulations have proven to be extremely useful in membrane research [54–58]. They have the capability of providing direct information about atomic level mechanisms not accessible by any of the current experimental techniques. Additionally, MD simulation measurements do not perturb the system as no additional probes are needed — it is even possible to study what effects probes may have on experimental results [59–61]. MD simulations track the positions and momenta of every single atom throughout the whole duration of each simulation and that provides a unique way to 'see' inside the simulated system. An example of such an observable, which provides important information yet is experimentally very hard to access directly is the lateral pressure profile inside a membrane [62]. Furthermore, combining MD simulations and experiments directly provides a unique way to gain insight into different

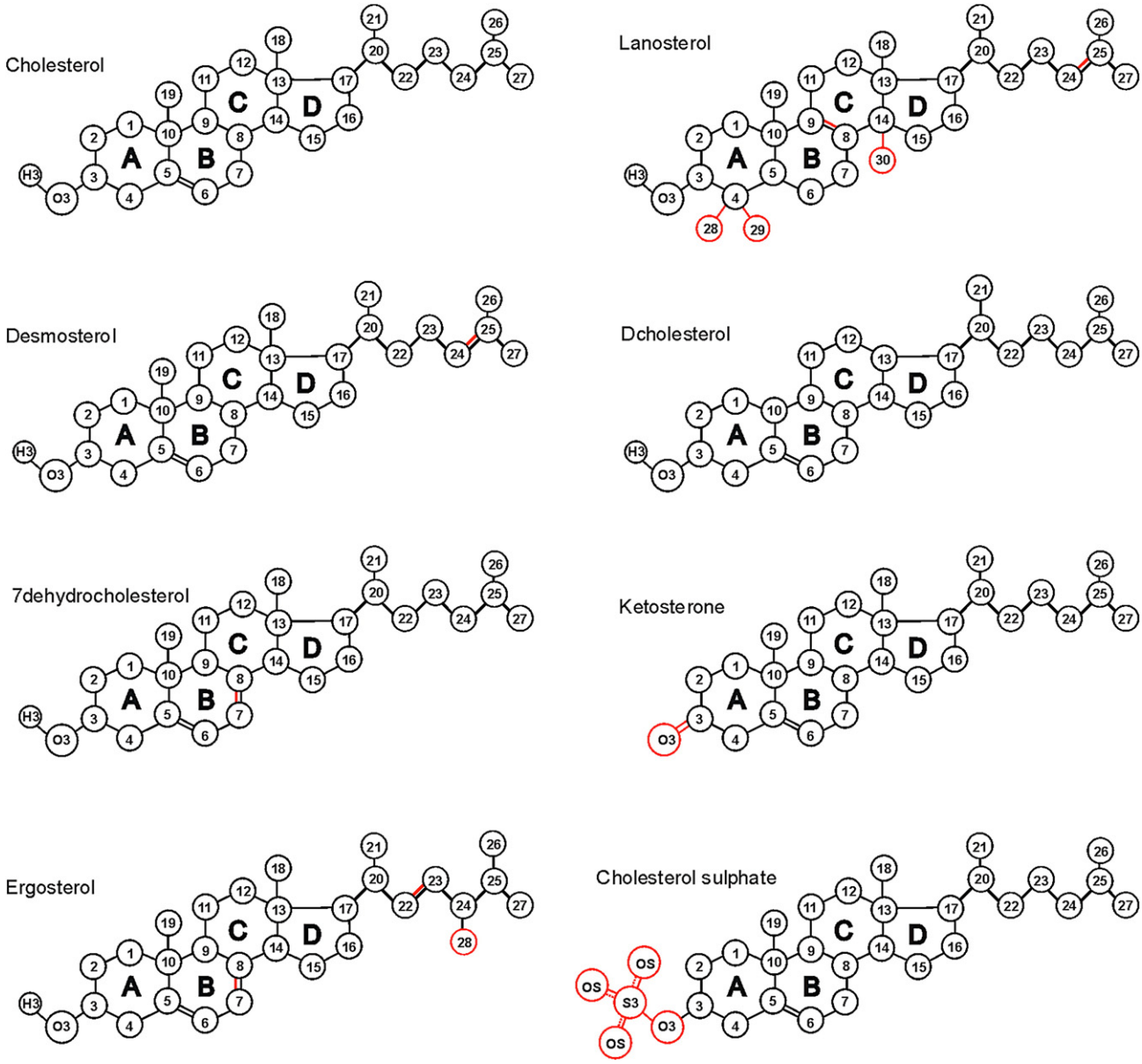

Fig. 2. Chemical structures of sterols discussed in this paper. The modified parts are marked in red.

properties at different time and length scales as the different methods can be correlated with each other [63–69]. Hence, it is not surprising that MD simulations have quickly become a widely used method also in the studies of the behavior of cholesterol in lipid bilayers [70–72].

In this review we concentrate on atomistic simulations of cholesterol in lipid bilayers performed with the molecular dynamics simulation method. Yet, there is reason to point out that a number of other computational and theoretical methods have also been applied to related systems. In particular, Monte Carlo simulations [73–76], combined Monte Carlo–molecular dynamics simulations [77,78], force-field studies [79], coarse-grained simulations [80,81], QSAR modeling [82], and continuum models [83,84] have been successfully applied to elucidate the behavior of cholesterol and other sterols. More coarse-grained aspects have even been studied with Master-equation type approach [85] and Ginzburg–Landau formalism [86].

The MD simulation method has been used in studies of macromolecules for a relatively short time. Thus, it is not surprising that even the computational methods themselves are under rapid development; in our own first simulations we were able to cover a time scale of 5 ns [87], while in the most recent simulations 100 ns has become the standard [88,89], and from bilayers composed of 72 lipids [87] we have moved to systems with more than 1000 lipids [90,91]. Due to possible artifacts [92,93], there has been considerable progress from using faster cut-off methods for electrostatic interactions to slower but more reliable and accurate techniques such as the particle-mesh-Ewald method [94]. Parameterization has also evolved; from the initially used united atom OPLS (Optimized Parameters for Liquid Simulations) parameterization [95] or the so-called Berger lipids [96], which is also a united atom description, we are slowly moving to a more accurate OPLS all atom [97] parameterization [98,99] and polarizable force fields [100]. This progress has allowed one to describe more complex phenomena occurring in greater time and length scales, such as collective oscillations in lipid bilayers [101], or the mechanisms of lipid diffusion characterized by large scale collective flows [90].

2. Cholesterol in saturated phosphatidylcholines

In this chapter we will describe effects of cholesterol on saturated phosphatidylcholines (PCs). Saturated PCs are not the most common lipids in nature as PCs in cell membranes are typically unsaturated,

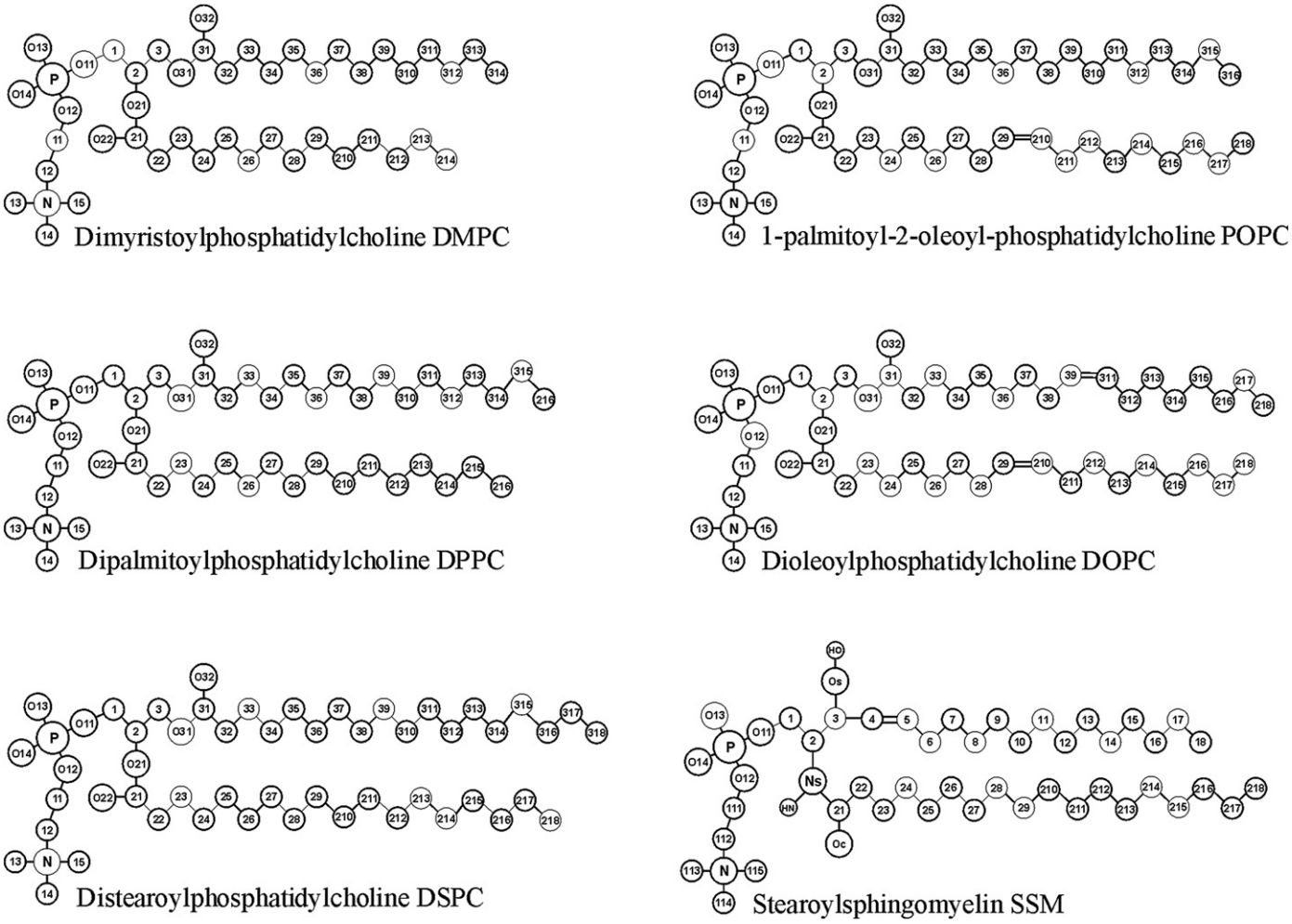

Fig. 3. Chemical structures of phospholipid discussed in this paper.

while the majority of saturated lipids are sphingolipids. Studying saturated lipids is, however, a very natural starting point for many reasons. First, majority of experimental data has been obtained for saturated PCs and thus, to be able to quantitatively compare the results, and to improve the force-field parameterizations, it is essential to start from saturated PCs. Second, parameterization of double bonds is not a straightforward matter due to the lack of detailed experimental data; hence investigations of the simpler saturated lipids are essential. Third, since most lipids have at least one saturated chain, knowing the interactions of cholesterol with saturated hydrocarbon chains is vital.

In our studies we have used two different saturated lipids as a matrix. The first one is dimyristoylphosphatidylcholine (DMPC), which has 14 carbon atoms in each of its acyl chains. As the second lipid we have used dipalmitoylphosphatidylcholine (DPPC), which has two acyl chains of length 16. At a first glance the difference between DMPC and DPPC seems almost negligible, but careful studies have shown it to be essential; cholesterol molecule is shorter than the chains of DMPC in a liquid bilayer, and hence cholesterol is able to intercalate between the membrane layers [102]. The difference of two extra carbons in the acyl chains of DPPC turns out to be crucial as no intercalation has been observed for DPPC [88]. In simulations, we have observed that cholesterol indeed has a stronger effect on a DPPC than a DMPC bilayer, which is in excellent agreement with experimental data [33,41].

2.1. Ordering effects

The cholesterol induced increased ordering (as compared to the situation in the absence of cholesterol) of the PC acyl chains is generally called as the "ordering effect", which is one of the generic effects cholesterol has on a lipid bilayer in the physiologically relevant fluid phase. The ordering effect has been measured and quantified by various experimental techniques including NMR, EPR, fluorescence spectroscopy, as well as others [19,20,103–110].

Robinson et al. [71] were the first ones to show the existence of the ordering effect in MD simulations. To quantitatively evaluate the order of lipid chains, one can use the molecular order parameter, S_{mol}:

$$S_{\mathrm{mol}} = \frac{1}{2}\langle 3\cos^2\theta_n - 1\rangle, \tag{1}$$

where θ_n is the instantaneous angle between the n^{th} segmental vector, i.e., (C_{n-1}, C_{n+1}) the vector linking $n-1$ and $n+1$ carbon atoms in the hydrocarbon chain and the bilayer normal. The angular brackets,<>, denote both the ensemble and the time averages. S_{mol} is closely related to the deuterium order parameter (S_{cd}) as measured in NMR spectroscopy: $S_{\mathrm{mol}} = -2S_{\mathrm{cd}}$ [111,112], which holds for saturated chains. For regions close to a double bond, the so-called S_{cd} order parameter is more appropriate.

In line with other studies, e.g., [113–116], MD simulations of DMPC and DPPC bilayers containing cholesterol have shown a clear increase in the order of the PC chains relative to the respective pure PC bilayers; Fig. 4 shows snapshots from MD simulations of DMPC and DMPC-Chol bilayers, and Fig. 5 displays the S_{mol} profiles for pure PC bilayers and those containing Chol and its analogues.

The order of the PC chains can also be evaluated by other parameters, such as the number of *gauche* rotamers/chain or the average tilt angle of the chains. The conformational state of a saturated

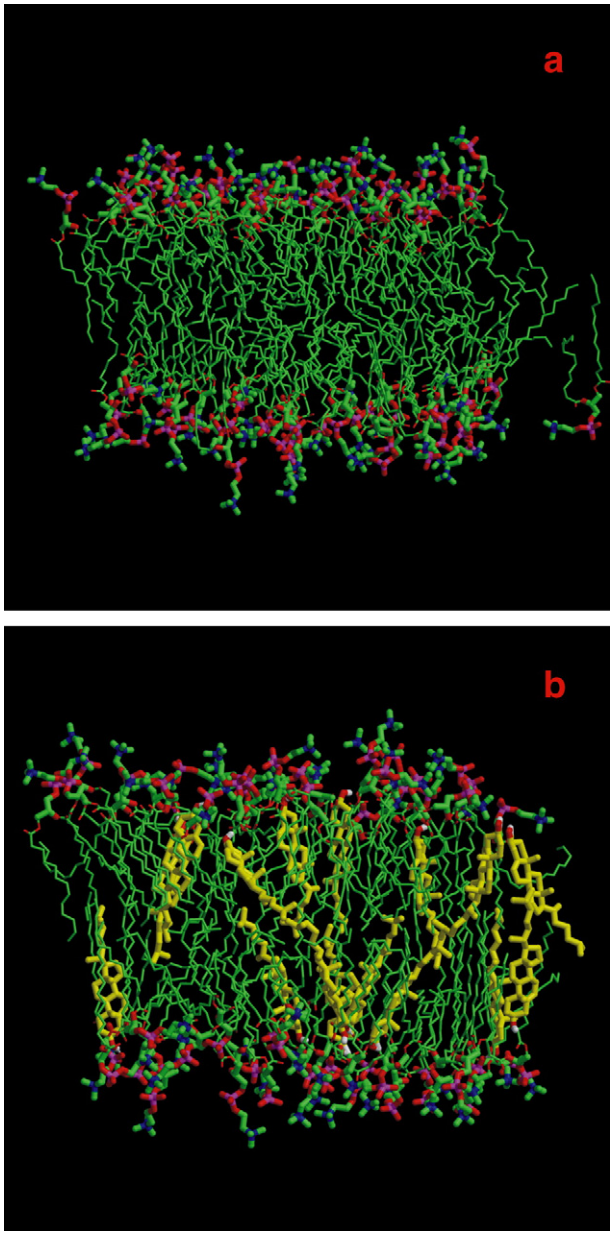

Fig. 4. Snapshots of DMPC and DMPC-Chol bilayers. The Chol molecules are yellow, and all other atoms, including the OH group of Chol, are in standard colors. Water is removed to better show the location of the polar groups [116].

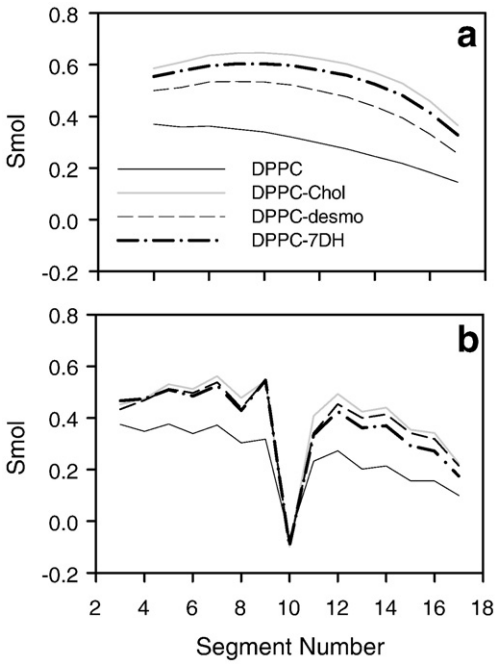

Fig. 5. Molecular order parameter profiles of the of the *sn*-1 tails for (a) DPPC, DPPC-Chol, DPPC-Desmosterol, and DPPC-7-dehydrocholesterol. (b) DOPC, DOPC-Chol, DOPC-Desmosterol, and DOPC-7-dehydrocholesterol. Line codes: pure PC (solid black), PC-Chol (solid gray), PC-Desmosterol (dashed), PC-7-dehydrocholesterol (dot–dashed).

acyl chain is determined by the *trans* and *gauche* conformations of the chain's torsion angles, see Fig. 6. The *trans* conformation corresponds to the torsion angle of 180±30°, while *gauche* corresponds to 60±30° (g^+) and 300±30° (g^-) [117,118]. A typical distribution of torsion angles illustrating the occurrence of *trans* and *gauche* conformations in the chains as well as a corresponding potential energy profile is shown in Fig. 6. We would also like to point out that when there is a double bond in the acyl chain or carbonyl group, stable conformations of the single bonds next to the double bond correspond neither to *trans* nor *gauche* conformations [118,119].

The number of *gauche* rotamers/chain can, in principle, be obtained from infrared spectroscopy. The results of different studies are, however, conflicting [120–125]. The effect of cholesterol on the number and distribution of *gauche* conformations along the chain differs between the DPPC and DMPC bilayers. In the DMPC bilayer, one has observed a redistribution of *gauche* rotamers along the chain without changing their number [118]; while in a DPPC bilayer their number was reduced [88]. We attribute these differences to the difference in the chain lengths, although further studies are needed to elucidate this. In addition, cholesterol clearly reduces the rate of *trans-gauche* isomerization in both bilayers [88,120].

The tilt angle of a lipid chain represents the amplitude of rigid body-like angular fluctuations of the chain and can be calculated from the average *cosine* square of the angle between the bilayer normal and the vector indicating the chain's orientation (a vector connecting the first and the last atom of the chain or an average segmental vector, as described in Ref. [126]). The tilt angle can also be measured

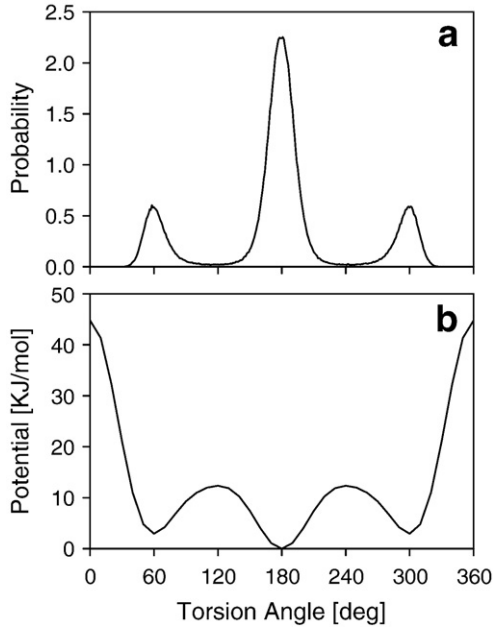

Fig. 6. Populations of a torsion angle in (a) the saturated acyl tail and (b) the corresponding torsional energy profile.

experimentally by fluorescence or EPR spectroscopy, when rigid fluorescence or paramagnetic probes, whose preferential orientation in the bilayer is parallel to the surrounding chains, are used [105,127,128]. The value of the tilt angle depends strongly on the conformation of the first torsion angles of the chain — this dependence explains why a redistribution of *gauche* conformations along the chain can affect its order and thus the tilt. Examples of tilt distributions for a DPPC bilayer without and with cholesterol, and a graphical interpretation of the tilt angle, are shown in Fig. 7. Not surprisingly, cholesterol significantly reduces the tilt of a chain [88,118].

Simulations provide direct information about the tilt angle of the cholesterol ring, which is defined as the angle between the bilayer normal and the C3–C17 vector (see Fig. 1) [118]. The Chol tilt in the DMPC-Chol bilayer is 17° [118] and in the DPPC-Chol bilayer 20° [88], close to the values measured experimentally [129].

To gain a better understanding of the ordering effect in the DMPC-Chol bilayer, one has analyzed effects of the smooth (α-) and rough (β-) faces of cholesterol on DMPC chains as well as that of the chain's distance from a cholesterol ring [118]. In the DMPC-Chol bilayer, one selected the chains neighboring the α- (group 1) and β-faces (group 2) of a cholesterol molecule as well as molecules not being in contact with any of the cholesterols in the system (group 3). The analysis was performed within a two nanosecond time window [118]. The order of the chains in all of the three groups is higher than in the pure DMPC bilayer, which means that the ordering effect is not limited only to the nearest neighbors [118]. Examples of the DMPC molecules from the first and second groups are shown in Fig. 8. A similar observation was made by Scott and McCullough in his MC studies [75]. Nevertheless,

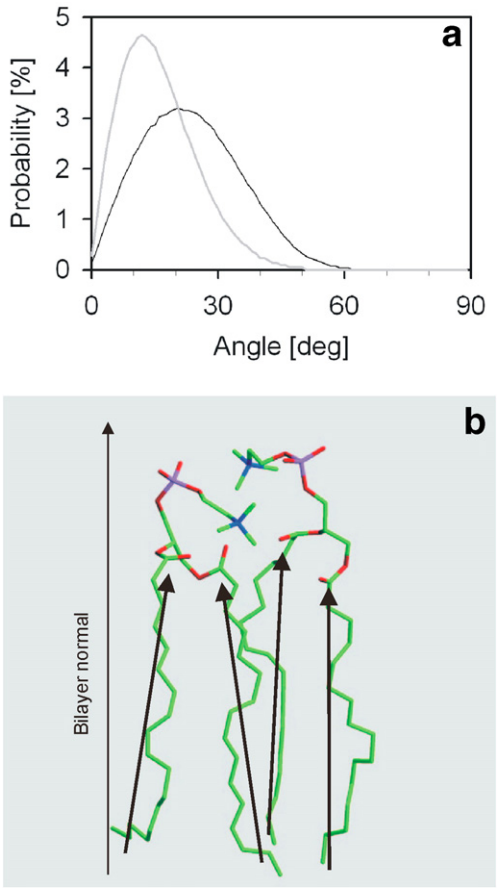

Fig. 7. (a) Distribution of tilt angles in DPPC (gray line) and DPPC-Chol bilayer (black line) [46]. (b) Examples of two PC molecules from a simulated PC bilayer with graphical representations of the tilt vector.

the chains neighboring the cholesterol α-face display the highest order. The exchange among the groups is slow and takes place mainly between groups 1 and 2 due to the rotation of cholesterol molecules; the exchange of lipids from groups 1 and 2 with those from group 3 is very limited, which agrees well with other studies on lateral diffusion of lipids [90].

2.2. Condensing effect

The second widely discussed and documented effect of cholesterol is the so-called condensing effect. Initially, it was defined as a cholesterol induced increase of the membrane surface density [21], but recently it has been re-defined as a decrease of the surface area occupied by PC molecules in mixed lipid bilayers containing cholesterol [22]. Probably the most systematic studies of the condensing effect have been performed on monolayer systems by Smaby et al. [42,130,131]. The condensing effect is closely related to the ordering effect (Section 2.1) and S_{cd} can be directly used to calculate the surface area per PC in a bilayer [132].

In a single component PC bilayer the average area per PC is calculated simply by dividing the total cross-sectional area of the bilayer by the number of PCs in one leaflet. To estimate the magnitude of the condensing effect, one has to calculate independently the areas per PC $\langle A_{PC}\rangle$ and cholesterol $\langle A_{Chol}\rangle$, and this is not an easy task. One possibility is to follow the approach used by Smaby et al. [42,130,131] where $\langle A_{PC}\rangle$ is derived by subtracting from the total area of the bilayer the total area of the cholesterol molecules, assuming that $\langle A_{Chol}\rangle$ is constant. Monolayer studies have provided support for that assumption and values in the range of 0.39–0.41 nm^2 have been measured for $\langle A_{Chol}\rangle$ [133,134]. The value of 0.39 nm^2 was used in rather recent simulations [118] as well as in recent X-ray studies of Hung et al. [135].

Using a constant value for $\langle A_{Chol}\rangle$ is only an approximation and not fully justified at all times. That is easy to understand since the cross-sectional area of the steroid ring is larger than that of the chain, and hence the distribution of the free area must depend on the distance from the bilayer centre; the amount of free area is the highest in the bilayer center and the lowest at the distance of 1–1.5 nm from the centre [136]. In addition, using $\langle A_{Chol}\rangle$ from monolayer studies is not generally justified since those results have been obtained in pure cholesterol monolayers, whereas in a bilayer with PC present, the flexible PC chains can pack tightly around a cholesterol molecule, which is not possible in a pure cholesterol monolayer. In addition, direct comparison of monolayer and bilayer systems is not straightforward and has to be done with care [137].

Keeping the above in mind, let us next discuss simulations of mixed cholesterol-PC systems. In their MD study of DPPC-Chol bilayers, Chiu et al. [138] varied cholesterol concentration in the range 0–50 mol%, and were able to show that $\langle A_{PC}\rangle$ is linearly dependent on cholesterol concentration in the range of 12–50 mol%, suggesting that $\langle A_{Chol}\rangle$=0.223 nm^2, which is significantly smaller than 0.39–0.41 nm^2 from monolayer studies. In another study, Hofsäß et al. [139] calculated $\langle A_{PC}\rangle$ from the formula:

$$A_{PC} = \frac{2A}{N_{PC}}\left(1 - \frac{N_{chol}V_{chol}}{V - N_w V_w}\right), \quad (2)$$

where A and V are the area and volume of the simulation box, N_{PC} is the number of PC molecules, N_w is the number of water molecules, V_w corresponds to the volume occupied by a water molecule, N_{chol} stands for the number of cholesterol molecules and V_{chol} is the volume of a cholesterol molecule. The above equation takes into account the volume occupied by the cholesterol and water molecules; the difference between A and $\langle A_{PC}\rangle$ allows one to calculate $\langle A_{Chol}\rangle$. Using Eq. (2), Hofsäß et al. found that $\langle A_{Chol}\rangle$ is 0.27–0.29 nm^2 with a slight dependence on cholesterol concentration.

An extensive discussion on the subject has been provided by Edholm and Nagle [140]. They re-analyzed MD simulation data for

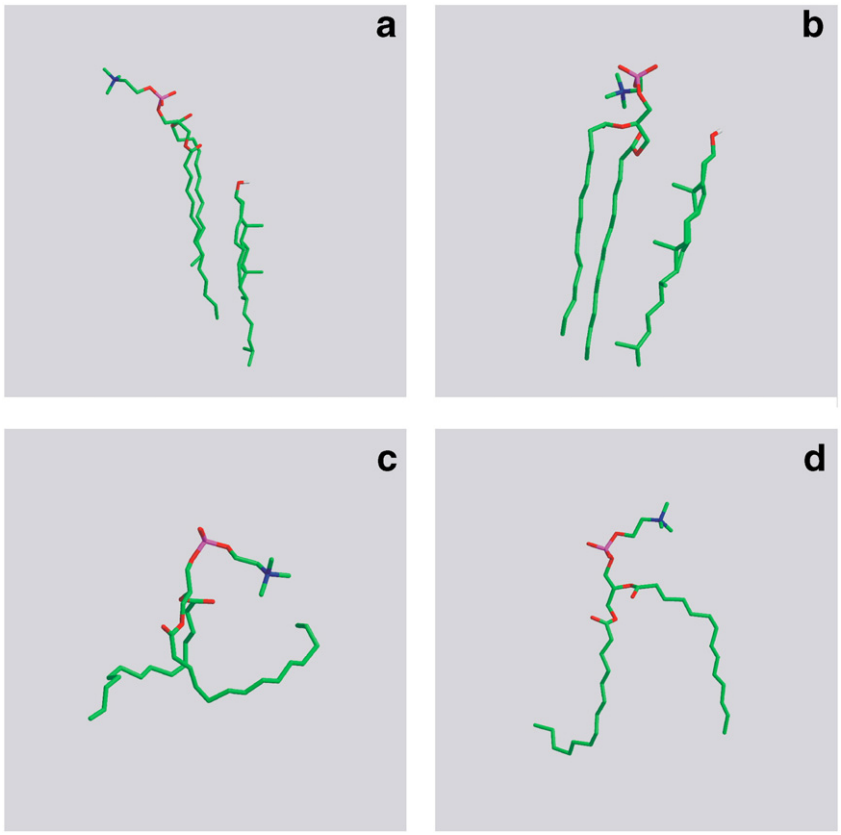

Fig. 8. Examples of DMPC molecules in the DMPC-Chol bilayer located next to the cholesterol α- (a), β-face (b) and not neighboring cholesterol molecule (c, d) [116].

DPPC-Chol bilayers with cholesterol concentrations ranging between 0 and 50 mol% and proposed a definition based on thermodynamics for the partial specific areas per cholesterol and DPPC in a mixed bilayer. In their definition, $\langle A_{Chol}\rangle$ depends on cholesterol concentration, and its values range from negative up to 0.27 nm^2 when cholesterol concentration is high. They interpreted the negative values as indications of the strength of the cholesterol condensing effect. Another way to calculate $\langle A_{Chol}\rangle$ is to use Voronoi tessellation, which essentially defines a Wigner–Seiz cell for a molecule in real space, i.e., it defines the area that is closer to a chosen molecule than any other molecule in the system. In the study of Pandit et al. $\langle A_{Chol}\rangle$ was estimated to be 0.296 nm^2 using Voronoi tessellation [141].

On the experimental side, the recent X-ray diffraction data of Hung et al. [135] enable comparison with MD simulations in the case of DMPC-Chol bilayers. The best way to compare experimental and theoretical data would be to compute the experimentally accessible observables, e.g., scattering form factors (for example see [63]) from MD simulations. In most cases, however, such parameters are not available. Hung et al. calculated the area per PC in the DMPC-Chol bilayer by the subtraction method assuming a constant $\langle A_{Chol}\rangle$ of 0.39 nm^2. They obtained $\langle A_{DMPC}\rangle$=0.61 nm^2 at 303 K in the pure DMPC bilayer, and 0.49 nm^2 in the DMPC-Chol bilayer at 20 mol% of cholesterol. The values are close to simulation results [118] of 0.60 and 0.53 nm^2, for the pure DMPC and DMPC-Chol (22 mol% Chol) bilayers, respectively, at 310 K (7 K temperature difference can be responsible for ~0.02 nm^2 difference in the area per lipid).

From a set of experimental data for DMPC-Chol bilayers with varying cholesterol concentration [135], the area per lipid can be calculated and plotted as a function of the cholesterol concentration (Fig. 9). For concentrations above 35 mol%, a linear dependence is observed. Following Chiu et al. [138] one can assume that $A_{av}=xA_{Chol}+(1-x)A_{PC}$ (where x stands for the Chol concentration). From this, one finds that $\langle A_{Chol}\rangle$ is 0.378 and $\langle A_{DMPC}\rangle$ is 0.45 nm^2 (above 35 mol% Chol). The value for $\langle A_{Chol}\rangle$ is close to that obtained in the crystal of 0.37 nm^2 [142] and $\langle A_{DMPC}\rangle$ at high Chol concentration is close to that in gel phase of lipid bilayers of 0.42–0.47 nm^2 [143,144]. Using 0.378 nm^2 for $\langle A_{Chol}\rangle$ in the DPPC-Chol with 50 mol% Chol [136], $\langle A_{DPPC}\rangle$ would be 0.39 nm^2. In fact, such values have been obtained for that concentration using the Voronoi tessellation method [136]. These two facts suggest that $\langle A_{Chol}\rangle$, at least in higher cholesterol concentrations, is definitely larger than proposed by Chiu et al [138] and Hofsäß et al. [139] and also suggest that the degree of condensation observed in DPPC bilayers is a bit too high (area per DPPC should not be below the area observed in the gel phase). However, as already pointed out, these considerations are based on indirect experimental data and on a system where condensation is expect to be weaker. Methodological issues, which can cause too strong a condensation, are briefly discussed in Section 5.

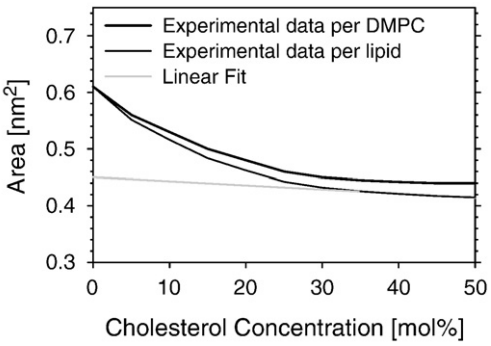

Fig. 9. Area per molecule in the DMPC-Chol bilayer as a function of cholesterol concentration obtained in experimental studies [133], area per DMPC with area per cholesterol of 0.39 nm extracted (black thick line), area per all lipids (black thin line), and a linear fit for the linear part of the curve.

In the above discussion, the presence of free volume was implied to but not discussed. To quantify the condensing effect, both the free area and free volume have been analyzed in detail. The results show clearly that the area and volume occupied by cholesterol vary significantly as a function of distance from the bilayer centre [136,145,146]. Furthermore, the effect of cholesterol on free volume (in terms of inter-atomic voids) is complicated [147]. Voids, or free volume pockets, may obtain different sizes and adopt various shapes and they are also dynamic entities. In general, cholesterol reduces the number of voids, especially the large ones, and with increasing cholesterol concentration the voids tend to become oriented parallel to the membrane normal and assume more elongated shapes. The effect is particularly strong in the vicinity of the steroid ring [145,147]. Similar conclusion was drawn from Voronoi tessellation studies of Jedlovszky et al. who showed that decrease of the DMPC molecular area is strongest for molecules close to cholesterol molecules [148]. Experimental studies on bimolecular collision rate measured with spin labels attached to the stearic acid at selected position of the chain showed reduction of collision rate for the positions 5 and 7 which correspond to the position of the cholesterol ring and increase of the collision rate for the positions 10 and 16 which are below cholesterol ring in the DMPC-Chol bilayer [149]. These results indirectly confirmed our observation of a strong decrease of free volume and voids close to the cholesterol ring.

The condensation is also reflected on membrane thickness. Membrane thickness is not a uniquely defined parameter, as several alternative definitions exist. In simulation studies the most often used one is likely the P–P distance (distance between the average positions of phosphate atoms in opposite leaflets). A commonly used alternative definition is to estimate the thickness as the distance between points (in opposite leaflets) at which the water and membrane densities are equal. In simulations of DPPC and DMPC bilayers containing cholesterol one observed an increase of membrane thickness, which agrees with experimental data [150,151]. Fig. 10 shows density profiles of DPPC and DPPC-Chol bilayers to demonstrate the observed increase in membrane thickness. There is, however, an important subtlety: the effect of cholesterol on membrane thickness depends on unsaturation [135,150] and chain length [151]. According to the data of McIntosh, thickness may be reduced when the chains are longer than 18 carbons. This can be due to the fact that cholesterol is shorter than those chains and the space under cholesterol molecule has to be filled, which leads to a decrease in thickness [151]. This is also reflected in the phase behavior of a mixture of cholesterol with a series of PCs with various chain lengths [152]. In unsaturated bilayers, the effect is less pronounced [135,150].

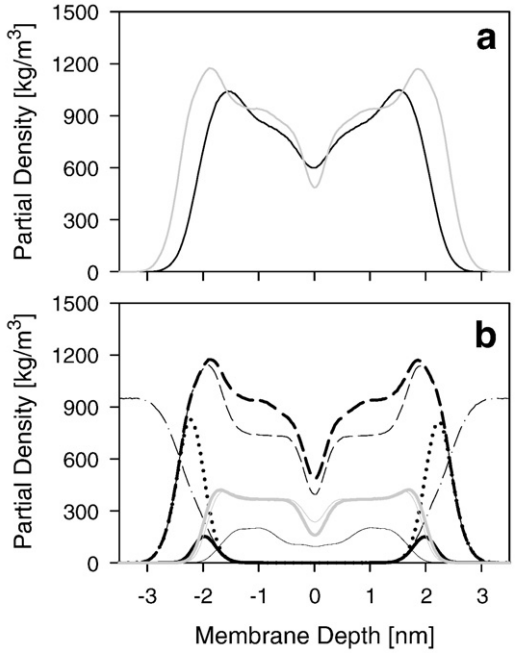

Fig. 10. Partial density profiles along the bilayer normal. (a) All bilayer atoms in DPPC (black line), and DPPC-Chol (gray line) bilayers. (b) In the DPPC-Chol bilayer: all bilayer atoms (thick dashed line), DPPC (thin dashed line), DPPC head groups (dotted line), DPPC *sn*-2 (gray thin line) and *sn*-1 chain (gray line), glycerol backbone (black thick line), cholesterol (black line), and water (dash–dot line) [46].

2.3. Polar interactions

The water–membrane interface is a very special region that strongly determines the properties of the whole membrane [153,154]. This is mainly due to two facts: First, there is a very large change in the dielectric constant around the interface as it goes from about 80 (bulk water) to about 2 (hydrocarbon region in a membrane) in just a few nanometers. Second, in this region there are polar and/or charged groups that generate long-range electrostatic fields and they also participate in shorter ranged interactions such as hydrogen bonds. Here, we concentrate on the following interactions: 1) those between lipids and water (hydration), 2) among lipids (direct hydrogen (H-) bonds and charge pairs), and 3) indirect, water mediated lipid–lipid interactions (water bridges) [87,155–158]. An example of the pairs of cholesterol and DMPC hydrogen bonded, water-bridged and charge pairs is shown in Fig. 11.

In our previous studies, we established a set of rules to evaluate the occurrence of such interactions [87,155,156]. For a H-bond, we use the following geometric criteria: the distance between a hydrogen donor (D) and a hydrogen acceptor (A) has to be less than 0.325 nm and the angle between the H–D bond and the vector connecting D and A has to be less than 35°. These criteria were established based on the radial distribution function of the donor atoms relative to acceptor atoms; the radial distribution function has a clear minimum at 0.325 nm, and the modified limiting value for the H-bond angle that was obtained from a screening of crystallographic data [155]. This set of H-bond criteria is commonly used. Alternative definitions do exist and are discussed elsewhere [159]. Interfacial water molecules participate in formation of H-bonds and may form clathrate-like structures around choline groups [155]. A charge pair is formed between a positively charged choline (N-CH3) group and a negatively charged lipid oxygen atom that are not farther than 0.4 nm from one another [156]. Again, the definition of a charge pair is based on the respective radial distribution function, which has a clear minimum at 0.4 nm. Short-range interactions between N-CH3 and phosphate groups were detected experimentally already in the 1970s [160] but at the time the results did not attract much attention. The importance of those early experiments was shown by MD simulations [87,156].

Charge pairs are considered as weak H-bonds by some authors [161,162]. Detailed quantum mechanical calculations for the particular groups in the presence of water molecules remain to be done, however. In general, C–H···O H-bonds have been shown to form under certain conditions [163–166]. Our studies show extensive formation of charge pairs in lipid membranes [156] resembling salt bridges found in proteins. A water molecule can be simultaneously H-bonded to two lipid polar groups either within the same molecule or belonging to two molecules and thereby creating a water bridge. Water bridges stabilize the membrane structure [155] similarly to “structural” water in proteins. The water–membrane interface is also the location at which ion binding occurs [167–169].

Stability of the above-mentioned interactions was evaluated by calculating their lifetimes. The lifetime was defined as a period between the first and the last observation of the given interaction allowing for short breaks that were ignored in the case of longer-lived interactions [155]. The range of lifetimes ranges from a fraction

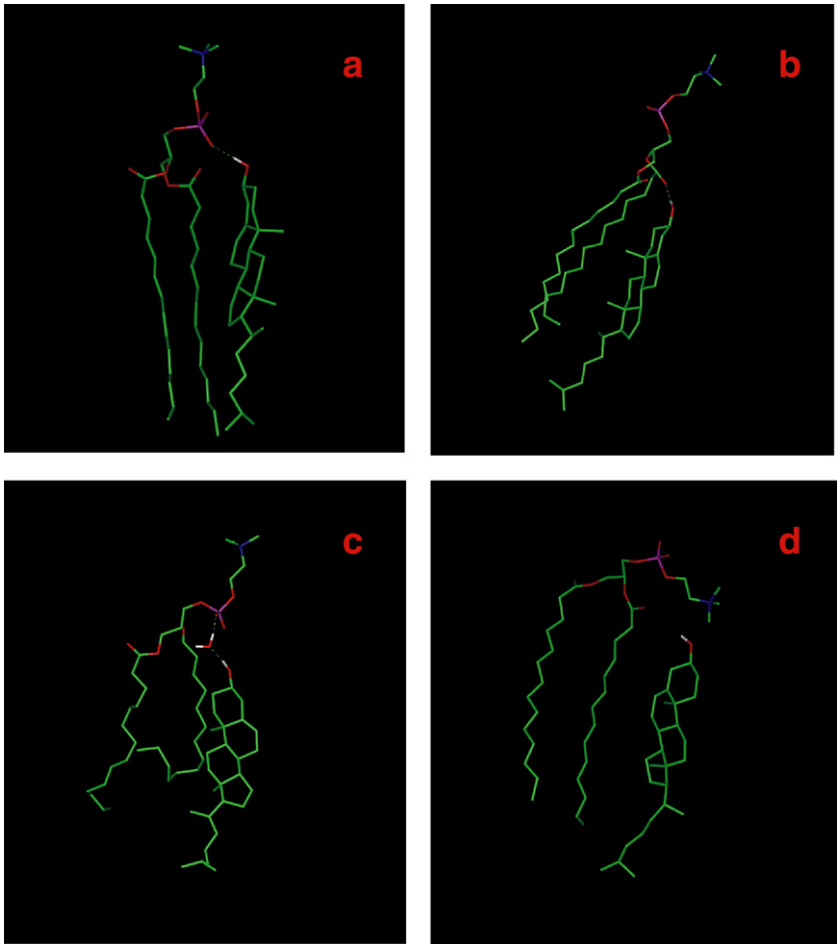

Fig. 11. Examples of DMPC-Chol interactions in the DMPC-Chol bilayer. Direct H-bonds (a, b); water bridge (c); and charge pair (d).

of a picosecond [170,171] to over tens of nanoseconds [87,155,156]. The long lifetimes make accurate determination difficult and the values obtained should hence be considered as lower limits only [87,157,158].

The cholesterol hydroxyl group can act both as H-bond donor and acceptor, besides which it can also participate in charge pairing. That gives cholesterol the versatility to form numerous different types of bonds in the interfacial region. Unfortunately, direct detection of H-bonds in hydrated lipid bilayers is not an easy task and older experiments have provided conflicting results (see Discussion in [162]). Here, we concentrate only on recent experimental and MD simulation data. As generally observed in MD simulation studies, cholesterol forms H-bonds mainly with carbonyl oxygen atoms (Oc). The exact numbers of Chol···Oc pairs differ from study to study. In our investigations of DMPC-Chol bilayers, we observed only 0.11 Chol···Oc per cholesterol [87]; while in a DPPC-Chol bilayer the number was 0.58 [87]. Cholesterol's preference to H-bond with Oc rather than the phosphate oxygen atom (Op) has also been observed in other MD simulations [116,172–174] and energy minimization studies [175]. The only exception, to our knowledge, is the study of Henin and Chipot, which showed cholesterol to prefer Op instead [176]. That is at variance with recent experimental data [174,177].

The cholesterol hydroxyl group (OH-Chol) also forms H-bonds with water. In MD studies, the number of Chol···water per Chol is typically ~1.1 in the case of a DMPC-Chol bilayer [87] and ~0.4 in case of a DPPC-Chol bilayer [88]. For the DMPC-Chol bilayer, the number of Chol···water per Chol agrees well with NMR results, which give 0.93 [174]. The observed differences in the numbers of H-bonds in DMPC-Chol and DPPC-Chol bilayers showed that cholesterol prefers interactions with DPPC rather than water. The deeper location of cholesterol molecules in the DPPC-Chol bilayer seems a reasonable explanation for such a preference. The lifetime of Chol···Oc in the DMPC-Chol bilayer is of the order of 70 ps, whereas that of Chol···water is ~40 ps [87]. In both bilayers we observed that 30–40% of the Chol molecules are water-bridged with PC molecules. The lifetime of these water bridges is of the order of 500 ps [87].

The number of PC-Chol charge pairs detected in the DMPC-Chol and DPPC-Chol bilayers differs as well. In the DMPC-Chol bilayer ~50% of Chol molecules are charge-paired with N-CH3 groups, their lifetime being around 900 ps [87]. The number of charge pairs in the DPPC-Chol bilayer is higher, and practically speaking all cholesterol molecules are charge-paired [88]. Pandit et al. consider charge pairs as weak H-bonds, using an energetic cut-off of −2.8 kcal/mol as a criterion — in terms of distance that matches well with a cut-off of 0.33 nm [161]. Their view was recently supported by a Raman spectroscopy analysis of Chol-PC mixture [162] as well as quantum mechanical calculations of other systems [163–166]. These calculations show that the strength of C–H···O depends on the presence of an electronegative atom next to the donor C–H group. The question of whether H-bonding between N-CH3 and Op, Oc or OH-Chol prevails in excess water remains open. Most likely, the interaction may be considered a charge-pair strengthened by H-bonds as it is in the case of a salt-bridge between Glu342 and Lys290 in α_1-antitrypsin [178]. Nevertheless, a distinct clathrate-like structure of water is formed around the choline group showing its inability to form strong H-bonds with water molecules.

A schematic representation of cholesterol interfacial interactions with DMPC and DPPC is shown in Fig. 12.

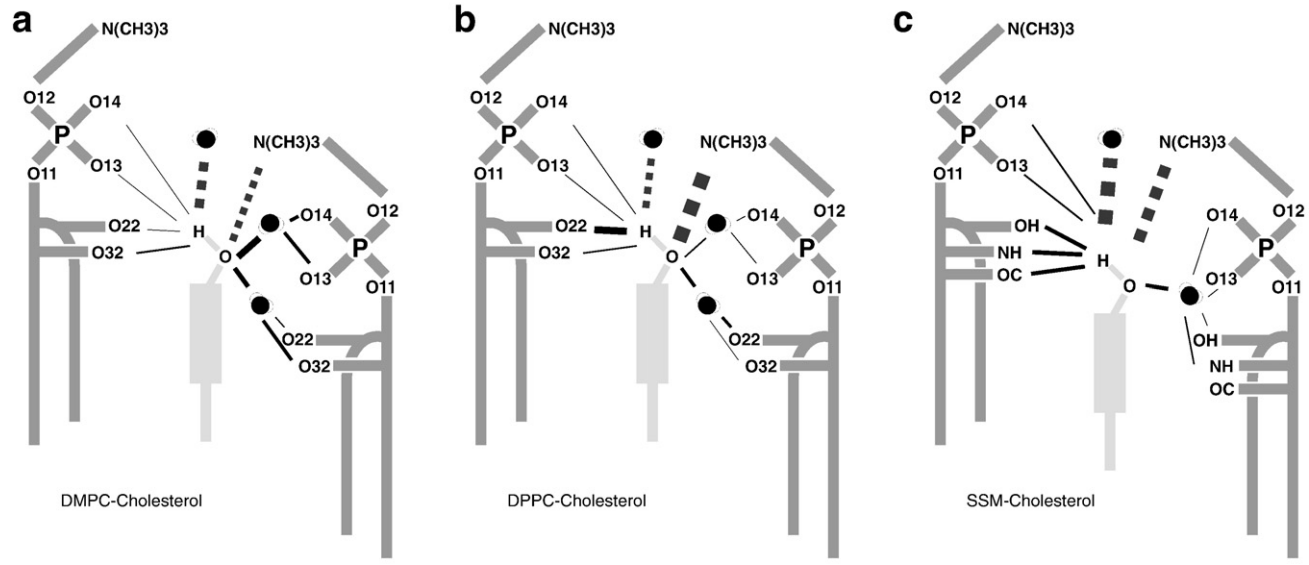

Fig. 12. Schematic representation of interfacial interactions between cholesterol and DMPC (a), DPPC (b), and SM (c). Probability of interactions is represented by the respective line thicknesses.

2.4. Non-polar interactions

Non-polar interactions are more difficult to evaluate numerically than the polar ones. This is mainly due to the fact that they are weaker and non-specific. In a lipid bilayer, non-polar interactions are predominantly of the van der Waals type. They determine, to a large extent, the structure and physico-chemical properties of the bilayer including condensing cholesterol effects [179,180]. Unfortunately, there are no experimental techniques to directly measure them. In MD simulations, non-polar interactions are estimated by evaluating the packing of atoms in the non-polar region of the bilayer [181]. This can be done by calculating the radial distribution function (RDF) or the number of neighbors (NS). The RDF of carbon atoms relative to each other (C–C RDF) is given by:

$$\mathrm{RDF} = \frac{V}{N}\left\langle \frac{n(r)}{4\pi r^2 dr} \right\rangle, \tag{3}$$

where $n(r)$ is the number of particles β in the spherical ring of radius r and width dr around the particle α, $4\pi r^2 dr$ is the ring volume; $\langle\ \rangle$ denotes the time and ensemble average. A C–C RDF for carbon atoms of the PC acyl chains in the DMPC bilayer is shown in Fig. 13. Its shape is very similar for DMPC, DPPC, POPC, DOPC, and sphingolipid bilayers, and thus there is reason to consider it to be typical for lipid bilayers in general. The typical features of the C–C RDF are the two maxima at ~0.5 and ~0.9 nm, and a minimum at 0.7 nm. The distance of 0.5 nm is close to the sum of the van der Waals radii of two CH_2 groups in the all atom parameterization, so the presence of the maximum indicates close contacts between the CH_2 groups in the bilayer core.

To obtain detailed information about the effect of cholesterol on atom packing in the bilayer core, the RDF of all carbon atoms in the core relative to a selected atom or a group of atoms has been calculated. The RDF of the carbon atoms relative to a cholesterol atom (pairs of atoms belonging to the same molecule were omitted when calculating RDF) (shown in Fig. 13) significantly differs from that of the acyl chains' carbon atoms relative to each other. The height of the first maximum is clearly reduced, indicating that the chain–Chol van der Waals interactions are weaker than chain–chain ones. Using the same approach, we calculated the RDFs of the carbon atoms for each atom of the cholesterol ring separately and, then, decomposed it into two components; the first calculated for the atoms located on the α-face side of the ring and the second for the atoms located on the β-face side. The two components of the RDF obtained for atom C8 are shown in Fig. 13. As Fig. 13 shows, packing of the chains' atoms on the Chol α-face side is regular and tight, while that on the β-face is less regular and less tight — that is evidently due to the two methyl groups protruding from the cholesterol β-face [102]. Detailed analysis of the RDFs showed that their shape is very sensitive to the position of the atom along the ring, its chemical character, and its position along the bilayer normal [102]. Furthermore, recent studies of cholesterol analogues [182] and membrane peptides [183] in the bilayer indicate that packing of atoms in the bilayer core is very sensitive to the structural details of the inserted molecule and, in many cases, is the key to understanding the global properties of the whole bilayer.

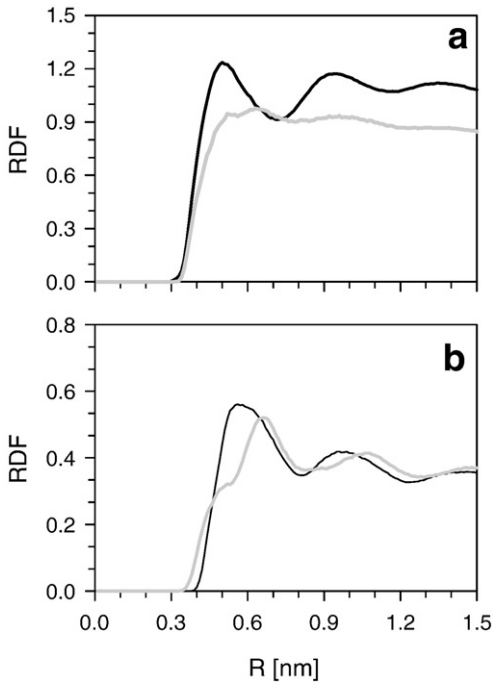

Fig. 13. Three-dimensional radial distribution functions (RDF) of (a) the carbon atoms in the bilayer core relative to a carbon atom of the DMPC acyl chain (black line) and Chol ring (gray line). (b) RDF of the carbon atoms in the bilayer core relative to CH group C8 α (black line) and β (gray line) components.

To evaluate the packing of atoms in a more quantitative way, the number of neighbors for selected atoms or groups of atoms has been evaluated. A neighbor is an atom located within a shell of 0.7 nm radius (which is the position of the first minimum in the C–C RDF) around a selected atom in the bilayer core and belonging to a different molecule. We found its average value (averaged over all acyl chain atoms) to increase from 38.82±0.05 in the DMPC bilayer to 40.15±0.05 in the DMPC-Chol bilayer. A better insight into the membrane structure is provided by a profile of the number of neighbors for the carbon atoms along an acyl chain (Fig. 14). The comparison between pure PC and mixed PC-Chol bilayers shows a substantial increase in the number of neighbors in the chain fragment, which penetrates to the same depth as the cholesterol ring. The neighbor analysis for PC chain atoms on the α- and β-face sides of cholesterol indicates that the packing of atoms on the α-face is similar to that of the PC chains relative to each other (similar values for a cholesterol α-face and an acyl chain). In general, an increase of packing originates from interactions between chains, not between chains and the ring of cholesterol. The C–C RDF analysis above shows that the PC acyl chains surround cholesterol molecules in the bilayer core. This was convincingly illustrated by Pitman et al. [184]. Their analysis indicates at least three shells of chains around a Chol molecule as well as zones of denser packing on both faces. Their bilayer contained, however, PCs with mixed chains (saturated and polyunsaturated). Our recent unpublished results are in line with those of Pitman et al. [184] and also show irregularly shaped shells of PC chains. This implies that the cholesterol β-face should in fact be considered as two separate surfaces, and thus the shape of the cross-section of a cholesterol molecule in the bilayer plane can be approximated by a triangle.

3. Effects of modification of cholesterol structure

Although cholesterol is a member of a larger sterol family, only cholesterol and the structurally similar ergosterol appear in larger quantities — cholesterol in animal and ergosterol in fungal membranes [185]. Not surprisingly, the diversity of possible sterol structures and the high selectivity have attracted considerable experimental and theoretical interest, as well as speculations of the biological selection process [4,5,29,66,186,187]. Modification of its basic structural elements (see Introduction) decreases cholesterol's ability to modify membrane properties [188] and has direct biological effects such as decrease of cell viability [189,190]. As far as a sterol's ability to modify the physical properties of membranes is concerned, only ergosterol has been found to be more or equally effective to cholesterol [20,43,191]. In most studies, sterols originating from natural sources were used. More recently, however, cholesterol stereoisomer (ent-cholesterol) was synthesized and shown to be less effective than cholesterol [192,193]. Among the sterols studied in the context of their membrane properties are the sterols from the cholesterol biosynthetic pathways [66,194], cholesterol polar esters [195], oxygenated sterols [196,197], plant sterols with various tail modifications [198–200], as well as some others [201–203]. Another interesting aspect are properties of the fluorescent and spin labeled steroids that are used to study sterol behavior in membranes [105,127,128,204], namely, how well do they mimic cholesterol?

In this section, we discuss studies of membrane properties of cholesterol analogues. The chemical structures of the discussed sterols are shown in Fig. 2.

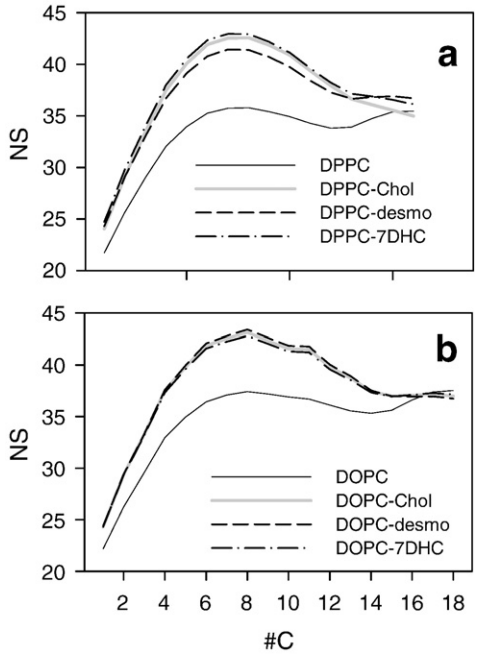

Fig. 14. Profiles of the number of neighbors (NS) along (a) DPPC and (b) DOPC *sn*-1 chain in PC (black line), PC-Cholesterol (gray line), PC-Desmosterol (dashed line), and PC-7-dehydrocholesterol (dashed–dot line) bilayers.

3.1. Cholesterol precursors

The cholesterol biosynthetic pathway consists of two possible routes, both starting from lanosterol, the first sterol with a steroid ring system, and ending with two direct precursors, desmosterol with an additional double bond in the isooctyl chain (cholesterol tail) between atoms C24–C25, or 7-dehydrocholesterol with a double bond in the ring system between atoms C7–C8. All together, there are 19 enzymatic reactions and 12 identified intermediates between lanosterol and cholesterol.

Lanosterol differs from cholesterol by having 3 methyl groups, the position of the double bond in the ring system being different, and there is an additional double bond at the end of the hydrocarbon chain (see Fig. 2). In one of the very first MD studies of sterols' effects on lipid bilayers, Smondyrev and Berkowitz found that at low concentrations cholesterol and lanosterol have similar ordering effects but have different tilt angles [205]. In later studies, Cournia et al. [206] showed that packing next to the lanosterol ring is not as tight as in the case of cholesterol, thus identifying a possible reason for the larger sterol tilt and lower ordering ability of lanosterol, both of which have been experimentally observed [20,207,208]. Interestingly, at the phase diagram of lanosterol-phospholipids no stable region of coexisting Ld–Lo phases (liquid disordered–liquid ordered), typical for most of the sterols, was observed [209,210].

Desmosterol is the direct precursor of cholesterol on the so-called Bloch pathway, and it differs from cholesterol only by one double bond in the sterol tail (see Fig. 2). Desmosterol was found as a minor component in cell membranes, with the exception of LM cells, astrocytes and spermatozoa where its amount is elevated [211–213]. Desmosterol is also of medical interest since there are several rare and serious disorders including desmosterolosis, a rare humane disorder caused by the body's inability to transform desmosterol into cholesterol [214]. From the biophysical point of view, experimental and theoretical studies express a clear picture: desmosterol influences a saturated bilayer less than cholesterol does [66,215], while their effects on unsaturated bilayers are almost identical [47,194,216,217]. Recent simulations provide an explanation for the experimental observations: in saturated bilayers one observed lower ordering due to the larger tilt angle of the desmosterol ring, which in turn is related to changes of packing relative to the desmosterol tail region [47,66,194]. One also observed tighter packing at the end of the tails and looser packing in its beginning. In unsaturated bilayers, packing in the center of a bilayer is higher than in saturated bilayers, thus packing relative to the tail end is not altered any further [194]. Fig. 15 shows

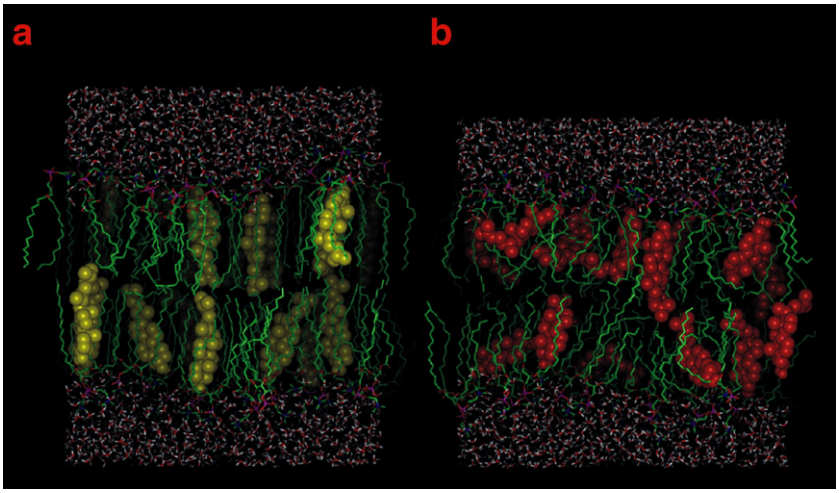

Fig. 15. Snapshot of (a) DPPC-Cholesterol and (b) DPPC-Desmosterol bilayers. Cholesterol is shown in yellow and desmosterol in red.

DPPC-Chol and DPPC-Desmosterol bilayers and visually demonstrates the difference in tilts of the two sterols.

7-Dehydrocholesterol, the direct precursor of cholesterol along the Kandutsch–Russel pathway, has an additional double bond in its ring system (see Fig. 2). Similarly to desmosterol it is found in elevated quantities in specialized cells like rat epididymis [218] and in patients with rare disorders (such as the Smith–Lemli–Opitz syndrome) where the conversion of 7-dehydrocholesterol to cholesterol is blocked or less efficient [214]. MD simulations showed that in both saturated and unsaturated bilayers 7-dehydrocholesterol has a lesser effect on membrane order and condensation than cholesterol. The difference between two sterols is, however, small [194]. As in the case of desmosterol, this difference is associated with larger tilt of the 7-dehydrocholesterol ring than that of cholesterol. Change of the tilt is associated with looser packing next to the modified part of the ring. In contrast to desmosterol, experimental data concerning the effect of 7-dehydrocholesterol on membrane properties are conflicting. Results of simulations are in line with monolayer studies on saturated and unsaturated PCs, which showed less condensation when 7-dehydrocholesterol was added instead of cholesterol [219,220]. It has also been shown that cholesterol increases membrane rigidity more than 7-dehydrocholesterol does [221] but fluorescence studies showed that relative effects of both sterols vary and depend on sterol concentration and temperature [222,216]. For the raft forming systems, fluorescence studies have shown that 7-dehydrocholesterol has a higher [223] or equal [224] ability to form domains compared to cholesterol. Spin label methods [225] and antibody based assay studies [226] indicate the opposite, however. These ambiguous results can be partly explained by the sensitivity of 7-dehydrocholesterol to oxidation related to the conjugated double bond structure of its ring that is not shared by either cholesterol or desmosterol.

3.2. Ergosterol

Ergosterol is the main sterol in fungi as well as in some protozoa and insects [185,227]. It differs from cholesterol by two additional double bonds of which the first one is in the ring system between C7 and C8, and the second one in its tail between atoms C22–C23. There is also an additional methyl group in the tail attached to C24 (see Fig. 2). Although ergosterol is a common and important sterol, its effects on a lipid bilayer are not well understood. In the few existing studies it has been shown both experimentally and computationally that ergosterol's ordering effect is greater than that of cholesterol's [20,191,207,228]. The essential difference between the behaviors of the two sterols has been associated to the ergosterol tilt angle and the higher stiffness of the ergosterol's tail [228,229].

3.3. Polar modifications

The cholesterol headgroup is comparatively small, as it is a hydroxyl group in the β conformation (located on the β-face). The importance of this part of a sterol molecule has been shown in numerous experimental studies and a few MD simulations including studies of cholesterol sulphate [230], epicholesterol [231], and ketosterone [89].

Cholesterol sulphate, ester of sulphuric acid and cholesterol, is a natural sterol, which can be found in sperm acrosome membranes [232] and erythrocyte membranes [233]. Strott and Higashi [195] provide a review of its physiological role. In both experimental [234,235] and theoretical studies [230] it has been observed that cholesterol sulphate affects lipid bilayer less than cholesterol. In contrast to cholesterol, the cholesterol sulphate headgroup is large, charged and fully hydrated thus effectively acts as a spacer between PC molecules [230]. Similar mechanism of decreasing sterol induced effects can be postulated for other sterols with large and bulky headgroups such as glycosylated sterols, which have been shown to be less effective than cholesterol in ordering acyl chains [236].

Epicholesterol is the epimeric form of cholesterol with its hydroxyl group in α conformation. Although epicholesterol is structurally similar to cholesterol, it is practically absent in nature. Experimental studies have shown that epicholesterol's effects on membrane properties are weaker in terms of the ordering effect [237] and reduction of membrane permeability [238]. In line with experiments, modeling studies have shown weaker ordering and condensing effects [231], and that at the molecular level epicholesterol's hydroxyl group protrudes more into the water phase. Due to this, epicholesterol is more prone to form hydrogen bonds with phosphate groups of PCs. The reason for that behavior is likely the different molecular shape of epicholesterol, which is less commensurate with the membrane environment and hence is being pushed upwards [231].

Ketosterone is an artificial steroid with the hydroxyl group substituted with a ketone group. The 3-ketone group is typical for steroid hormones but is not present in sterols. The presence of 3-ketone group was, however, observed in metabolism of membrane sterols where it is quickly reduced to hydroxyl [239,240]. Extensive simulations of ketosterone in lipid bilayers [89] showed, for the first time, rapid flip-flops between the bilayer leaflets. For comparison, flip-flops of cholesterol have never been reported in atomistic simulations. An example of a ketosterone molecule involved in a flip-flop process is shown in Fig. 16. The reason for ketosterone's ability to undergo flip-flops in very short time scales is that the ketone group has weaker polar interactions, it is not able to hydrogen bond with PC, it forms less charge pairs, and it prefers to be located deeper inside the membrane; all of these properties lead to weaker ordering and condensing effects demonstrated by a large tilt angle [89]. Biologically,

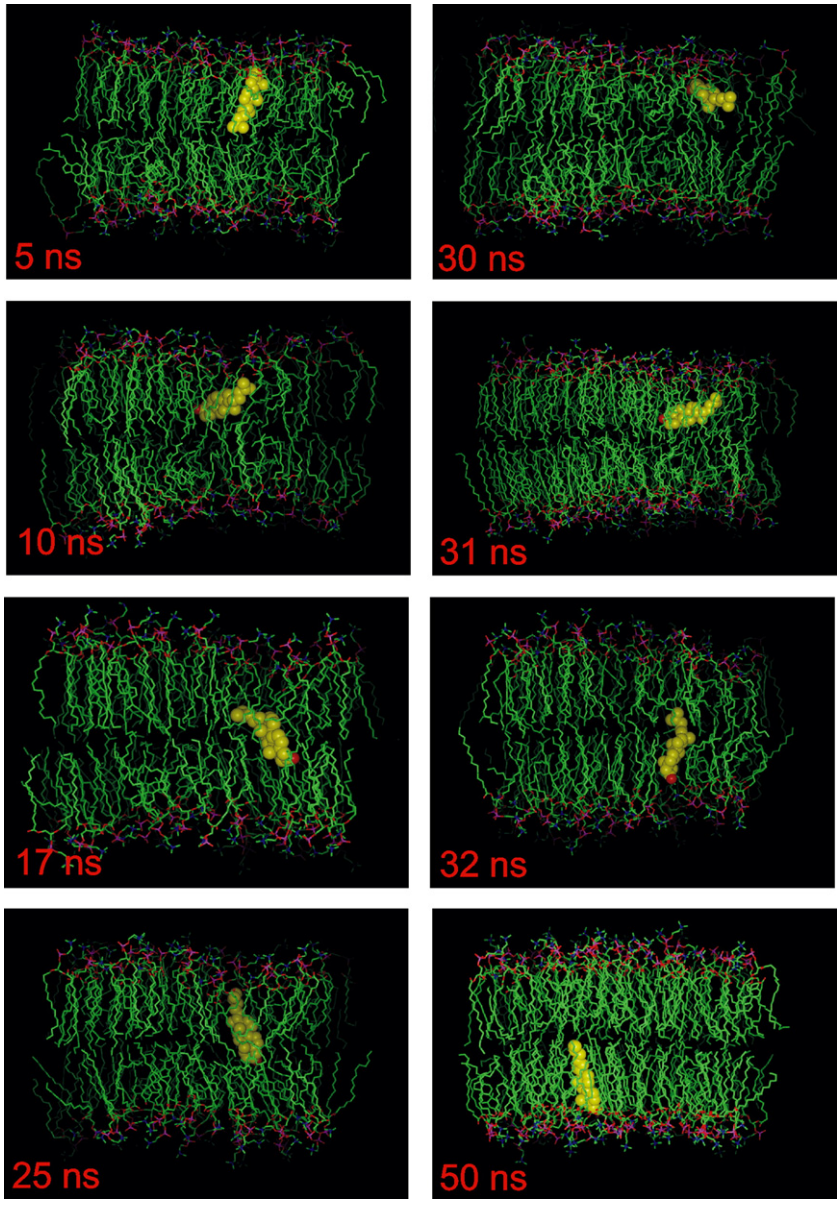

Fig. 16. Snapshots of a ketosterone molecule during a flip-flop process [89].

the results indicate that the presence of the ketone group can facilitate hormone diffusion through lipid bilayers as indicated also by free energy calculations of cholesterol and testosterone [241].

Another polar modification of the cholesterol molecule studied by MD simulations is cholesterol oxygenation [242]. Smondyrev and Berkowitz showed that the presence of an additional ketone group at position 6 affects the sterol's position in the bilayer — it is moved more towards the water phase allowing for better hydration of the additional ketone group. This decreases membrane order and condensation [242].

The final example of polar modification is cholate — a sterol molecule with two additional hydroxyl groups at positions 7 and 12, and with a carboxyl group at the end of the tail. Due to this extensive modification cholates in a mixture with phospholipids lead to formation of micelles with the cholates located at the interface as has been shown in extensive simulations of Marrink and Mark [243].

3.4. Cholesterol methyl groups

One of the intriguing features of cholesterol is the presence of methyl groups at the ring system. It is tempting to imagine that the removal of those groups would improve the ordering capability of cholesterol. Such conclusion could be drawn from the biosynthetic pathway of cholesterol — in the process of transforming lanosterol to cholesterol three methyl groups are removed (2 from the α-face and 1 from the β-face). The biosynthetic pathway of cholesterol is believed to reflect evolutionary optimization of cholesterol molecule [5,186,187] and is clearly associated with an increasing ordering capability towards the end of the pathway [20,207,208].

The first studies of the ordering effect of cholesterol clearly showed that in saturated lipid bilayers, the α-face orders neighboring lipids more than the β-face [118] and that the packing of hydrocarbon chain atoms next to the α-face is tighter than next to the β-face [102]. All this supports the idea that cholesterol's smoothness is important for its ordering capability. Following this idea, one has performed simulations of modified cholesterol with methyl groups C19 and C18 removed (Dcholesterol) [88]. Fig. 17 shows three-dimensional structures of lanosterol, cholesterol and Dcholesterol. Surprisingly, one has found that such a sterol has weaker effects on membrane order and condensation than cholesterol. That was related to an increase of 6° in the sterol ring tilt angle. Then one has constructed a set of 5 new

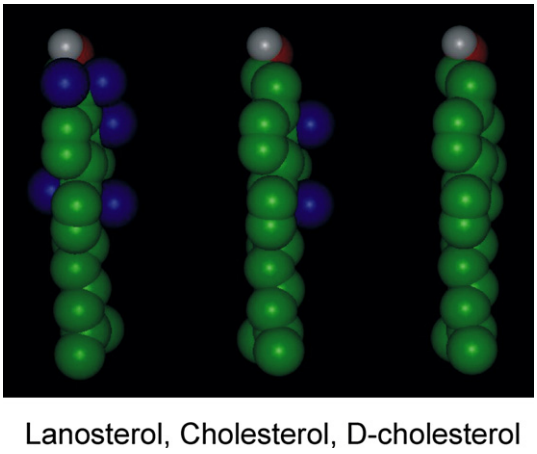

Fig. 17. Three-dimensional structures of lanosterol, cholesterol and Dcholesterol [86].

sterols with selectively removed methyl groups from the ring and from the beginning of the tail: sterol1 with removed C19, sterol2 with removed C18, sterol3 with removed C21, sterol4 with removed C19 and C21, sterol5 with removed C19 and C21 as well as with a single bond C5–C6 instead of the double bond [182]. Chemical structures of these sterols are shown in Fig. 18. Sterols with removed either C19 or C21 methyl groups were almost equivalent to cholesterol, while removing the methyl group C18 or two methyl groups at the same time were strongly affecting the sterol's ordering properties. In all cases, an increase of the sterol tilt was observed, the change varying between one and six degrees.

These results show that the smoothness of the sterol ring can compensate for the increase of the tilt and is one of the factors affecting a sterol's ordering abilities, but it is a less important one. An interesting problem would be to clarify which interactions actually affect sterol tilt. At present, our conclusion is that change of the balance of interactions between the sterol α- and β-faces and surrounding lipids mostly affects the sterol ring tilt. For each modified sterol it was observed redistribution of interactions from the α-face to the β-face, which can be driven by hydrogen bonding between the OH group located on the β-face and the PC carbonyl groups. This emphasizes once more the importance of the hydroxyl group conformations. Modifications of the sterol chemical structure also have a weak effect on ring structure, although that seems to be a less important factor [182]. The simulation results have also shown that the methyl groups are important structural elements and cannot be considered as evolutionary fossils. This is illustrated by the fact that none of the known sterols lacks the methyl group C18 that, in the light of our results, is crucial for the molecule to maintain a proper tilt. Interestingly, there are some sterols in marine invertebrates, which do not have C19, yet C18 is always present [244].

3.5. Sterol tilt, key parameter

Most of the work performed on various sterols has shown that their ordering and condensing abilities correlate with a single parameter — the tilt of the sterol ring. The smaller the sterol tilt, the more ordered and condensed the bilayer is [47,194]. The important role of cholesterol location and orientation in the bilayer was also shown via free energy calculations [245]. At the level of individual molecules, the tilt of a sterol molecule correlates with the order of the neighboring PC chains (see Fig. 19). In Table 1 we have gathered data from all the sterols used in our simulations — all simulations have been performed at the same temperature and using the same methodology, and thus they can be treated at an equal footing. Tilt of the sterol ring depends on all interactions between the sterols and other lipids in the bilayer. In our MD simulations we have been able to identify which interactions are responsible for tilt modulations in each particular case; e.g., due to changes in the polar part which decreases the strength of hydrogen bonding and charge pairing (ketosterone) or changes in packing in the cases of ring and tail modifications. Analysis of packing has been particularly successful in explaining the reason for changes in tilt. We have shown that redistribution of packing between the sterol ring faces, and those changes in the relative balances between them can strongly affect the tilt angle [88,182].

4. Effects of matrix lipid structure

In the previous section we concentrated mainly on saturated phosphatidylcholines. Biological membranes are, however, much more complex as they can be composed of about 100–1000 different lipid species, each having a certain function. Some of the lipid groups are typical for specific membranes, e.g., cardiolipins are located mainly in mitochondria and thus have virtually no contact with cholesterol [246]. Unsaturated PCs are, however, probably the most common lipids in nature. And, as mentioned in the Introduction, together with sphingolipids and cholesterol they are one of the three main components of the outer leaflet of eukaryotic cellular membranes. Unlike saturated lipids, the unsaturated ones have at least one double bond along at least one of their chains. Here, we focus on monounsaturated lipids, i.e., lipids having only one double bond.

4.1. Unsaturated lipids

Over the last eight years, we have performed a series of computational studies on the effect of cholesterol on saturated and unsaturated lipids. As a clear trend, the fully saturated DPPCs have the strongest interactions with cholesterol. Furthermore, in perfect agreement with experimental studies, we have found that the effect of cholesterol on a monounsaturated POPC bilayer is weaker than on a fully saturated DMPC bilayer even though the DMPC chains are shorter [247]. This is also similar in the case of di-unsaturated DOPCs on which the effect is weaker than on fully saturated DSPCs (in this case the chains are of equal length) [44]. A straightforward analysis showed immediately that a lesser degree of membrane condensation and a lower degree of ordering was induced by cholesterol in membranes consisting of unsaturated lipids (see Figs. 5, 14, 19 and Table 1). A more detailed analysis of the effects of the cholesterol α- and β-faces on chain ordering also showed that POPC chains behave very differently from the DMPC ones; the α-face induced less order on the unsaturated chains than the β-face did [247]. This probably is of importance in

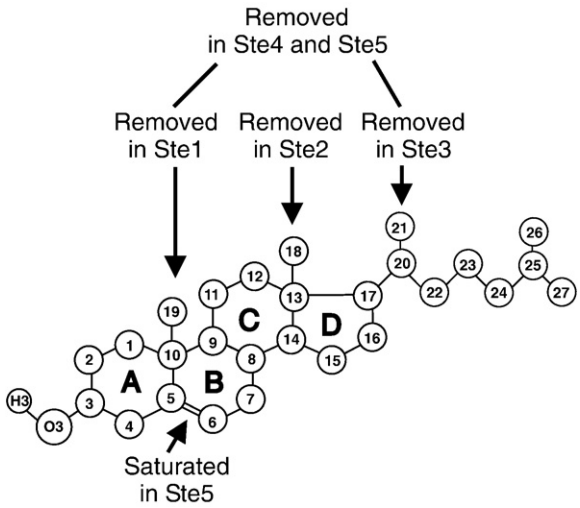

Fig. 18. Chemical structure of cholesterol molecule and its demethylated derivatives studied in [180].

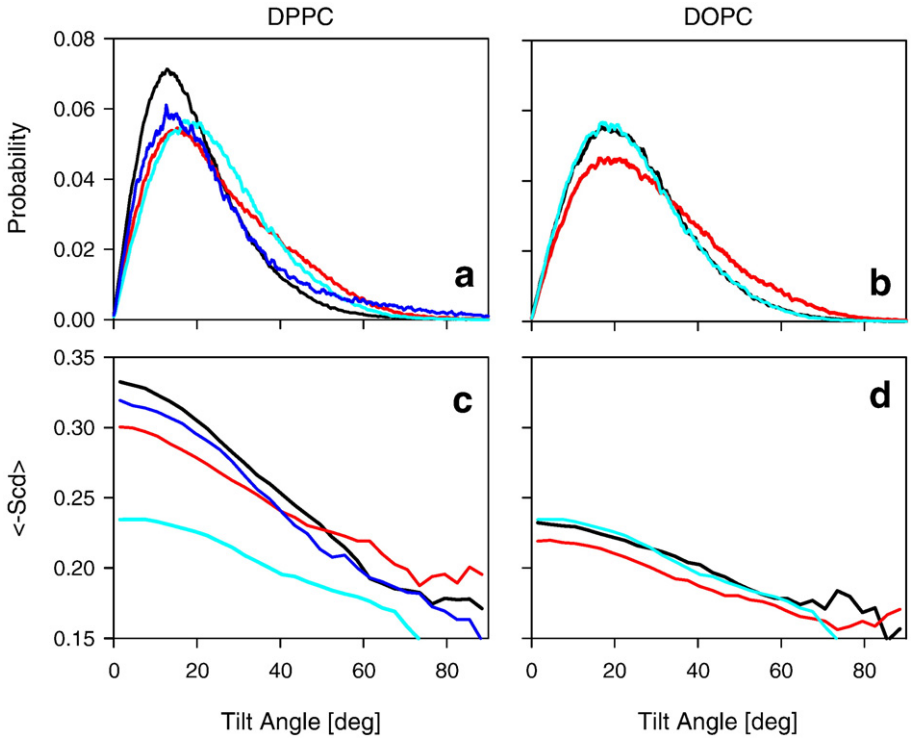

Fig. 19. Sterol tilt angle distributions in (a) DPPC and (b) DOPC bilayers. Panels (c) for DPPC and (d) for DOPC illustrate the correlation between the instantaneous tilt angle of a sterol and the corresponding average molecular order parameter $<-S_{cd}>$ of *sn*-1 chains neighboring the sterol ring. Color scheme: cholesterol — black, desmosterol — light-blue, ketosterone — dark-blue, dchol — red, sterol free systems — green.

higher cholesterol concentrations where the POPC *sn*-1 chain is equally ordered as the DPPC chains, and the POPC *sn*-2 chain is more ordered than the DOPC *sn*-2 chain as has been shown by Pandit et al. [248].

As already discussed in Section 3.5, the tilt angle is a good characteristic of a sterol's ability to induce order in a membrane [66,47] and it is also an experimentally accessible quantity. Consistently with weaker ordering, we have found that the tilt angle of the cholesterol ring system with respect to the membrane normal was significantly larger in POPC bilayers (33°) in comparison with the DMPC systems (17°) [118]. In the case of DPPC and DOPC matrices, the angles were 20° and 24°, respectively [47]. The results presented in Table 1 strongly suggest that the unsaturated bilayers are less sensitive to sterol structure compared to the saturated ones [47,194].

The most common unsaturated phospholipids, such as POPC or DOPC, have a double bond located at the middle of (at least) one of the acyl chains. This specificity about the location of the double bond has attracted relatively little attention, yet its abundance indicates that nature has preferred that location to all the others. Calorimetric studies have shown that the temperature of the main phase transition is the lowest for a PC having a double bond located in the middle of a chain [249,250]. To assess the above quantitatively, we have performed a study on a series of PCs with the double bond moved systematically along the chain. We studied both pure PC systems (as control systems) and PC membranes with cholesterol added in them. The results in pure PC membranes showed a non-monotonic behavior of the surface area and chain order when the double bond was moved from the beginning to the end of the chain [251]. The maximum surface area per lipid was observed for the double bond at the 9th position, and it decreased about 0.02 nm^2 when the bond was moved to the beginning of the chain. When the bond was translated to the end of the chain, the area decreased even more by 0.05 nm^2. This tendency became much stronger when cholesterol was added: the condensing and ordering effects were the weakest for the chains with the double bond located in the bilayer center [44,119]. We found that this effect is correlated with co-localization of the double bond and the methyl groups of the cholesterol ring.

In a separate study, Zhu et al. [252] used 200 ns simulations to analyze the effect of different starting configurations on the properties of a membrane comprised of POPCs with 40 mol% of cholesterol. They started from two different initial conditions — in the first one cholesterol and POPC molecules were distributed in the membrane plane according to the superlattice model [76], whereas in the second case the molecules were distributed randomly. Even after such long simulations they found the two simulated systems to be different. For instance, the area per molecule differs more than 0.01 nm^2, much more than the error range. Furthermore, some other properties showed a small systematic drift over the whole simulation period. It should be kept in mind, however, that the high cholesterol

Table 1
Average surface area, order parameter Scd, and sterol tilt in DPPC and DOPC based bilayers

Sterol	DPPC			DOPC		
	Area [nm^2]	Scd	Sterol tilt [°]	Area [nm^2]	Scd	Sterol tilt [°]
None	0.66	0.28	–	0.69	0.27	–
Cholesterol	0.60	0.55	19.7	0.65	0.42	24.7
Desmosterol	0.65	0.45	26.9	0.65	0.42	24.9
7-Dehydrocholesterol	0.62	0.51	21.9	0.66	0.41	25.8
Dcholesterol	0.62	0.50	25.3	0.67	0.32	28.5
Ste1	0.60	0.54	22.1	–	–	–
Ste2	0.64	0.48	26.0	–	–	–
Ste3	0.60	0.54	20.7	–	–	–
Ste4	0.62	0.50	24.3	–	–	–
Ste5	0.61	0.54	23.0	–	–	–
Ketosterone	0.63	0.51	28.1	–	–	–

Surface area in mixed systems is obtained by dividing the total area by the number of PC molecules without extracting sterol area to avoid arbitrary definitions as discussed in Section 2.3. For our purposes, this suffices to evaluate the sterols' condensing strength. The numbers of the PC and sterol molecules are the same in all systems.

concentration of 40 mol% affects the diffusion of the lipids quite strongly [136,253,254], thus slowing down the lateral dynamics of the system. In DPPC simulations the difference in lateral diffusion rates between the pure DPPC bilayer and the same system with 40% cholesterol was as large as a factor of six, and one should keep in mind that the neighbor exchange inside the lipid bilayer is even slower [90].

In the above discussion we have considered only lipids with monounsaturated chains (DOPC and POPC). A large portion of lipids, however, are polyunsaturated with the *sn*-1 chain being saturated and the *sn*-2 chain being polyunsaturated (such as docosahexaenoic acid with 6 double bonds). From the point of view of computer simulations, relatively little work has been done on this type of lipids [62,184,255], and even less on their mixtures with cholesterol. In an early study Pitman et al. [184] used membranes composed of SDPC and showed that cholesterol interacts preferentially with saturated chains. Recently, Marrink et al. [255] performed simulations using coarse-grained models of DAPC (diarachidonylphosphatidylcholine), SAPC (1-stearoyl-2-arachidonyl-phosphatidylcholine) and POPC bilayers with cholesterol. They showed a high rate of flip-flop motions of cholesterol in the polyunsaturated DAPC bilayer, and a limited number of flip-flops in the POPC bilayer. They also observed through coarse-grained simulations a high fraction of cholesterol molecules located in the DAPC bilayer interior oriented parallel to the membrane surface, though atomistic simulations were not able to confirm this. The unusual orientation of cholesterol was previously reported experimentally [256].

4.2. Sphingolipids

Sphingolipids, the second major lipid class in the outer cellular membrane, differs structurally from the PCs in its backbone region. PC is based on a glycerol backbone while SM has a sphingosine backbone (Fig. 3) and has various possible modifications of the basic structure [41,257]. The most typical sphingolipid, sphingomyelin, has a phosphatidylcholine headgroup, but various glycolipid headgroups are also possible [258,259]. The most important difference between the SM and PC backbone regions is the presence of hydrogen bonding donor groups (hydroxyl and amide) in SM. PCs cannot act as donors, but as acceptors only. The second important difference is the level of unsaturation. PCs are predominately unsaturated while SM chains are usually saturated. In the sphingosine structure there is usually one *trans* double bond between C4–C5. If the C4=C5 bond on SM (cf. Fig. 1b) is not considered, the occurrence of *cis* double bonds in PC chains is 5–10 times higher than in SM chains [41]. If a *cis* double bond occurs in the acyl chain of SM, it is usually located further away from the headgroup than is the case in a PC [41], and its effect on the properties of a SM bilayer is small [260]. In SM and PC, the chains are commonly 16–24 and 16–18 carbon atoms long, respectively. The chain length, the level of unsaturation, and the possibility for H-bonding as a donor are believed to be the most important factors in explaining why cholesterol interacts preferentially with SMs as compared to PCs [41].

The obvious first question that needs to be quantified is if cholesterol can form hydrogen bonds with SM. The ability of SMs to form H-bonds has been postulated as being one of the driving forces in raft formation [48]. To study the differences between PCs and SMs, we have performed MD simulations of SM containing membranes for two concentrations of cholesterol, 20 and 50 mol% [261]. We observed numerous H-bonds, 0.91 and 0.64 per cholesterol, respectively. In the case of PCs mixed with cholesterol (20 mol%) the number was significantly lower, 0.28. When the temperature was raised from the physiological temperature of 310 K to 333 K, we observed a reduction of H-bonds from 0.91 to 0.78 at 20 mol% concentration [261]. Khelashvili et al. [262] observed 0.57 H-bonds at cholesterol concentration of 31 mol%. The above difference originates from the bonding of cholesterols with the phosphate oxygens. That was observed more often in our studies [261], which can result from the shorter SM chain (16 versus 18). Small differences in hydrogen bonding patterns were observed depending on cholesterol concentration and temperature, but in both studies all SM polar groups located in the linking region, i.e., (equivalent to glycerol region) OC, OH and NH, participated in H-bonding with cholesterol. We also observed a significant number of charge pairs between cholesterol and SM [261]. Schematic representation of cholesterol interfacial interactions with SM compared to DMPC and DPPC is shown in Fig. 12. An example of the hydrogen bonded pairs of cholesterol and SM molecules are shown Fig. 20.

Interestingly, in a dilute mixture of cholesterol and SM in a POPC matrix, we observed a completely different picture [263]. The number of H-bonds with cholesterol was negligible, but charge pairs were numerous and there was a clear preference for cholesterol to form charge pairs with SM over POPC. This difference seems be related with the structural differences between PCs and SMs: the SM headgroup is

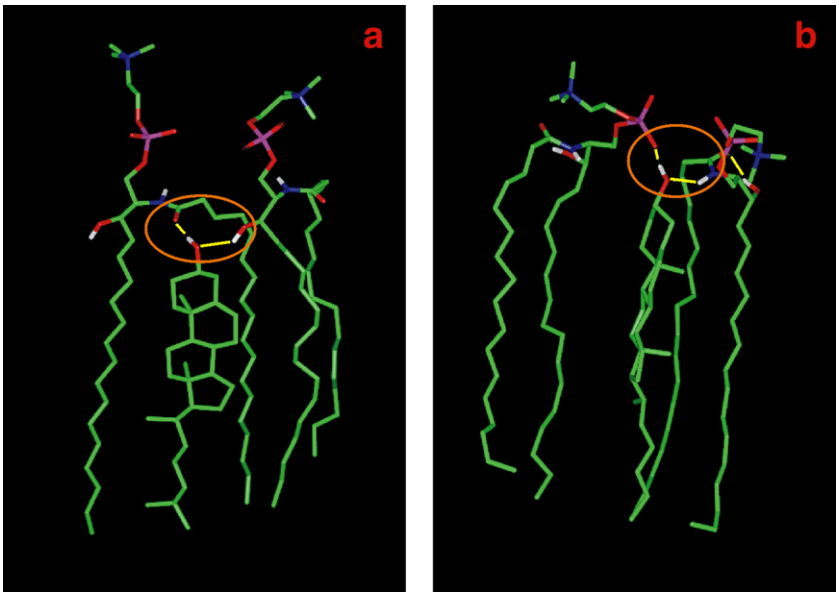

Fig. 20. Examples of H-bonds between the OH-Chol group of one Chol molecule and two SM molecules (thin yellow lines). (a) OH-Chol···SM-OH and OH-Chol···SM-OC H-bonds; (b) OH-Chol···SM-NH and OH-Chol···SM-OP H-bonds in the SM-Chol20 bilayer [258].

more flexible which makes it easier for it to bend and to form charge pairs with cholesterol molecules located deeper inside the bilayer. Importantly, these studies show that observations from binary systems cannot be simply extrapolated to more complicated systems [263]. The bonding patterns observed in our binary systems can be indicative to raft systems composed mainly of cholesterol and SM, but they do not provide insight into raft formation in a ternary mixture.

The above observations suggest that not only particular interactions such as hydrogen bonds are of importance but also the overall shape and elasticity of the molecule should be accounted for. Similar conclusion can be drawn from experimental studies of SMs with various linkage groups between the phosphate and sphingosine groups [264]. This particular linkage is not a major target of hydrogen bonds for cholesterol but it nevertheless has an influence on domain properties.

Rafts have been for a while, and remain to be, one of the most discussed topics in molecular biology see e.g., [46,48–50] and references therein. On the computational side they present a formidable challenge as the requirements even for a minimal system are demanding: the systems should be preferably (at least) ternary, the number of lipids should be at least thousands, and the time scales should be at least of the order of 100 ns. To-date, there are two extensive simulations of lipid rafts. In the first one, Pandit et al. [265] performed an extensive 200 ns simulation of a raft system using an equimolar mixture of DOPC, SM and cholesterol. Despite its extraordinary length, the time scale of the simulation turned out to be insufficient to study the actual raft formation process although they managed to observe the initial stages. Interestingly, cholesterol showed preferences to be located at the domain border with its α-face oriented towards SM and the β-face towards DOPC. This is in excellent agreement with previously described results showing higher ordering of saturated chains at the α-face [118] and unsaturated at the β-face of cholesterol [247]. In another extensive simulation, Niemelä et al. [91] performed 100 ns simulations of three different POPC-cholesterol-SM systems comprised of more than 1000 lipids. Like Pandit et al., they were not able to observe the actual raft formation but they focused on showing how the elastic and dynamic properties depend on the composition and how the raft composition exerts a distinct lateral pressure on membrane proteins such as the mechanosensitve MscL channel. A different approach to study rafts was taken by Zhang et al. [266,267]. They considered interaction energies between cholesterols, SMs and POPCs. They found little differences between the energies of interactions of cholesterol with various neighbors [266]. Calculations of the free energy showed favorable changes in lipid–lipid interactions near cholesterol molecules but not in actual energies of cholesterol–lipid interactions [267]. This is in line with our previous work, which showed that an increase in packing, and thus in the van deer Waals interactions is due to interactions between the acyl chains and not between chains and cholesterol [181].

Using an asymmetric setup, Bhide et al. [268] performed studies of asymmetric bilayers with one leaflet composed of SM and cholesterol, and the second one of phosphatidylserine and cholesterol. Such a choice of lipids was due to the asymmetric composition of cell membranes: in the outer leaflet the majority of lipids are SMs and PCs while in the inner one they are mostly PSs and PEs [269]. Due to this, in biological membranes rafts rather exist in the extracellular layer and the problem of what is in the intracellular layer remains unsolved. The authors compared results from asymmetric systems to those from analogous symmetric systems and found only small differences between them. They found that the hydrogen bonding network in the SM-Chol system is more extended than in the PS-Chol system even though the average number of H-bonds was observed to be similar. This suggests that SM promotes the creation of domains much more strongly than PSs [268].

Although not specific to SM, we would like to mention yet a different computational approach by Fan et al. [86]. Instead of having actual molecules as in a MD simulation, they devised a field theoretical model in terms of the concentrations of the liquid-ordered (raft) and liquid-disordered phases in the spirit of the Ginzburg–Landau formalism. This approach allows studies of the dynamics and domain size distribution not accessible by any other current computational approach. In particular, they were able to show that the domain, or raft, size distribution and lifetimes may be explained in terms of the competition between local interaction with membrane proteins and lipid transport in the membrane.

4.3. Peptides and proteins

Membrane proteins are a large and important group of proteins. Not surprisingly, molecular dynamics simulation studies of membrane proteins are widely described in the literature [270,271]. Taking into account the important role of cholesterol in membranes, it is surprising how little has been done about interaction studies between proteins or peptides and cholesterol [270,271].

Membrane thickness plays an important role in protein and peptide interactions with lipid bilayers due to the so-called hydrophobic mismatch [272–274]. Hydrophobic mismatch is the situation where the hydrophobic profile of the peptide does not match to the hydrophobic thickness of the lipid bilayer. As already discussed above, cholesterol modulates membrane thickness (ordering and condensing effects) and its effect depends on the lipid chain length and the level of unsaturation [150].

In our simulations, we have directly analyzed the effect of hydrophobic mismatch on peptide structure and behavior in lipid bilayers with various thicknesses due to different cholesterol concentrations as well as variations in lipid matrices (unsaturated PC versus sphingolipids) [183]. As a model peptide we have used the *trans*-membrane fragment of the EGF (epithelial growth factor) receptor, which is a single helix spanning the membrane. In MD simulations, we observed two distinct effects: first, the whole peptide was tilted in all membranes to compensate for the hydrophobic mismatch, and second, in thinner POPC membranes the helical structure was locally deformed to make it easier to hide the hydrophobic residues in the membrane core. Snapshots of the POPC and POPC-Chol bilayers with inserted peptides, snapshots of the structure from all studied systems and detailed view of deformed part of the peptide are shown in Fig. 21.

While hydrophobic mismatch can be considered as an unspecific cholesterol effect, in our simulations we also considered the specific interactions between cholesterols and lipids. We found that the peptide interacts preferentially with the chain atoms, and that interactions with cholesterol were not favorable [183]. Similar observations have been made in simulations of rhodopsin in a mixture of cholesterol and lipids with a single polyunsaturated chain [275]. It was shown that polyunsaturated chains prefer to be next to the protein while cholesterol is rather excluded from protein surroundings.

Cholesterol can also influence interactions between peptides and the water–membrane interface. In our preliminary studies on interactions of the antimicrobial peptide magainine-2 with lipid bilayers, we showed large differences in peptide behavior in model bacterial membranes (composed of POPE and POPG in ratio 3:1) and in model eukaryotic membranes (compose of POPC and cholesterol). In a bacterial membrane the peptide preserved its helical structure while in the animal membrane the helical structure was lost due to strong H-bonding between peptides and cholesterol [276]. Snapshots of the POPC-Chol bilayer with magainine are shown in Fig. 22.

Another important effect is the lateral pressure inside the membrane — a topic that has become of increasing interest lately [91,277–282]. As was shown by Lindahl and Edholm [281], the local lateral pressure in a membrane can be of the order of 1000 bar and analysis of the involved forces shows that it can most likely have a

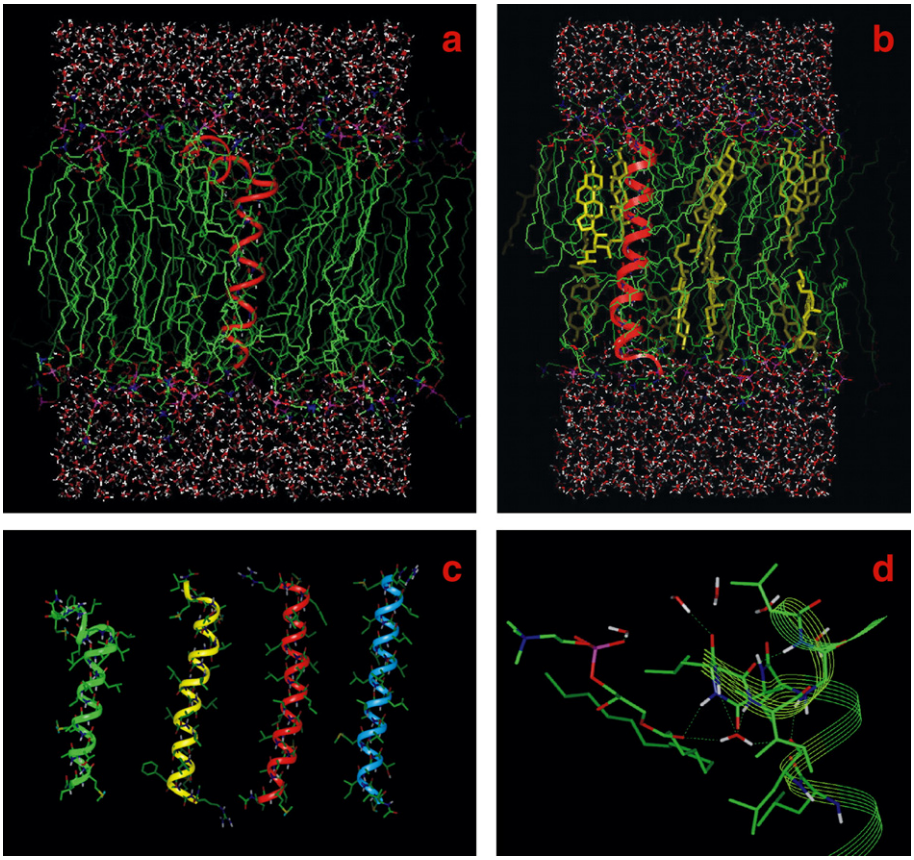

Fig. 21. Snapshots of the structure of (a) POPC and (b) POPC-Chol bilayers with the *trans*-membrane EGF receptor fragment. Color scheme: carbon — green, oxygen — red, hydrogen — white, nitrogen — blue, phosphorus — purple. Chol molecules are shown in yellow. (c) Snapshots of the peptide structure at the end of simulation, green — POPC, yellow — POPC-Chol, red — SM-Chol20, blue — SM-Chol50 bilayer. (d) Snapshot of a peptide fragment which adopts a non-helical structure in POPC bilayer with surrounding lipid and water; hydrogen bonds are marked.

direct impact on protein structure [277–280]. In our simulations we have concentrated on cholesterol and other sterols' effects on the lateral pressure profile in both saturated and unsaturated bilayers [280]. Characteristic features of the pressure profile are the positive peak in the headgroup region, a negative peak between water and the hydrophobic region, and a shallow positive peak in the bilayer center [277]. The presence of cholesterol affects the lateral pressure profile both quantitatively and qualitatively: the amplitude of the peak is doubled and additional peaks appear. Similar effect of cholesterol on a saturated bilayer was previously observed by Patra [282], who studied the lateral pressure as a function of cholesterol concentration.

Finally, the effect of other sterols on lateral pressure profile is similar to cholesterol; desmosterol, 7-dehydrocholesterol and ketosterol have similar qualitative effects but the quantitative changes are distinctly clear [280].

4.4. Other bilayer environments

In addition to the much studied bilayers with cholesterol, SM and PC, there are a few studies with less typical lipid matrices. In one of them, Höltje et al. [283] performed simulations of fatty acids mixed with cholesterol to mimic the behavior of *stratum corneum* — the outermost layer of human skin. They used a mixture of cholesterol, palmitic acid, and stearic acid organized into a bilayer structure showing a highly ordered structure at body temperature [283]. From the methodological point of view important applications of the MD simulations include investigations of the molecular probes used in fluorescence [59,60] and EPR [61,284] spectroscopies. These studies have provided information about the degree of membrane perturbations, probe locations and the dynamics, and can be useful in direct interpretation of experimental data. In addition, in a related study Garay and Rodrigues [285] consider the effects of spin labels on highly ordered lipid bilayers in the gel state or bilayers with high cholesterol concentration in the liquid-ordered state. They showed that the presence of labels in the gel state, even in small concentrations, induces global changes in the structure. This effect was not observed in liquid-ordered bilayers where the probes are localized below the cholesterol ring system having little effect on the bilayer structure.

4.5. Non-membrane simulations

We will finish our discussion with a few notes on non-membrane systems. The interest in cholesterol is not limited to lipid bilayers [286–289] and not even to biological systems [290–292]. Cholesterol is one of the crucial components of pulmonary surfactant, probably the only monolayer in the human body. Importance of cholesterol in the functioning of lung surfactant has been a subject of recent coarse-grained simulations [286]. Interactions of cholesterol with cyclodextrins, cyclic carbohydrate known to desorb cholesterol from membranes, have been studied via MD simulations and free energy calculations by Yu et al. [287].

Other cholesterol-containing systems of importance, particularly biological and medical are the so-called good (HDL — High Density Lipoprotein) and bad (LDL — Low Density Lipoprotein) cholesterol. These are complexes of lipids, proteins and cholesterol, and are responsible for the transport of cholesterol in the circulation. Computational modeling of HDL and LDL is challenging since they are multicomponent systems and relatively large (diameters range from about 9 nm to 25 nm). There are a few recent MD and coarse-grained studies addressing various problems related to (mostly) HDL,

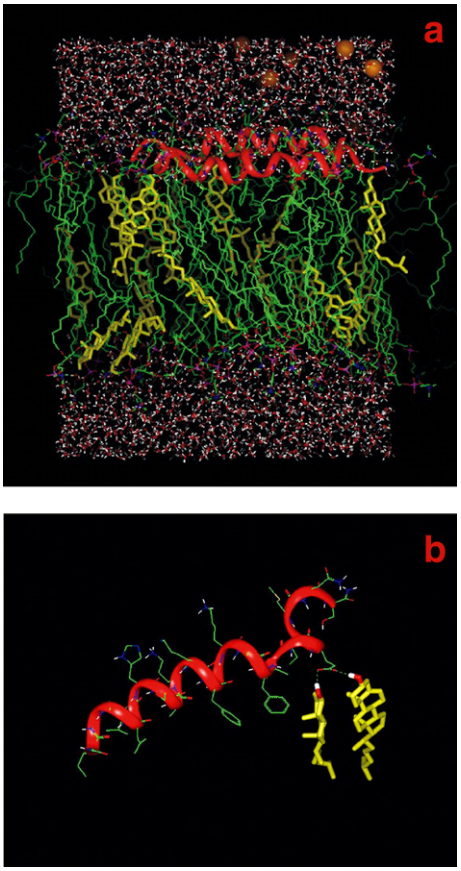

Fig. 22. Snapshot of magainine molecule hydrogen bonded with two cholesterol molecules.

either in its discoidal shape [293,294] or its spherical form [289]. An important related aspect is modeling cholesteryl esters, which are one of the main components of lipoproteins [288], and triglycerides, which are also abundant in the hydrophobic core of lipoproteins [295].

5. Notes on computational aspects

Thus far, we have discussed mostly results from simulations of cholesterol-containing systems, but not much has been said about the simulations themselves. We will not try to review the simulations methods here, but simply provide a short account of some aspects that should be noticed when comparing simulations to others or/and experiments. In addition, the details of the simulation protocols can be found from the cited papers and we will not discuss them here.

First, there is the issue of force fields. All of them have their strengths and weaknesses, and force-field development has been, and remains to be, an active area of research on its own. For those interested in force fields, we simply refer the readers to recent articles (and references in them), which can be used as starting points [95,97,296–301]. The same applies to computational methodology, as it is a very active field and we refer the readers to two different aspects, the former providing insight into bilayer specific issues, and the other one to the more general issues in simulations of soft and biological matter [57,302]. We would like to mention that progress in computational modeling relies on advances in the development of algorithms. Effective algorithms and methods provide more speed-up than any reasonable progress on the hardware side. That is particularly true when it comes to electrostatic interactions, which are the most important and computationally expensive ones in biomolecular simulations. For a recent review covering both reciprocal and real space methods and different boundary conditions, see [303].

We finish with a couple of comments on simulations. When comparing results from different simulations, the numerical values of, say, the areas per lipid, may depend on the methodology used in that particular MD simulation. For example, in our DPPC bilayer simulations we have used the Berger parameters for acyl chains [96], while for the cholesterol molecule the van der Waals parameters originate from the GROMOS force field. Both force fields are also commonly used in membrane protein simulations [304]. Nevertheless, recent studies of the VALP peptide in the lipid bilayer have shown that the same combination of force fields causes a small condensation effect [304], while the OPLS parameterization does not. Thus, that is to indicate that while small differences exist, yet the differences may well be within experimental errors and sometimes indistinguishable.

In another study, Chiu et al. [138] applied a surface tension of 80 dyn/cm and obtained a reasonable value, 0.63 nm^2, for $\langle A_{DPPC} \rangle$ in a pure DPPC bilayer — a method sometimes used in connection with the CHARMM force field. One can consider the surface tension term as a correction to the used force field, but then there is still an open question if the same surface tension should be used in all systems [206]. Improvement cannot be achieved without experimental results provided for the same systems studied under the same conditions as the MD simulated systems. For example, currently there are no data available for DPPC-Chol bilayers.

It is important to keep in mind, though, that when the force field is well proven, simulations are a method to study the systems directly without disturbing it with additional probes as all the momenta and coordinates are known at all times — that provides a unique and powerful starting point for analysis and comparison with experiments, which do, of course, have their own error sources and should also be kept in mind when comparing with simulations. The simulations are, at least as far as the most common force fields go, able to produce real quantitative information, which is undoubtedly valuable on its own right.

6. Summary

The main focus of this article has been on the role of cholesterol structure on its interactions with phospholipids. In particular, the role of cholesterol methyl groups appears to be an important and interesting issue, which has not received much attention. There is no existing experimental data concerning the role and effect of the methyl groups, which is, most likely, due to the limitations of current experimental techniques and difficulties in novel sterol synthesis due to high stereo specificity.

Molecular dynamics simulation studies fill this gap in an excellent manner, as – within the framework of a proven force field – it is possible to construct new molecules relatively easily. Our initial studies [102,118] show that the disordering effect of the methyl groups are in agreement with predictions based on experimental data when comparing cholesterol with its more methylated precursor, lanosterol, as well as with speculations on sterol evolutions drawn from cholesterol biosynthetic pathway. Surprisingly, we found that the removal of the methyl groups, especially C18, does not improve sterol properties in terms of the ordering and condensing effect [88,182]. This was due to the increase of sterol tilt, which is correlated with the decrease of the acyl chains' order [47]. Additional studies on unsaturated lipids showed that the β-face has a stronger ordering effect on unsaturated chains than the α-face does [247]. In agreement with that, Chiu et al. found that cholesterol prefers the interface between the ordered saturated and the unordered unsaturated phase with the methyl groups oriented towards unsaturated lipids [265]. Our latest studies show that the role of the methyl groups is even more far reaching. Due to the threefold symmetry of the steroid ring (due to methyl groups cholesterol has a triangular shape) cholesterol can promote formation of quasi-long-range lateral order forming honeycomb like lattice, which does not appear in the case of demethylated sterols (to be published).

Second interesting problem, which can be addressed with MD simulation studies is the atomic level mechanisms of the ordering and condensing effects. For the condensing effect it seems that the mechanism is related to the increase of van der Waals interactions between acyl chains due to the increase of the order. On the other hand, the mechanism for the increase of the acyl chain order seems to be dependent on the chain lengths and the level of unsaturation, and cannot be simply described in terms of the chain conformation (*trans*/*gauche*) or its tilt. We have found, however, a clear correlation between a given sterol tilt and the induced ordering effect. In addition, the pattern of hydrogen bonding between cholesterol and phospholipids is dependent on matrix lipid structure.

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Appendix A. Additional information

Many of the parameters and equilibrated membrane configurations are freely available for downloading at www.softsimu.org.

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