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Review

Detailed molecular dynamics simulations of model biological membranes containing cholesterol

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ABSTRACT

Detailed molecular dynamics simulations performed to study the nature of lipid raft domains that appear in model membranes are reviewed in this paper. The described simulations were performed on hydrated bilayers containing binary mixtures of cholesterol with phospholipids and also on ternary mixtures containing cholesterol, a phospholipid with a high main transition temperature T_m, and a phospholipid with a low transition temperature T_m. These simulations provide qualitative and semi-quantitative information about cholesterol–lipid interactions and also a testing ground for major assumptions made to explain the nature of lipid rafts in model membranes.

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1. Introduction

Biological membranes are complicated entities containing self assembled mixtures of different lipids and proteins. Among all these lipids, cholesterol is playing quite a special role due to its role in the creation of membrane rafts [1]. The issues related to the nature and organization of rafts in natural membranes are far from being clarified and agreed upon [2], although a definition of what is a raft exists. According to the Keystone Symposium on Lipid Rafts and Cell Function in 2006, "Membrane rafts are small (10–200 nm) heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes" [3]. To understand why cholesterol enrichment is present in raft domains, we need to understand in detail the character of specific interactions between cholesterol and lipids and this is the reason why it is studied so intensely [4–6].

Since biological membranes are complicated mixtures that are very difficult to analyze, many investigations are performed on model membranes containing either pure components or well-controlled mixtures of two-three components [7]. Especially interesting are synthetic membranes containing three components: saturated phospholipids, unsaturated phospholipids and cholesterol. In these model membranes one can observe raft like domains enhanced with cholesterol and saturated phospholipids, domains ranging in size from nanoscale to microscale. Study of the interactions between cholesterol and lipids in these model membranes can also shed light on the nature of lipid rafts in biomembranes.

Measurements performed on synthetic membranes containing two or three lipid components (one of them cholesterol) provided information used to construct phase diagrams for these mixtures. For

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binary mixtures the phase diagrams depend on the value of the T_m, the temperature of the gel to liquid crystal phase transition of the phospholipid. For mixtures containing phospholipids with high T_m, such as saturated phosphatidylcholine (PC) or sphingomyline (SM), two possible phase diagrams were proposed. These are shown in Fig. 1 reproduced from the review of Veatch and Keller [8] that gives an excellent introduction into the subject of phase diagrams of two and three component mixtures in general and mixtures with cholesterol specifically. The diagrams from Fig. 1 demonstrate that three phases can exist in such mixtures. These are: L_a or L_d – liquid disordered phase in which both translational and conformational degrees of freedom for phospholipids molecules are disordered; L_o – liquid ordered phase that contains lipids that are translationally disordered and conformationally ordered; and finally, S_o phase – solid ordered phase where both translational and conformational degrees of freedom of lipids are ordered, as in a gel phase of a one component phospholipids bilayer. The phase diagram from panel 1a shows that at temperatures above T_m, regions of liquid–liquid coexistence are present in the two component lipid bilayers with cholesterol as one of the components, while diagram from Fig. 1b discards the liquid–liquid coexisting region. The nomenclature for the bilayer phases was established by Ipsen et al. [9] who also demonstrated that it is possible to obtain the phase diagram shown on panel 1a from a specific model that can be considered to be an example of a "coarse-grained" model, i.e. a model containing only gross features of the system. Recent experimental work [10,11] indicates that it is the diagram depicted in Fig. 1b that should be used to describe the binary mixture of cholesterol and high T_m phospholipid. While latest results show that in binary mixtures of cholesterol and phospholipids there are no liquid–liquid coexistence regions, work on ternary mixtures of cholesterol with two phospholipids, one with a high T_m and one with a low T_m, demonstrates that liquid–liquid coexistence regions are indeed present in these systems [8]. It seems that, in this case, cholesterol displays selective properties by creating liquid ordered domains—L_o, regions enriched in cholesterol and high T_m lipids. These domains "float" in the liquid disordered phase (L_d) of the rest of the membrane. Micron-scale liquid domains (rafts) and liquid–liquid phase demixing in artificial membranes were observed in giant unilamellar vesicles (GUVs) [8,12] when vesicles contained three lipid components. One of the earlier studied ternary mixtures contained a relatively saturated lipid such as sphingomyelin (SM), cholesterol and an unsaturated lipid such as dioleoylphosphatidylcholine (di(16:1)PC or DOPC) in ratio 1:1:1. For this mixture the lipid raft domains in L_o phase were observed to be enriched by SM and cholesterol, while the liquid disordered phase outside the rafts was enriched by DOPC [13]. Later, the phase diagram for the mixture containing palmitoyl (16:0SM or PSM), DOPC and cholesterol was mapped out and it displayed the coexistence of L_o and L_d phases [14,15]. The choice of the SM molecule as a saturated lipid in a mixture was perhaps dictated by the observation that natural membranes contain a large proportion of

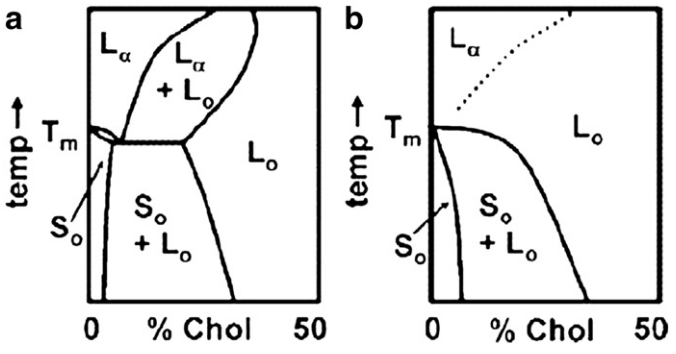

Fig. 1. Two possible phase diagrams for the binary mixture of cholesterol and high T_m phospholipid. The figure is reproduced from reference [8].

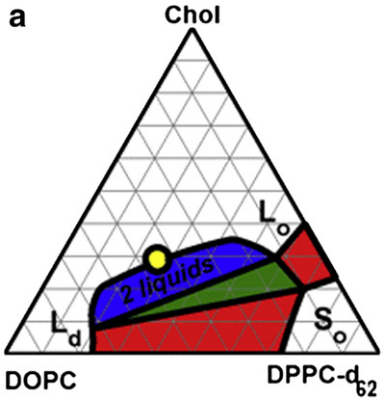

Fig. 2. Phase diagram for a ternary mixture of DOPC/DPPC/cholesterol at 10 °C. The L_d–L_o coexistence region is in blue. Adapted from reference [70].

SM. Instead of SM one can choose another saturated lipid with high T_m, such as the well studied dipalmitoylphosphatidylcholine (di(16:0) PC or DPPC), and still observe the liquid–liquid coexistence region in the phase diagram of a ternary mixture of DOPC/DPPC/Chol [8]. Fig. 2 depicts such a phase diagram. Obviously, one wants to know what kind of interactions between cholesterol and phospholipids are responsible for the liquid–liquid coexistence regions in these mixtures. Are they very specific? What is the role of the phospholipid headgroup/cholesterol headgroup interaction and phospholipid tail/cholesterol tail interaction? The answers to these kinds of questions can be obtained from computer simulations that include explicit representations of all atoms in the simulated system, although to obtain a phase diagram from such detailed simulations is not possible today.

Mixtures of cholesterol with phospholipids can be studied not just in bilayers, but also in monolayers. Thus McConnell and his collaborators [16–18] observed that for monolayers containing a binary mixture of cholesterol and low T_m, for example cholesterol/di(10:0)PC or cholesterol/di(12:0)PC or cholesterol/di(14:0)PC at low surface pressures, two coexisting liquid phases were present. At higher pressures, the two phases merged into one phase, and the diagram displayed an upper miscibility critical point. When the mixture contained cholesterol and a lipid with a higher T_m, for example di(15:0), two upper critical points appeared. The phase diagrams for monolayers of chol/di(14:0)PC and chol/di(15:0)PC mixtures are shown in Fig. 3. According to McConnell and collaborators, the existence of two critical points in mixtures of di(15:0)PC phospholipids with cholesterol indicates that formation of a "condensed complex" between cholesterol (C) and phospholipid (P) is taking place. This process can be described by the reaction:

$$nq\mathrm{C} + np\mathrm{P} \Leftrightarrow \mathrm{C}_{nq}\mathrm{P}_{np},$$

where q and p are stoichiometric integers and n is the measure of the size of the complex. n also shows the degree of cooperativity in the complex formation. Using the notion of the condensed complex, we can assign one region of the phase diagram from Fig. 3, region α, as due to immiscibility of pure phospholipid and complex and the other region, β, as due to immiscibility of complex and cholesterol. What is the molecular structure of the complex? It was pointed out in the work of McConnell's group that no molecular picture of a cholesterol/phospholipid complex exists at the present time. There is also no clear understanding what are the values of q, p and n, although some indications are that $q=1$ or 2 and $p=2$ or 3 and $n\sim 2$–10.

Is there a connection between the proposed existence of complexes in binary monolayers and existence of the domains in ternary mixtures of bilayers? Recently McConnell, using a modified theory of regular solutions, has suggested that the liquid–liquid coexistence region in phase diagrams of ternary mixtures and the

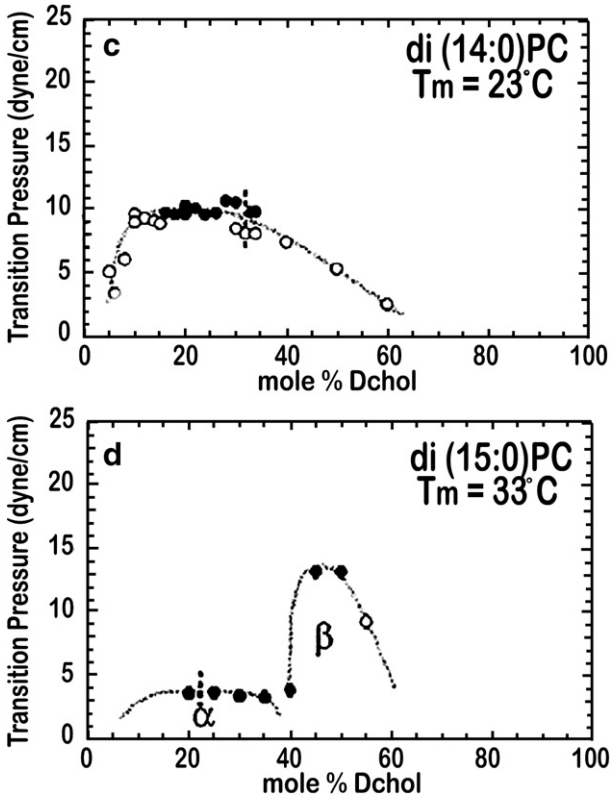

Fig. 3. Phase diagrams for monolayers of chol/di(14:0)PC and chol/di(15:0)PC mixtures. Adapted from reference [16].

existence of two critical points in phase diagrams for binary monolayers (see Fig. 3) are due to the same phenomenon: creation of a complex between cholesterol and high T_m lipids [19]. The complexes, as it was proposed, are not stationary and may have structures that fluctuate rapidly over a range of conformations. Further, the existence of complexes is closely connected to the existence of rafts. It seems that computer simulations describing phospholipid–cholesterol interactions in full molecular detail should be able to help us understand the validity of this proposal, and be able to identify these complexes.

Another model that was proposed to explain the properties of mixtures containing cholesterol and phospholipids is an Umbrella model [20]. According to this model cholesterol is shielded from water by a large headgroup of the neighboring phospholipids to prevent the non-polar part of cholesterol from the exposure to water. Since every cholesterol molecule wants to be covered by its own umbrella made of phospholipids, cholesterol molecules avoid clustering and create regular patterns to minimize cholesterol–cholesterol interactions. Recent experiments that measured cholesterol activity with the cholesterol oxidase activity essay were interpreted as supporting the Umbrella model [21]. Relevant to the Umbrella model with its proposed suggestion that cholesterol–cholesterol interactions are minimized when regular patterns of cholesterol are created, is the theory of cholesterol superlattices [22].

2. Simulations

2.1. Earlier simulations using simple models

Computer simulations of mixtures containing cholesterol and phospholipids employ different models that describe the molecular interactions on a different level of detail. The goal of the simulations is to provide a description of the phenomena observed in experiments. Ideally, full molecular details embedded in a specific force field used in the simulations should be employed to accomplish this goal. But enormous computational demands, especially in the field of membrane simulations, require "coarse graining" of the details to produce a "coarse grained" force field. Simulations using a coarse-grained description can be performed over longer time periods and larger spatial domain regions. Recent reviews by Muller et al. [23] and Venturoli et al. [24] provide comprehensive description of coarse-graining work performed on membranes. Because these reviews on coarse-graining of membranes are available in the literature, we will place here an emphasis on simulations that were done using molecular detailed description of phospholipids.

The earlier simulations of lipid mixtures were actually done using pure phenomenological coarse grained models, where the connection between the parameters of the models and atomistic properties were weak. Nevertheless, the simulations played an important role in our understanding of phase diagrams in lipid mixtures [25]. It was pointed out in that work that phase transitions in bilayers involve two distinct, but coupled order–disorder processes: one related to the translational degrees of freedom and the other one to the conformational degrees. In a two component lipid bilayer containing cholesterol as one of the components, the decoupling between these degrees of freedom takes place and a liquid ordered phase appears. Nielsen et al. [26] performed Monte-Carlo simulations on a specifically designed off-lattice model where lipids and cholesterol molecules were represented as hard-core particles with internal degrees of freedom that also had specific nearest-neighbor interactions. According to Nielsen et al., the model used represented the minimal model needed to describe the appropriate physics of the problem. The large number of conformational states of lipids in the model was reduced to just two conformational states and cholesterol was treated as a substitutional impurity. The interactions between particles were designed to contain the features needed to produce the simple phase behavior of one component lipid bilayer and the dual role of cholesterol as a "crystal breaker" and a "chain rigidifier". It was shown that using this minimal model, a phase diagram, such as the one depicted in Fig. 1a, could be obtained. Since obtaining a phase diagram in simulations is quite a complicated task, the authors used histogram reweighting method, finite size scaling and non-Boltzmann sampling techniques.

In addition to phase diagrams for the investigated cholesterol/phospholipid bilayer, the structural properties of different phases were also studied by Nielsen et al. The calculated structure factors clearly showed that the L_o phase is a liquid phase. It was also observed that lipid chains in direct contact with cholesterol molecules tended to align with their own kind, making a "treadlike" structure. The propagation of these "threads" was rather short, involving just a few molecules. These "threads" could be considered as hints pointing towards the existence of cholesterol/phospholipid complexes. All together, the results from the work of Nielsen et al. based on a physically simple (but in no way computationally simple) model demonstrate how much information one can obtain from such a model. Still, it is not clear how well justified are the forms of interactions assumed in the model. Such a justification should be the task of detailed molecular simulations.

2.2. Simulations using detailed molecular models

2.2.1. Simulations of bilayers containing binary mixtures of cholesterol and phospholipids

Computer simulations of lipid mixtures containing cholesterol were performed using different levels of model Hamiltonians and applying both Monte Carlo (MC) and Molecular Dynamics (MD) simulation techniques. Simulations of lipid bilayers containing just one phospholipid component that employed detailed atomic level force fields (we consider a united atom description of the chain methylene and methyl groups used in many simulations as also belonging to the type of detailed atomic simulations) started in

earnest in early nineties and, during a five year period, reasonable success was accomplished in the building of force-fields that could reproduce many properties of such bilayers [27–29]. These simulations also provided a molecular level description of properties of water next to the bilayers [30]. Earlier simulations with atomic resolution force field performed on the binary cholesterol/phospholipid system showed that properties of phospholipid molecules in bilayers strongly depend on the amount of cholesterol. Tu et al. [31] performed a 1.4 ns MD run at a constant-pressure on a system containing 12.5 mol% cholesterol in DPPC bilayer at temperature of 50 °C. In the simulation of Tu et al., the total size of the system was relatively small as it contained only 64 lipids in the bilayer. The results from this simulation were compared with the results obtained from a simulation on a pure DPPC performed under the same conditions [32]. It was observed that in the bilayer interior, cholesterol at this concentration did not significantly affect the conformations and packing of the hydrocarbon chains, only slightly reducing the free volume. The simulations revealed that cholesterol had a significant effect on lipid dynamics on the sub-nanosecond range, slowing down the C–H reorientational motion along the lengths of the hydrocarbon chains. To understand the influence of cholesterol on the neighboring phospholipids when cholesterol concentration can be low and high, Smondyrev and Berkowitz performed molecular dynamics simulations at 11 mol% and 50 mol% concentrations [33] and compared the results with the results of their previous simulation on pure DPPC [34]. In the simulation of Smondyrev and Berkowitz, bilayers also contained only 64 lipids. Two simulations were performed at high cholesterol concentration that differed by the initial arrangement of cholesterol in bilayers. Since the total run time for each simulation was only 2 ns, the arrangements of lipids in bilayers did not change in any substantial way during the time of the simulations and, therefore, the results showed dependence on the initial structural arrangement. Smondyrev and Berkowitz observed that cholesterol molecules displayed a tilt, so that they could be accommodated in membranes. While the tilt was larger in bilayers with small amount of cholesterol, it decreased in bilayers with 50 mol% cholesterol since the size of the hydrophobic region in this bilayer increased. Smondyrev and Berkowitz also observed that the area of the bilayer decreased substantially at a large cholesterol concentration, thus confirming the condensation effect that cholesterol produces in the bilayer. Assuming that the area of cholesterol remained the same (0.32 nm^2) as the concentration of cholesterol changed, they estimated that the area per DPPC decreased from the value of 0.616 nm^2 for pure DPPC to 0.58 nm^2 at 11% cholesterol to ~0.45 nm^2 at 50% cholesterol. They also pointed out that the value of the area of the lipid in the mixture depends on the distance from the bilayer center.

Computer simulations aimed at a systematic study of the properties of the cholesterol/DPPC bilayer as a function of cholesterol concentration were performed by Chiu et al. [35], Hofsass et al. [36], and Falck et al. [37]. In these simulations authors used different methods to estimate the values of the area per lipid in the mixture and establish a connection between these areas and condensation effect.

Chiu et al. [35] performed a series of simulations on bilayers containing mixtures of DPPC/cholesterol at different composition ratios such as 24:1, 47:3, 11.5:1, 8:1, 7:1, 4:1, 3:1, 2:1 and 1:1. Clear onset of the condensation effect beyond 10 mol% fraction of cholesterol was observed in their simulations by analyzing the behavior of the area per molecule. When the calculated area per molecule was plotted as a function of the cholesterol fraction (in the range of 0.1 to 0.5) a linear plot emerged. This plot was fitted to a straight line given by Eq. (1)

$$a_{\mathrm{mol}} = x_{\mathrm{DPPC}} a_{\mathrm{DPPC}} + x_{\mathrm{chol}} a_{\mathrm{chol}} \tag{1}$$

where x_i represented the molar fraction of species *i*. The following values for areas were obtained using Eq. (1): a_{DPPC}=0.507 nm^2 and a_{chol}=0.223 nm^2. One can think of these areas as partial areas that had composition independent values when cholesterol composition was in the range of 10 to 50 mol%. The partial areas per lipid obtained from procedure proposed by Chiu et al. required that simulations be performed in a range of different fractions of lipid components. The method described in Hofsass et al. [36] provides the values of areas per lipid at a given composition from the simulation data performed at that composition only. According to Hofsass et al., the values for areas that can be assigned to different molecules in the mixed bilayer, can be obtained from an analysis based on simple geometry using the following equations:

$$a_{\mathrm{PL}} = \frac{2A}{(1-x)N_{\mathrm{lipid}}}\left[1-\frac{xN_{\mathrm{lipid}}V_{\mathrm{chol}}}{V-N_W V_W}\right] \tag{2}$$

$$a_{\mathrm{chol}} = \frac{2AV_{\mathrm{chol}}}{V-V_W N_W} \tag{3}$$

where A is the cross section area of the simulation box, N_{lipid} is the total number of molecules in the bilayer, x is the mole fraction of cholesterol: $x=N_{\mathrm{chol}}/N_{\mathrm{lipid}}$, V is the volume of the simulation box, N_W is the number of water molecules in the system. The volume per cholesterol molecule, V_{chol}, is taken to be 0.593 nm^3 and that of water, V_W, is 0.0305 nm^3. Hofsass et al. [36] performed a series of simulations on bilayers with different cholesterol content in the range of 0–40 mol %. Their simulations were performed on larger sized patches of bilayers containing 1024 lipid molecules and over time periods of 10 ns, which improved the statistics. Hofsass et al. observed that both areas per DPPC and cholesterol were composition dependent, although the dependence for cholesterol was very weak. The calculated area per cholesterol of 0.27 nm^2 obtained by Hofsass et al. for larger cholesterol content was somewhat larger than the one obtained by Chiu et al., but still was much smaller than the value of 0.38 nm^2 obtained from calculations performed on a cholesterol crystal.

Falk et al. [37] demonstrated, by using geometrical constructions based on values for van der Waals radii of atoms in lipids, that indeed the area per lipid in a cholesterol/phospholipid binary mixture depends on the distance from the bilayer center. Using this construction Falck et al. were able to divide the volume in the bilayer into volume belonging to lipids and "free" volume. This allowed them to investigate the validity of the main assumptions made in the diffusion theory based on a "free" volume theory and find them to be unsatisfactory. In addition, they were able to study the behavior of average areas per lipid in the mixture; the results were qualitatively similar to the results found by Hofsass et al. and Chiu et al. at cholesterol mole fractions above 0.1.

Geometrical construction was also used by Jedlovszky et al. [38] to analyze the results from their Monte-Carlo simulations of dimyristoylphosphatidylcholine (DMPC)–cholesterol mixture [39]. They projected the centers of mass of molecules in the simulated membranes to the plane of membrane and performed a two dimensional Voronoi tessellation of the resulting projections. By analyzing Voronoi polygon area distributions in bilayers obtained from simulations with and without cholesterol it was concluded that lateral condensation of the membrane upon addition of cholesterol is solely due to the observed specific interaction occurring between neighboring DMPC–cholesterol pairs [38].

More recently Edholm and Nagle critically analyzed the data from the simulations of Chiu et al., Hofsass et al., and Falck et al. and proposed their own way of these data analysis [40].

Edholm and Nagle proposed to define partial areas in lipid mixtures in close analogy to the definition of partial volumes in simple mixtures. Their analysis showed that partial areas for a binary mixture of DPPC/cholesterol can be conveniently obtained from a plot where the total area of the simulation box divided by the number of

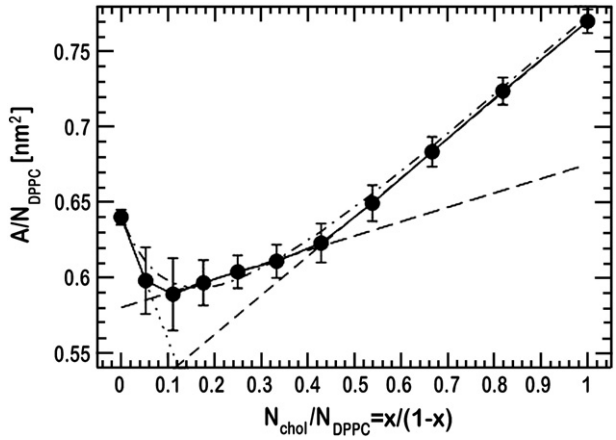

Fig. 4. The total area $A(x)$ divided by the number of DPPC molecules (i.e., $a(x)/(1-x)$) versus $x/(1-x)$. The black circles with error bars are simulation results. The three straight lines indicate possible fits for small, large, and intermediate x. The figure is reproduced from reference [40].

DPPC molecules is plotted as a function of a ratio of number of cholesterol molecules to the number of DPPC molecules. From such a plot the area per cholesterol at any concentration point is obtained from a value of a tangent to this point and the area per DPPC is given by an intersection of the tangent with the y ordinate axis, when the fraction of cholesterol is zero. The plot from the work of Edholm and Nagle, reproduced in our Fig. 4 illustrates nicely how with the addition of cholesterol the partial area per DPPC molecule decreases, in agreement with the condensation effect. One can see from this figure that in case of DPPC/cholesterol mixture roughly three different regimes of condensation exist. Notice also, that the partial area of cholesterol is negative at small cholesterol concentrations, which is again serving as a confirmation of the condensation effect. The existence of a negative partial area of cholesterol should not be surprising; it is analogues to the existence of the negative partial volume for ions in dilute aqueous solutions due to the electrostriction effect.

The condensing effect of cholesterol can also be seen from the change in the chain order parameters when cholesterol is added to the phospholipid bilayer. The chain order parameters that can be measured by the deuterium NMR are defined as

$$S_{CD} = \frac{1}{2}\left(3\langle\cos^2\vartheta_{CD}\rangle - 1\right) \quad (4)$$

where θ_{CD} is the angle between a CD bond (in simulations a CH bond) and the membrane normal. Order parameters measured and calculated for every carbon atom of the DPPC chains provide a detailed picture of the chain ordering. We display calculated order parameters obtained from simulations in Fig. 5. In simulations, for pure DPPC bilayer the order parameter changes between −0.1 and −0.22 with the more ordered (more negative) values displayed by the chain carbons located closer to the headgroup region, where a plateau-like behavior is observed for carbons 2 to 8. For the hydrocarbon chains with random orientation of CD bonds, $S_{CD}=0$, while for the perfectly ordered chains, the order parameter is $S_{CD}=-0.5$.

The plateau region is preserved, although at a more ordered value, when the amount of cholesterol is ~10 mol%, but it disappears when the amount of cholesterol increases. The order is increased by around a factor of two when cholesterol fraction is around 40 mol%. (Fig. 6 displays a snapshot from a simulation performed at 40 mol% cholesterol; the high degree of phospholipid chain ordering can be observed in this picture). The largest order is observed for DPPC carbons that face the ring system of cholesterol and the order is decreased rapidly towards the tail region of phospholipids. It is clear that the change in the ordering observed by the measurement of the

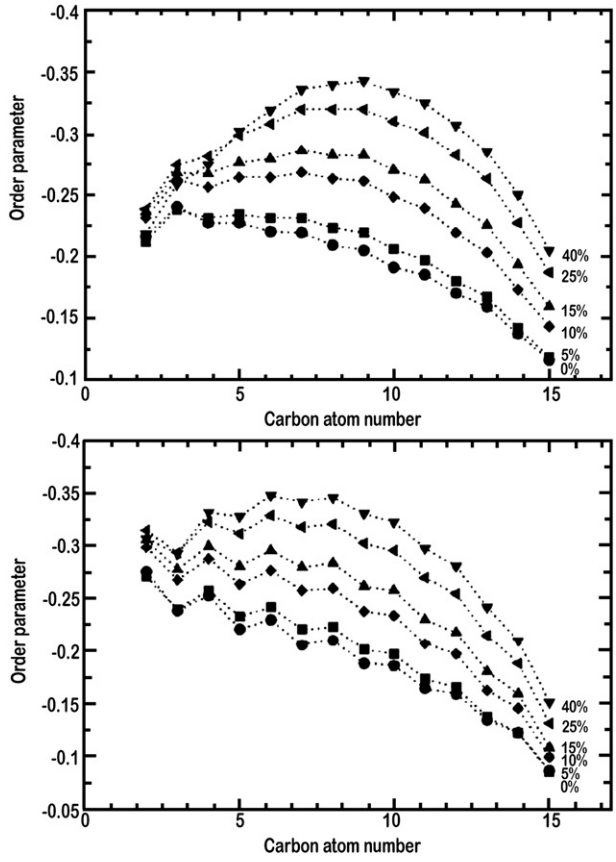

Fig. 5. Calculated order parameters versus chain position for different cholesterol concentrations (top for the Sn1 chain and bottom for the Sn2 chain). Reproduced from reference [36].

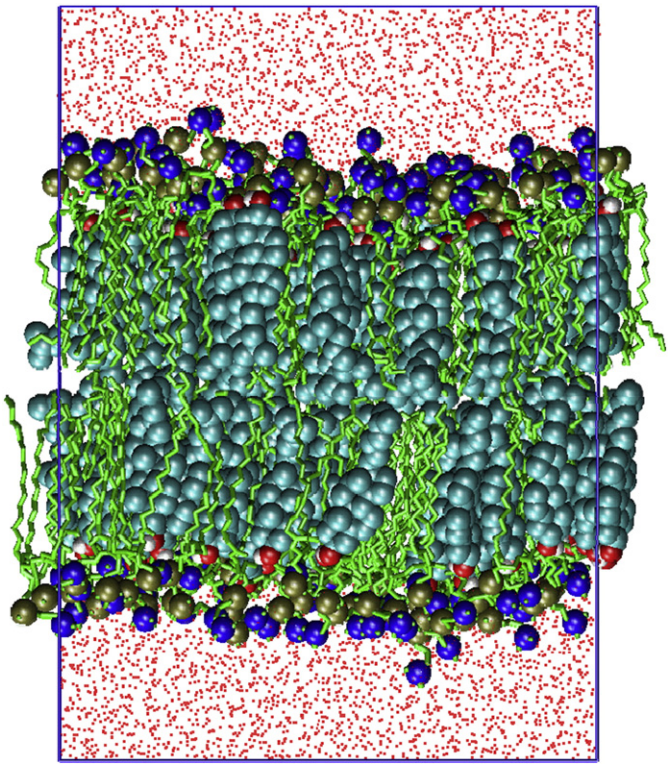

Fig. 6. Snapshot from a simulation performed on the cholesterol/DPPC mixture at 40 mol% cholesterol.

deuterium order parameter and the change in the area per lipid molecule are due to the same physical phenomenon and therefore, these changes should be connected. Indeed, the simulations of Hofsass et al. support this assertion.

Another means of studying the ordering of the tails in phospholipid molecules is to measure the fraction of gauche defects in these tails. This was also calculated and, again, it was observed that cholesterol increases the tail ordering by decreasing the gauche fraction in lipid tails, especially in the region of carbons 4–10 where carbons face cholesterol rings.

As we can see, earlier simulations on DPPC/cholesterol bilayers we mentioned above clearly demonstrated the condensing effect of cholesterol and a strong increase in tail ordering at larger mol fraction of cholesterol. The results on chain ordering and condensation effect were in an agreement with the experimental conclusions. Simulations can also provide molecular details related to specific interactions such as hydrogen bonding in bilayers and how it is affected when the bilayer composition is changed. Thus, Smondyrev and Berkowitz determined the average number and the specific atoms of phospholipids that formed hydrogen bonds with cholesterol [33]. Since, traditionally, these are considered to be O–H type bonds, for the DPPC/cholesterol/water system, it was observed that the hydroxyl hydrogen of cholesterol was engaged in hydrogen bonding with oxygens in carbonyl groups and one of the oxygens of phosphate group. It was also observed that cholesterol makes hydrogen bonds with water molecules. Chiu et al. found that when the cholesterol fraction in the bilayer is below 50 mol%, cholesterol displays a clear preference for hydrogen bonding with carbonyl oxygens over the phosphate oxygens [35]. The patterns of direct and indirect (through water) hydrogen bonding between cholesterol and PC molecules was investigated in detail in works of Rog and Pasenkiewicz-Gierula [41] and Pasenkiewicz-Gierula et al. [42], where results from 14 ns MD simulation of dimyristoylphosphatidylcholine (DMPC) and cholesterol mixture containing 56 DMPC and 16 cholesterol (22 mol%) were presented.

The cholesterol molecule has a smooth α-face and a rough β-face with two protruding methyl groups. This kind of a special molecular structure is believed to be important in shaping the interaction of cholesterol with lipid molecules containing both saturated and unsaturated chains. To study this situation Pitman et al. performed a simulation wherein the binary bilayer contained a 1:3 mixture of cholesterol and stearoyl-docosahexanenoyl -phosphocholine (SDPC) lipids [43]. One of the SDPC chains is strongly unsaturated. Pitman et al. observed that cholesterol had a preference for a solvation by a saturated fatty acid chain, especially the smooth side of the cholesterol. This interaction bias created an inhomogeneous environment around cholesterol that could play an important role in the formation of microdomains. The picture that emerged from the simulation of Pitman et al. was consistent with the model of Mitchell and Litman [44], who proposed that formation of microdomains is solely due to the acyl chain–cholesterol interactions.

As we have seen, the definition of lipid rafts requires the presence of cholesterol and sphingomyelin molecules in the bilayers. Therefore, the emphasis of simulations shifted from simulations of binary mixtures containing cholesterol and PC lipids to binary mixtures containing cholesterol and SM lipids. Thus, Khelashvili and Scott simulated a bilayer containing 266 stearoyl SM (18:0 SM or SSM) and 122 cholesterol molecules at two different temperatures, 20 °C and 50 °C [45]. In experiments these two temperatures bracket the main transition in the pure SSM, but due to the presence of cholesterol this transition is eliminated. The results of the simulations were quite similar at two temperatures, despite the 30 °C difference. The comparison between the results from simulations performed on SSM bilayers with cholesterol and pure SSM bilayers showed that SSM lipid chains were more ordered when cholesterol was present in the system. A tendency to form hydrogen bonds between cholesterol and SM was observed. A more detailed study of cholesterol–sphingomyelin hydrogen bonding pattern and the difference between cholesterol–PC and cholesterol–SM pair interaction was presented by Rog and Pasenkiewicz-Gierula [46].

As we can see the simulations performed on bilayers containing binary mixtures of cholesterol and phospholipids demonstrated that a network of hydrogen bonds was present in these bilayers. Chiu et al. noticed that in their simulations each cholesterol molecule was hydrogen bonded to at least one PC phospholipid. They proposed that this bonding is responsible for the creation of a two molecule (cholesterol and DPPC) complex that serves as a "building block" for the creation of an oligomeric structure of complexes as was proposed by McConnell and co-workers. Pandit et al. moved this idea even further [47]. By noticing that complexes of cholesterol and phospholipids can contain 1 cholesterol and 2 phospholipid molecules, they suggested that cholesterol can hydrogen bond to DPPC lipids not just through a cholesterol headgroup hydrogen, but also through its oxygen, making a hydrogen bond to the methyl group of tetramethylammonia of the DPPC choline. The existence of hydrogen bonding of the O–CH_3 type is well accepted concept in chemistry [48], although the interaction between hydroxyl oxygen of cholesterol and methyl group of PC may be not exactly of hydrogen bonding type. Thus, Pasenkiewitz-Gierula et al. [49] described this interaction in a more conservative way as partial charge pair interaction. Only high level quantum chemistry calculations can establish if the above mentioned interaction represent a hydrogen bond. But since the force fields we use in most of membrane simulations do not have special terms for hydrogen bonds, for the purpose of our analysis we can consider these interactions as hydrogen bonds. The most probable number of hydrogen bonds per cholesterol in simulations of Pandit et al. turned out to be two when the bilayer contained a mixture of cholesterol and DPPC. When the phospholipid in the bilayer was DLPC (12:0, 12:0 PC), which contains shorter hydrocarbon chains, the most probable number of hydrogen bonds per cholesterol was just one. Pandit et al. speculated that with two hydrogen bonds per cholesterol, one can create a linear network of hydrogen bonded molecules Ph(phospholipid)-chol-Ph-chol … when Ph is a DPPC molecule. Such a network has a smaller chance to exist when Ph is a DLPC molecule. Therefore, if one assumes that presence of a hydrogen bonded network is correlated with the existence of oligomeric complexes, one finds that such complexes are more probable to exist in bilayers containing mixtures of cholesterol and DPPC compared to bilayers with mixtures of cholesterol and DLPC. According to Pandit et al, the reason that cholesterol is most likely engaged in two hydrogen bonds with DPPC and only one bond with DLPC is that the cholesterol axis had a larger tilt in DLPC to accommodate cholesterol in the thinner hydrophobic region of this membrane. Due to the larger tilt, cholesterol was not in a favorable position to engage in two hydrogen bonds with neighboring phospholipids. The phase diagrams obtained by McConnell and coworkers for cholesterol/DPPC and cholesterol/DLPC monolayers, and their interpretation of these diagrams, indicated that complexes exist for DPPC/cholesterol system and do not exist for DLPC/cholesterol case. Therefore, the interpretation of the results obtained from the simulations on DPPC/cholesterol and DLPC/cholesterol bilayers presented by Pandit et al. was consistent with the results observed in the experiment. In spite of this agreement, one should be careful when comparing results obtained from bilayers and monolayers, since these may display profound differences in their structures due to the presence of a dipolar layer in the monolayer.

Although simulations have shown that there is a difference in the interaction of cholesterol with SM compared to the interaction with PC, the issue of a specificity of cholesterol–SM interaction still remained unclear. While some experimental studies indicated the specific character of cholesterol–SM interactions [4], other studies found evidence for a lack of such interactions [50]. It was suspected that the specific nature of the interaction between cholesterol and SM is due to the direct hydrogen bonding between cholesterol and SM,

but in a simulation of Aittoniemi et al. [51] it was shown that this is not the case. According to Aittoniemi et al. the total interaction between SM and cholesterol is not determined by the hydrogen bonding only; it is more subtle and comprises of contributions from electrostatic, van der Waals and hydrophobic effects.

Therefore, if it is not just the hydrogen bond that determines the interaction between sphingomyelin (or another phospholipid molecule) and cholesterol, one needs to study the total energy of the cholesterol–phospholipid interaction to understand when and why the cholesterol–phospholipid complexes may be created. Thus, Zhang et al. [52] studied the energy of interaction between POPC and cholesterol and compared it to the interaction energy of SSM with cholesterol. Since POPC and SSM differ in the structure of both their tails and their headgroups Zhang et al. also studied the interaction of oleoyl SM (18:1 SM or OSM) with cholesterol. OSM is a molecule that is somewhat intermediary between POPC and SSM: it has the same headgroup as SSM and tails similar to tails in POPC.

Using the data obtained from their simulations Zhang et al. made an attempt to establish an energetic criterion that would distinguish between a cholesterol/phospholipid complex from the non-complex. In search for such a criterion they calculated the distribution for the lowest nearest neighbor interaction energies between cholesterol and POPC, SSM and OSM phospholipid molecules. The distributions for Chol/SSM and Chol/OSM interactions looked very similar, and the distribution for the Chol/POPC interaction did not differ much from them either (see Fig. 7). The calculated distributions of lowest energies for the triplet of molecules containing one cholesterol and two (but same type) phospholipids were also very similar to each other (also shown in Fig. 7). Moreover, the energy distributions did not display any pronounced bimodal structure that could be used to identify which of the cholesterol–phospholipid 1:1 pair or 1:2 triplet were engaged in a creation of a complex. The distributions also did not indicate, from the energetic point of view, that cholesterol should prefer SM over POPC. The results from Zhang et al. work show that one cannot establish a simple criterion for complex formation between cholesterol and phospholipids by looking at the corresponding total interaction energy between these molecules and therefore one needs to look for another criterion to prove or disprove complex existence from the simulations. These calculations also showed that, on average, the POPC/cholesterol interaction energy was slightly stronger than the SM/cholesterol interaction. It was observed that among three bilayers containing pure phospholipids (POPC, SSM and OSM) the larger change upon addition of cholesterol occured in the POPC bilayer. Specifically Zhang et al. observed a change in the tail ordering of the phospholipid molecules. Since the ordering of tails of phospholipid molecules is connected to the conformational entropy of lipids, it was concluded that the preference of cholesterol for a specific phospholipid is due to a balance between energy and entropy.

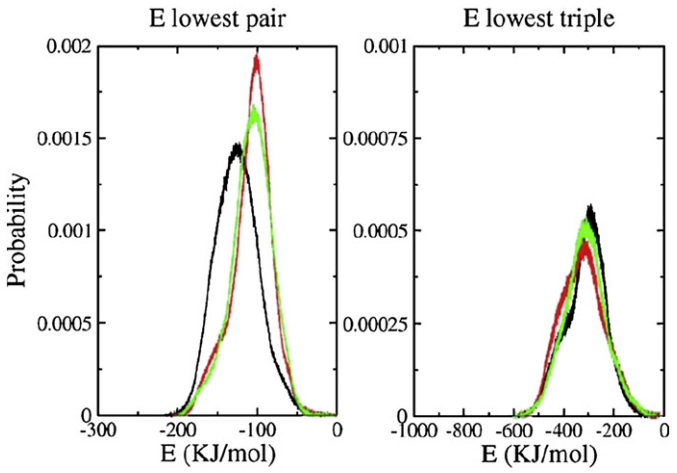

Fig. 7.

Based on the results from the simulation of Zhang et al. it is tempting to conclude that the favorable free energy of transfer of cholesterol from the environment where cholesterol is surrounded by unsaturated phospholipids (like POPC) to the environment where cholesterol is in the neighborhood of the saturated lipids, such as SSM is due to entropy. This transfer, one can argue, is favorable because the energy of cholesterol/phospholipid interaction is nearly the same for cholesterol/POPC and cholesterol/SSM interactions, but entropy is gained by removing cholesterol from the POPC environment and therefore removing an entropic penalty imposed on the conformational degrees of freedom of POPC chains. The entropy change due to conformational degrees of freedom for the SSM chain imposed by cholesterol is probably not large, since SSM molecules being saturated tend to keep their chains rather straight even without cholesterol. Similar arguments about the dominant nature of entropy in the energetics of cholesterol/phospholipid interactions were made by the proponents of Umbrella model to explain the nature of raft domains [53]. Also, because the results for OSM and SSM are similar, it is tempting to argue that cholesterol/phospholipid headgroup interaction is perhaps more important than cholesterol/phospholipid tailgroup interaction for the entropy-energy balance.

To obtain a better understanding of the balance of entropy and energy in the phospholipid/cholesterol interaction and also to get a quantitative measure of such a balance, Zhang et al., in another recent work, performed simulations where they calculated the free energies of cholesterol removal from the POPC (ΔG_1) and SSM (ΔG_2) bilayers and determined the free energy of cholesterol transfer from POPC to SSM as a free energy difference ($\Delta\Delta G$) between these quantities [54]. They chose to perform the simulations at limited cholesterol dilution to get a clear understanding of how cholesterol–phospholipid interactions affect the free energy of cholesterol transfer from the POPC to the SM bilayer. Zhang et al. used a biased sampling method (called umbrella sampling [55]) to calculate the potential of mean force (pmf), i.e. the free energy of the cholesterol removal, as a function of distance between the cholesterol center-of mass and the center of a phospholipid bilayer, as cholesterol was slowly removed from the bilayer. Since there was a need to perform a large number of runs due to different windows in the umbrella sampling, the size of the chosen systems was not that large; the systems contained only 35 phospholipid molecules and one cholesterol molecule in each leaflet of the bilayers, resulting in the presence of 72 lipid molecules in each bilayer solvated with 3600 water molecules. To avoid artifacts that could be created due to asymmetric amount of cholesterol in small sized systems, one cholesterol molecule was placed in every leaflet of the bilayer.

According to calculations, the difference in ΔG, $\Delta\Delta G$ that is equal to the free energy of cholesterol transfer from POPC to SSM at the limited cholesterol dilution is only -6.5 ± 3.33 kJ/mol at 319 K. This number is the measure of the relative affinity cholesterol has to SSM compared to POPC. To separate the free energy difference into its energetic and entropic parts, Zhang et al. used a numerical approximation to thermodynamic relationships. For this purpose, they calculated the free energy of cholesterol removal from the bilayer at few different (but close to each other) temperatures: 319 K and 329 K, and a simple approximation to the derivative that determines entropy at temperature T was used

$$-S(T) = \left(\frac{\partial G}{\partial T}\right)_P \approx \frac{G(T+\Delta T) - G(T)}{\Delta T} \tag{5}$$

It was determined from the simulations that at temperature $T=329$ K, $\Delta\Delta G$ is equal to -4.8 ± 2.7 kJ/mol and the entropic contribution to the free energy of transfer is unfavorable and equal to $-(54.3)$ kJ/mol. (It was observed that large error bars were present in the calculation of

the free energy difference and entropy difference. These large error bars appeared due to the accumulation of errors in the calculation of a difference between two similar in value numbers. Thus, although the error in the free energy calculation for the cholesterol removal (ΔG) is only 2–3%, the error in the difference of the free energies (ΔΔG) is already much larger and it is around 50%. Use of the Eq. (5) produces even larger relative error.) Since the free energy of cholesterol transfer from POPC to SSM is favorable, but entropy is not, this means that the transfer is promoted by the energetic component, i.e. transfer is exothermic. This is in a qualitative agreement with the recent results from the work of Heerklotz and collaborators [56].

At this point, we can only speculate why the transfer of cholesterol from the POPC to SSM bilayer at limited cholesterol concentration produces favorable change in energy and unfavorable entropy change. One of the possibilities of entropy loss is connected to change in the entropy of cholesterol. Zhang et al. observed that cholesterol orients itself in the phospholipid bilayer in such a way as to produce a small perturbation on the environment. In the case of SSM bilayer, cholesterol can achieve this by inserting itself in an almost vertical position into the bilayer, where it fits nicely due to its matching hydrophobic length. In the case of the POPC bilayer, cholesterol will try to orient itself to avoid the misfit of hydrophobic lengths and it can do this, since the free volume in POPC is larger than in SSM. By orienting itself at an angle to the bilayer normal, cholesterol interferes less with the tail conformational motion of neighboring POPC lipids and, therefore, causes a smaller reduction of the lipid chain conformational entropy. In Fig. 8 we present the distribution for the orientational angle, which is defined as the angle between the cholesterol axis and the normal to the bilayer. While this distribution is narrow for cholesterol in SSM, it is broad for cholesterol in POPC. The broadness of the distribution in the POPC that we observe at limited cholesterol dilution indicates that cholesterol undergoes rotational (librational) motion in this bilayer, when its concentration is small. Thus, when cholesterol is transferred from the POPC to SSM, its rotational entropy is reduced and, perhaps, a small relative change in the conformational entropy of the surrounding lipids is also produced. As a result, due to the loss in cholesterol entropy, there is a loss in total entropy. The balance in total energy also depends on the inclination of cholesterol, since it perturbs the tail ordering of neighboring lipids and subsequently the van der Waals interactions between them. We can speculate that the increased van der Waals interaction of the tails of neighboring to cholesterol SM molecules and a decrease in this interaction between neighboring to cholesterol POPC molecules produce a balance that results in exoenergetic transfer of cholesterol, i.e. the exoenergetic character of the cholesterol transfer is due to the change in the interaction energy of the tails in the neighboring to cholesterol phospholipids.

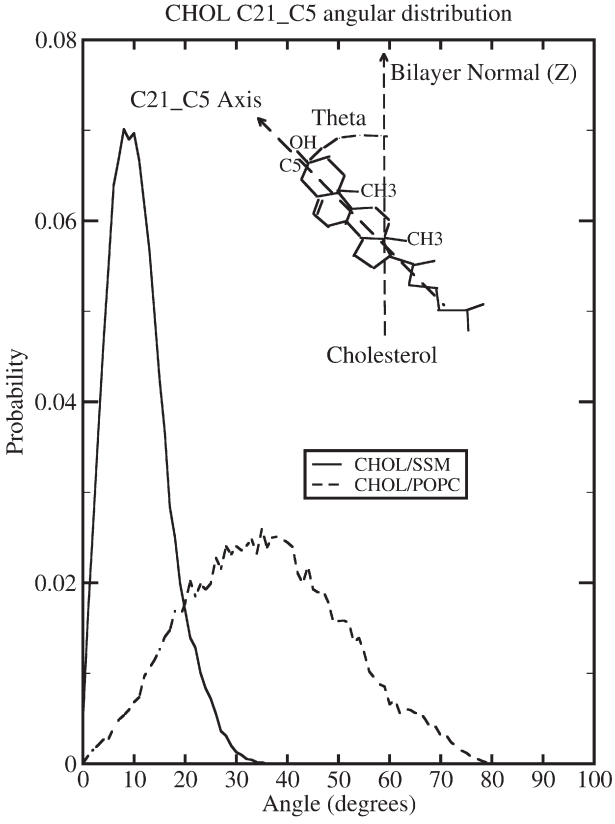

Fig. 8. Cholesterol principal axis tilt angle distributions with respect to bilayer normal in SSM (solid) and POPC (dotted) bilayers. The cholesterol principal axis is defined as the vector connecting carbon C21 (the carbon atom in the ring to which the short tail of a cholesterol molecule is attached) and C5 (the carbon atom to which the hydroxyl group binds).

To determine the values of the change in the conformational and rotational entropies and cholesterol–phospholipid and phospholipid–phospholipid interactions as a result of cholesterol transfer, one will need to perform very careful and detailed calculations of entropy from the first principles. The calculations of entropy by using the derivatives of free energy suffer from large uncertainties in the results and so do the calculations of energy contributions. In addition, the results of the calculations are very sensitive to the force fields used. Nevertheless, as we can see, the present calculations are in qualitative agreement with the experiment by determining that the transfer of cholesterol from the POPC bilayer to SSM bilayer has an exothermic character with a loss of entropy. The experiment also showed a dependence of the transfer free energy on cholesterol concentration. Thus, from experiment, the free energy change for a process of cholesterol transfer from the POPC bilayer to SM at 30 mol% cholesterol was −5 kJ/mol and it decreased to −8 kJ/mol when cholesterol concentration decreased to 20 mol%. We do not know if the measured free energy change decreases monotonically with the decrease in cholesterol concentration, but if it is, the calculated free energy change is having an absolute value that is below the experimental value, and a refinement of the force field for cholesterol interactions with phospholipid molecules is required. This indicates the need for further experiments and calculations that will produce consistent results. At this stage we want to point out that calculations and available experimental data show that the free energy of cholesterol transfer from POPC to SM is only ~5 kJ/mol. Assuming that this energy is equally distributed between ~4 phospholipid neighbors interacting with the cholesterol we get a change of ~1 kJ/mol per interacting cholesterol/phospholipid pair upon cholesterol transfer.

To add to the complexity of biological membranes, the identity of lipids in the leaflets of the bilayers that one finds in cell membranes is different. Thus, for example, lipids such as SM and PC can be often found in the outer leaflet of the plasma membrane, while two other typical lipids: phosphatidylserine (PS) and phosphatidylethanolamine (PE) are found in the inner leaflet of the membrane [57]. The asymmetry of the membrane presents us with the following question: if a domain is formed in the outer leaflet of the membrane, is there a domain formed in the inner leaflet underneath the outer leaflet domain? Is there a cross leaflet interaction? Just to get some feelings related to posed questions Bhide et al. [58] reported results from a molecular dynamics (MD) simulation performed on an asymmetric bilayer containing a mixture of cholesterol and SSM in one leaflet and SOPS (stearoyl-oleoyl-phosphatidylserine) and cholesterol in the other leaflet. The ratio of concentrations of cholesterol to SM (1:2) was chosen for the one leaflet and the same ratio between cholesterol and PS was chosen for the other leaflet. Bhide et al. also compared the results from the simulations on the asymmetric bilayer with the results from simulations on two symmetric bilayers containing binary

mixtures of cholesterol and SSM and cholesterol and POPS respectively. From these simulations it was concluded that properties of the monolayers in the leaflets did not change much when going from a symmetric to an asymmetric bilayer, indicating that cross-leaflet interactions such as interdigitation were unimportant for the bilayers containing these specific leaflets. The work of Bhide et al. also suggests that PS phospholipids may be engaged in the creation of lipid raft domains in the inner leaflet of biological membranes.

2.2.2. Simulations of ternary mixtures containing cholesterol, SM and PC

Simulations were also performed on systems containing lipid bilayers with three components, the same components that are able to create lipid rafts in model membranes: cholesterol, sphingomyelin and unsaturated phosphatidylcholine. One of the major problems with the simulations of bilayers with a ternary composition is that, due to inhomogeneous structure of these bilayers, it is hard to find proper starting configurations. This problem may be not that severe in simulations with coarse-grained potentials. In such simulations, one can start with a random distribution of lipids and observe how the system phase separates, since with a coarse grained potential one can perform simulations on a larger sized patches of membranes and over longer simulation times. The problem can be rather severe when a detailed microscopic description is used. In latter case, due to the slow diffusion of lipid molecules, the configurations change slowly and one may need to simulate the system containing a large number of particles over microseconds of time in order to establish an equilibrium distribution, which is impractical. To avoid this problem, it is possible to prepare the system in a special arrangement that may be closer to the assumed equilibrium state. Therefore, simulating a mixture containing 1424 DOPC, 266 molecules of SSM and 122 molecules of cholesterol, Pandit et al. [59] prepared a patch that contained all the SSM and cholesterol molecules. This patch was imbedded in the rest of membrane containing all the DOPC molecules. This way, the condition that the L_o phase of the SM-cholesterol mixture is imbedded in the L_d phase containing the majority of unsaturated PC molecules was reproduced. This solution of the initial problem is not perfect: in experiment, the L_o domain has much larger dimensions, and it is not clear if one can reproduce the correct physics of the inhomogeneous membrane by scaling down the patch size. In any case, it was observed that the SM-cholesterol domain was stable and remained stable for the length of the analyzed simulation run (8.5 ns). It was observed that the liquid-ordered patch was 0.45 nm thicker than the rest of the membrane. To understand what was happening during the initial stages of the L_o domain formation, Pandit et al. [60] also simulated a ternary mixture of DOPC/SSM/cholesterol in a proportion of 1:1:1 (100 molecules of each lipid) starting from a random initial distribution of lipids. The simulation run was performed for 200 ns and it was concluded that on the initial stage of possible phase separation between the L_o and L_d domains, cholesterol is located on the domain border, thus reducing the line tension due to the presence of the interface between the liquid phases. This can be also understood on the microscopic level. As was already mentioned, the structure of a cholesterol molecule displays two faces: a smooth one and a rough one. According to Pandit et al., due to steric constrains, cholesterol orients its smooth face to SM molecule and shows its rough face to DOPC, therefore acting as a two-dimensional surfactant and therefore reducing the line tension. This argument is quite similar to the argument invoked by Pitman et al. [43] that we discussed above.

Tension and other mechanical quantities play an important role in the stability of the membranes. It was recently proposed that it is not just the tension, which is an integral over the local pressure, but the whole distribution of the local pressure in the bilayer that is determining the functioning of membranes, including the functioning of proteins in membranes [61]. Niemela et al. [62] studied large sized patches (1024 lipid molecules) of ternary mixtures of POPC/PSM/cholesterol by performing molecular dynamics runs for relatively long (for systems of this size) time periods of 100 ns. Two raft-like concentrations were studied in two systems A and B at concentrations having a 1:1:1 ratio and 2:1:1 ratio of POPC:PSM:Chol, respectively, and compared the results to the results from a simulation on system C at a non-raft concentration mixture of 62:1:1, where only few PSM and cholesterol molecules were present. It was observed that elastic properties and the local pressure profiles of mixtures A and B differed substantially from properties measured in the mixture C. Full implications of this difference for the functioning of peptides and proteins in membranes is still not clear, although experiments [63] indicate that the transfer of peptide from the L_d domain to the L_o domain produces a change of 4–8 kT, thus substantially influencing the partitioning of peptides in membranes.

As we can see, while the issue of equilibration of membranes containing ternary mixtures is still not completely resolved, simulation of these mixtures has provided us with interesting observations and suggestions.

3. Discussions

Cholesterol plays an important role in the creation of liquid ordered domains in biological membranes. Experiments show that such domains can be created in synthetic membranes containing just three components: cholesterol and two phospholipids. What is the molecular detailed background that is responsible for the creation of these domains? Four different microscopic level models describing cholesterol/phospholipid interaction that can produce liquid ordered raft domains were proposed. These include a model that proposes a creation of a cholesterol/phospholipid complex, the Umbrella model, cholesterol as a two-dimensional surfactant model and finally a model that does not ascribe any special features to cholesterol/phospholipid interactions; instead it proposes that domains are created as a result of a subtle balance between entropy and energy change when cholesterol is transferred from the neighborhood where unsaturated lipids dominate to a neighborhood dominated by saturated lipids. What do computer simulations that take into account atomic detail of the lipid molecules tell us about the validity of the assumptions made in these models?

With respect to a proposed existence of cholesterol/phospholipid complexes computer simulations still did not come up with a proper definition of complexes that can identify them in a unique way. Similar to the situation in liquid water we observed from the simulations of bilayers that a network of hydrogen bonds exists in them. Therefore, it is tempting to assume that complexes in the bilayers are due to the network of hydrogen bonding. But while in water hydrogen bonding is a determining factor in the interaction, simulations in bilayers do not support the dominance of hydrogen bonding. Therefore one should consider the distributions of the total interaction energy between cholesterol and lipids, saturated and unsaturated. These distributions do not provide any criteria for separation of complexes from non-complexes.

Simulations support the assumption of the Umbrella model that cholesterol rings are situated in the bilayer in such a way that they are not wetted by water. At the same time it is not possible to claim that cholesterol is shielded from water by an umbrella created by the phospholipid headgroup, since the simulations show that hydrogen bonding exists between cholesterol hydroxide group and water. The assumption that follows from Umbrella model that cholesterol molecules strongly avoid each other in order to be under phospholipid umbrellas, and therefore that superlattices are created at small specific concentrations and regular patterns at larger specific concentrations, is hard to check in simulations with molecular level detail, since they require very long times for the randomly assembled system to find a regular arrangement (if indeed such exists). It is possible to preassemble special arrangements of cholesterol in

bilayers and compare the results from prearranged system with the system containing random arrangement. This was done recently in a set of simulations of bilayers containing a binary mixture of cholesterol and POPC [64]. The results from 200 ns simulations showed that the time evolution of the radial distribution of cholesterol hydroxy oxygen was more stable when a superlattice like arrangement was utilized. It was also observed in the simulation that a relatively long simulation run, longer than 100 ns was needed to accomplish equilibration of the bilayer containing a binary mixture in a random arrangement. These results are interesting, but still one needs to find out that regular arrangements correspond to lower free energies; the stability of the regular patterns during the simulation period may involve some kinetic issues. More research along these directions is needed.

The assumption that cholesterol is situated mostly on the border between the patch of saturated lipids and surrounding unsaturated lipids acting as a surfactant due to its non-symmetrical character of the ring structure was supported by simulations that used pre-arranged assemblies. In these simulations the patch of L_o domain containing cholesterol was inserted into the L_d surrounding containing unsaturated lipids. After 200 ns run it was observed that cholesterol moved to the border of the domain. The issue that needs to be investigated carefully in this case is the dependence of the results on the preassembled domain size. There is a large difference in the domain size in simulations and in experiments.

The model that does not require any special geometric arrangement of cholesterol or creation of special entities like complexes recently received support from theory, experiment and simulations. Phenomenological Landau type theory that explained the phase diagram of three component cholesterol/phospholipids mixture was constructed by Garbès Putzel and Schick [65]. It did not require any special arrangements of cholesterol, no complexation and no enhanced presence of cholesterol on the boundary of the domains (phases). The constructed Landau free energy in this theory depended in addition to order parameters such as concentrations, also on order parameters that described the structural properties of the tails. The theory indicated that the change in free energy of interaction between cholesterol and unsaturated lipid and cholesterol/saturated lipid is not large, of order kT. The same order of magnitude in free energy change was also obtained in the resent experiments performed by Frazier et al. [66] who studied BSM/POPC/cholesterol bilayer using Fluorescence Resonance Energy Transfer technique. This value of interaction is much smaller than the one that appears in the regular solution theory with the incorporated cholesterol/phospholipid complexes proposed by McConnell. Our simulations calculating the free energy change gives the same order of magnitude as from the work of Garbès Putzel and Schick, Frazier et al., and Heerklotz et al. In addition, our simulations together with the recent experimental and theoretical work indicate that subtle balance of energy and entropy exists in ternary systems. The delicate balance indicates a possibility that in ternary mixtures of sphingomyelin/phosphatidylcholine/Chol, the domain structure may exist when sphingomyelin has both saturated chains like in the SSM molecule, while the domain structure may disappear when the SSM is replaced by the OSM molecule. Indeed, Epand and Epand noted that this is the case for the ternary mixture of OSM/SOPC/Chol [67]. Because the change in free energy is small, the task of calculating this change from detailed simulations is difficult, since the results may be quite sensitive to the details of the simulations and the force fields used. This is why it is important to continue with the development of the all atom and united atom force fields used in the lipid simulations [68]. It is also important to develop computational techniques that can be used to calculate the energy and entropy components of free energy changes with higher precision, than it is done today, by using thermodynamic derivatives. Nevertheless, even today, detailed molecular simulations can provide a qualitative and even semi-quantitative insight into the complicated (and system sensitive) energetics of cholesterol transfer from one lipid bilayer to another. This can be very helpful for the understanding of lipid raft formation.

To observe self-assembly of rafts in simulations one needs to perform multi-scale simulations, where the information from detailed molecular simulations is passed to coarse grained simulations, so that larger sized and longer in time simulations can be performed. Some simulations that involve mixtures of cholesterol and phospholipids already used this multi-scale approach [69] and the reader can find a more detailed description of them elsewhere in this issue. This review does not represent an exhaustive description of all the simulations related to the subject of cholesterol/lipid interactions. There are other papers in this special issue that address other aspects of the same subject and the reader is referred to them to get a more complete picture. At this stage the author feels that the task of understanding why cholesterol creates domains in membranes, even in model membranes, is still not completely accomplished. More work, including work in both simulation and theory, is needed.

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