Scientific report

Role of cholesterol off-plane methyl groups in the formation of ‘condensed complexes’ and ‘superlattice structures’ in cholesterol/phospholipid membranes

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1 Purpose of the visit

Cholesterol (Chol) is the most common lipid component in mammalian cell membranes [1]. The role of Chol as an agent for promoting membrane order is well established, and it is involved in a large number of cellular functions. Experimental and theoretical studies have shown that Chol has a tendency to form regularly distributed lateral structures [2]. For example, the liquid-ordered (lo) phase in membranes is characteristic to Chol at sterol concentrations above ~25 mol%. Meanwhile, unlike many other sterols, Chol is able to induce the coexistence of lo and liquid-disordered (ld) phases at intermediate Chol concentrations, characterized by Chol-rich and Chol-poor regions. Why this happens is not well understood. Moving on, Chol is involved in formation of highly ordered domains called lipid rafts [3,4] which play a role in numerous cellular functions. While there are conceptual models like the *condensed complex* model [5] (based on low-energy lipid-Chol complexes) and the *superlattice* model [6] (based on the existence of extended ordered distributions) suggested for describing the formation of lateral structures due to Chol, the molecular mechanism associated with these structures has remained unclear.

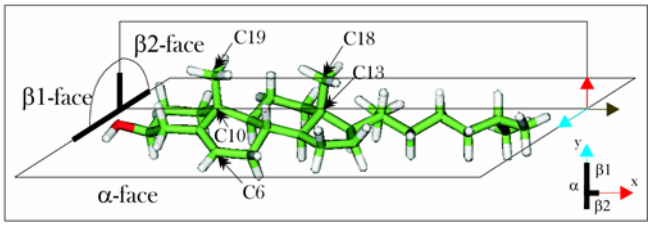

Figure 1. Molecular structure of cholesterol molecule and definition of its different faces. In the lower right part, a schematic upper view of the cholesterol molecule defining the reference axis system used for the 2d-density functions.

Cholesterol consists of a semi-rigid tetracyclic ring system with a 3β-hydroxyl group and a short 8-carbon atom. The ring system forms an asymmetric planar structure with two methyl substituents defining the *rough* side (β-face). Such β-face splits into the two sub-faces β1 and β2 due to the presence of the off-plane methyl groups. The *smooth* side has not methyl substituents (α-face). The Chol biosynthetic pathway shows a systematic removal of methyl groups from the steroid ring. Each removal step optimizes sterol properties in terms of ordering and condensing effects [7]. This observation could lead to the wrong conclusion that the remaining methyl groups are just evolutionary fossils: however, recent studies [8,9] have shown that their presence regulates the sterol ring orientation in the bilayer, which is a major determinant of its ordering properties.

Cholesterol lateral organization is a long-standing problem in membrane biology. Its biological importance is implicated in a wide range of cell biology studies [10-13]. Because cell membranes are extremely complex, model membrane studies are required for understanding the cholesterol lateral organization. Theoretically, the membrane components can be either segregated in domains, randomly distributed, or regularly distributed in the plane of a membrane [14].

The main goal of the visit was to bring some light on the interaction of cholesterol with other lipids (Phosphatidylcholines) in membranes at molecular level. From these interactions one can expect to find the key that explains the role of cholesterol in membranes and particularly its ability to induce order in the short/long range. This description is really needed to move forward in the complex puzzle given by the experimental results already available.

The recognized experience of the research Group leaded by Ilpo Vattulainen made the TUT (Tampere University of Technology) the perfect place to try to chase this problem with some guaranties of success.

2 Description of the work carried out during the visit

In this project, we have studied the role of the off-plane methyl groups of Chol by means of Molecular Dynamics (MD) simulations of membrane systems composed of either fully saturated distearyl phosphatidylcholine (DSPC) or di-unsaturated dioleoyl phosphatidylcholine (DOPC) in the fluid phase (338 K) with different molar fractions of Chol. Details of the used simulation protocol can be found in Ref. [15]. This work can be divided in two main parts:

First, a set of simulations has been performed for systems containing cholesterol molar concentrations about 10%, 20%, 30%, 40%, 50% with both lipids of as long as 300ns each. This was possible by the use of the BSC supercomputer (www.bsc.es) which granted us computational time with the common project with Tomasz Róg, former member of Ilpo Vattulainen's group. The superlattice structure has been proposed to show up more clearly at defined "magic" cholesterol concentrations

[5,6], and it is also expected to appear in the proposed set of simulations. For the same reason, we have carried out simulations for a large number of cholesterol concentrations and in this manner unravel the nature of “superlattice” structures under a multitude of conditions. The preliminary findings are truly exciting and imply that atomistic simulations are eventually at a stage where they can shed light on these fundamental issues that have been speculated in the experimental papers since the late 1980s[16]. The analysis of the trajectories indicates that the arrangement of cholesterols with respect to each other discloses a clear anisotropy and a well defined three-fold symmetry. Also the position of PC molecules around cholesterol is far from random, since five different preferential regions are found. All these effects are more evident for DSPC membranes than for DOPC, in agreement with the established view that Chol orders saturated chains more than unsaturated ones. Such findings seem to indicate a dynamic self-assembly of triangular *building blocks* (or *condensed complexes*) that eventually form superlattice-like structures and likely has an important role in lipid raft formation (manuscript in preparation). At this point the common effort with other members of the host group working with the same problem but with different lipids was also important to generalize some of the findings that has been obtained.

Second, a set of simulations with sterol molecules without the off-plane methyl groups (flat cholesterols) at the same 20% and 40% molar fraction as in the previous section also with DOPC and DSPC were performed. Comparison with the previous simulations will differentiate the effect of the planar structure and the off-plane methyl groups on the observed effects. Long simulations of about 400ns have been required. The analysis of the resulting trajectories is currently underway.

The main goal of the proposal has been tackle: to establish a connection between nano-scale molecular ordering phenomena with macroscopic observations related to the formation of *condensed complexes* and *superlattice structures*. From a general perspective, the objectives to fully understand the role of cholesterol in inducing ordered structures in its vicinity, and further to understand the underlying mechanisms related to raft formation have been mostly achieved.

3 Description of the main results obtained

Cholesterol (Chol) is the most common lipid component in animal cell membranes[1]. It increases the order of fluid-phase phospholipid acyl chains and determines the permeability, fluidity and mechanical properties of membranes[17]. It is also involved in formation of highly ordered domains called lipid rafts[3,4] which play a role in numerous cellular functions[18]. The role of Chol as an agent for promoting membrane order is well established and is involved in all the above functions. Experimental and theoretical studies have shown that Chol has the tendency to form regularly distributed lateral structures[19] that correspond in the macroscopic level to a liquid-ordered (lo) phase. While there are conceptual models like the condensed complex model[5] (based on low-energy

lipid-Chol complexes) and the superlattice model[6] (based on the existence of extended ordered distributions) suggested for describing the formation of ordered lateral structures due to Chol, the molecular mechanism associated with this lipid organization has remained unclear.

In this work we have clearly observed that the molecular structure of cholesterol induces a particular lateral lipid organization. First, the preference of cholesterol molecules to be placed in a second coordination shell (~1 nm), avoiding direct cholesterol-cholesterol contacts, is observed. Second, we show that cholesterol off-plane methyl groups induce a three-fold symmetry arrangement of proximal cholesterol molecules. Finally, we notice that the relative orientation of cholesterols is far from being random and displays clear preferential configurations, despite the liquid nature of the system.

The first observation reported here is provided in Figure 2 for DSPC/Chol membranes by the one-dimensional (1d) density functions, RDF(r), computed as the relative density of molecules at a radial distance r from a cholesterol molecule. Unlike DSPC lipids that display the highest peak at a distance ~0.5 nm, cholesterol molecules have a strong tendency to accumulate in a sort of second shell of coordination, and mostly refuse to be in adjacent positions. This behavior is rather constant for moderate cholesterol amounts, and only at high cholesterol contents (>30%) the occurrence of close contacts becomes relevant. This finding is related to jumps of cholesterol chemical potential when surpassing certain concentrations in PC/Chol bilayers[19]. Similar behavior is found for DOPC but with slightly higher occurrence of direct Chol-Chol contacts (see Table 1). This finding is a consequence of the fact that cholesterol hydrophilic headgroup is rather small, and therefore, a close contact of two sterol molecules is energetically non-favorable since too much hydrophobic membrane region is exposed to water. In bilayers with Dchol, close sterol-sterol contacts are also disfavored (see Table 1).

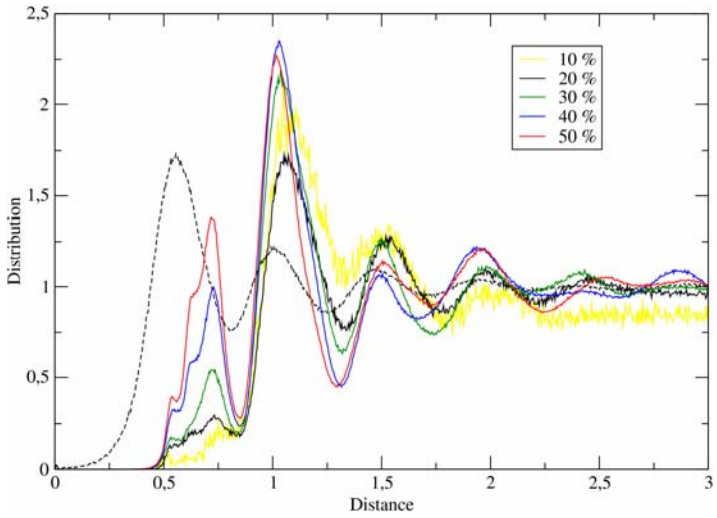

Figure 2: 1d-density functions for Chol (solid) and DSPC lipids (dashed) density functions for the simulated DSPC/Chol membranes. r stands for the radial distance to a Chol molecule. Color code indicates the sterol content.

DSPC +	**10% Chol**	**20% Chol**	**30% Chol**	**40% Chol**	**40% Dchol**	**50% Chol**
0.4-0.8 nm	0.253	0.228	0.277	0.480	0.441	0.670
0.8-1.1 nm	1.49	1.24	1.40	1.46	1.52	1.44
DOPC +	**10% Chol**	**20% Chol**	**30% Chol**	**40% Chol**	**40% Dchol**	**50% Chol**
0.4-0.8 nm	0.300	0.368	0.364	0.516	0.574	0.700
0.8-1.1 nm	1.51	1.39	1.46	1.47	1.55	1.41

Table 1: Ratio of cholesterol density averaged inside the first (0.4-0.8 nm) and second (0.8-1.1 nm) coordination shells respect to the total mean density.

The second observation reported here is related to the lateral spatial ordering of lipid molecules. We analyze this issue by computing the in-plane 2d-probability densities according to a reference axis system defined by the positions of several Chol groups. The origin is centered at the C13 group, the x-axis is defined by the C13-C18 direction, and the x-z plane is chosen to include the C13-C10 vector (Figure 1). Using this reference frame in each leaflet, we compute the 2d-probability density for carbon atoms in PC acyl tails (Figure 3a), and for atom groups of Chol (Figure 3b) in the DSPC membranes with 20 %mol Chol. As reported above, the first shell located at ~0.5 nm from the distribution center is occupied almost exclusively by PC molecules, whereas the second one at ~1 nm is where Chol molecules mostly reside. In both cases, however, it is observed that ordering is strongly suppressed in the positive direction of the x-axis due to the steric effects of C18 and C19 off-plane groups on the cholesterol rough β-face. For PC acyl chains, we observe at least three coordination shells, as a consequence of the strong ordering ability of Chol. A closer inspection of the first coordination shell reveals that PC acyl tails ordering is anisotropic in the bilayer plane and 5 clearly defined maxima can be observed. The arrangement of Chol with respect to each other is even more appealing. It discloses a clear anisotropy and a well-defined triangular symmetry with a clear peak at the α-face and two maxima at the β-face split into the two sub-faces β1 and β2 (see Figure 3b). The splitting on the rough β-face is due to the off-plane methyl groups. The 2d-density plots for DOPC bilayers display similar trends as for DSPC but with slightly less defined peaks and coordination shells, in agreement with the established fact that Chol orders saturated chains more than unsaturated ones. Finally, the importance of off-plane cholesterol methyl groups in the induced lateral ordering anisotropy is confirmed when computing the 2d-density functions for membranes with Dchol instead of Chol. In these systems, for both PC acyl tails and sterol distributions, a two-fold symmetry is observed with one broad peak at each side of the sterol plane.

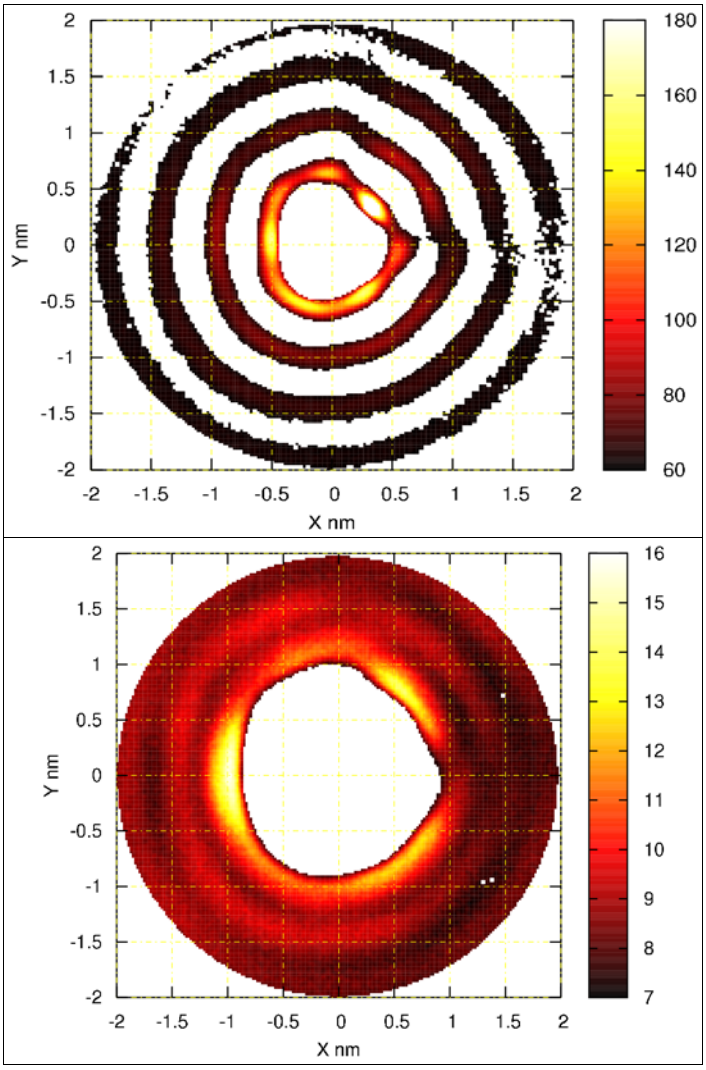

Figure 3: 2d-probability density functions for (a) DSPC acyl chains and (b) Chol molecules around a tagged Chol (see Fig. 1 for axis). Data shown for DSPC bilayers with 20 %mol of Chol.

The third finding reported here corresponds to the orientational order of Chol. We have already noticed that neighboring sterol molecules (corresponding to the 1 nm coordination shell) interact in such a way that a particular triangular anisotropy appears in their relative spatial distribution despite the fact that at least one PC molecule is intercalated between them. Cholesterol planar structure and off-plane methyl groups are fundamental to promote this organization. Moreover, our analysis shows that the relative orientation between two neighboring cholesterols is also constrained. We compute the occurrence distribution for the angle formed between the C6-C11 vectors of two neighboring cholesterol molecules at different sectors of the first Chol-Chol coordination shell. We do so by computing the relative angle of those Chol pairs at a radial distance in the [0.8-1.1 nm] range and a polar angle range that comprise a particular peak in the Chol-Chol 2d-density function (see caption of Figure 4). In Figure 4 relative angle distributions are plotted for the three preferred locations (a, b1 and

b2) and for different PC species and amounts. In all cases, we observe three maxima for each location that correspond to preferential relative orientations. These 9 preferred configurations are schematically pictured in Figure 4, but notice that some of them are equivalent. Particularly, the first peak for the a location is equivalent to the third peak in the b2 sector, third peak in a is equivalent to the first peak in b1, and third peak in b1 is equivalent to the first peak in b2. So finally, only six different configurations are selected for neighboring cholesterol molecules.

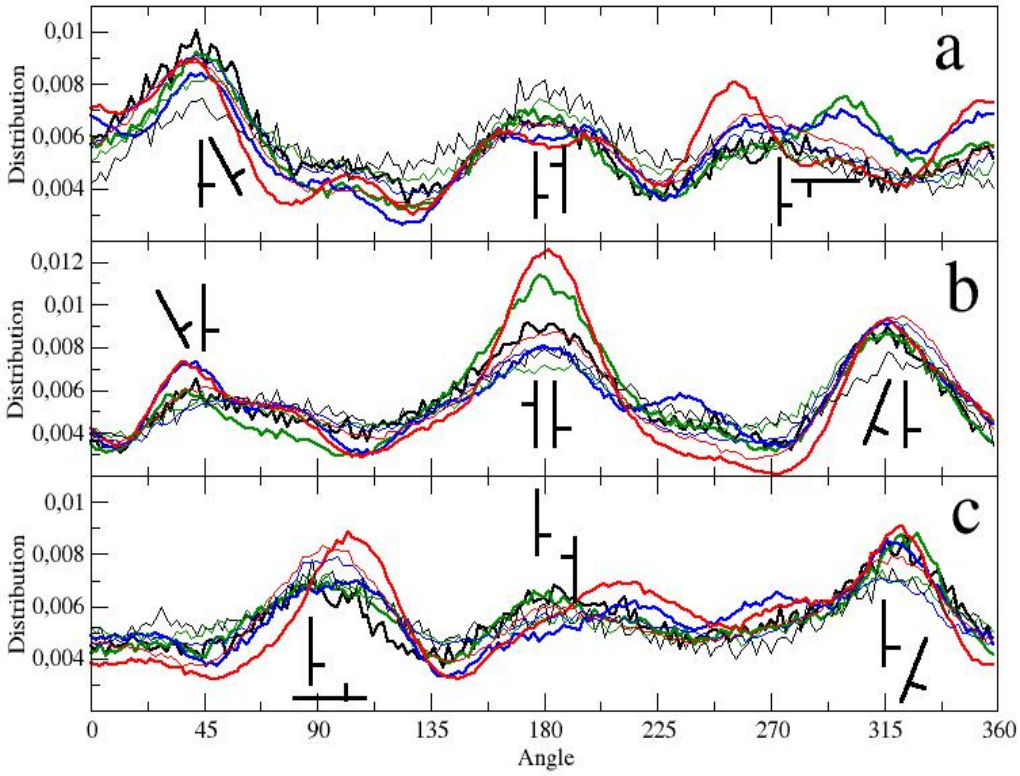

Figure 4: Angle distribution for cholesterol pairs corresponding to the three preferred locations displayed in the Chol-Chol 2d-density functions. The angle in the x-axis is the one formed by the C6-C11 vectors of each cholesterol molecule in the pair. Panel (a) refers to b1 peak (NE: Angle [22.5º-67.5º]). Panel (b) contains the data for the a peak (W: Angle [157.5º-202.5º]). Panel (c) corresponds to the b2 peak (SE: Angle [292.5º-247.5º]). Thick lines correspond to DSPC systems whereas thin curves correspond to DOPC membranes. Color code indicates cholesterol content: 20 % (black), 30 % (green), 40 % (blue) and 50 % (red). Simulations with 10 %mol of Chol are not provided here since they present poor statistics. Schematic preferred configurations are plotted at each distribution maximum.

Our results suggest that the particular characteristics of cholesterol molecular structure are largely responsible of its ordering inductive abilities. Its small headgroup disfavors direct sterol-sterol contacts in order to prevent the exposure of hydrophobic membrane regions to water. A PC lipid is, therefore, located in between two sterol molecules. Off-plane cholesterol methyl groups determine the ordering

of PCs around Chol, which in turn induces a triangular symmetry for the location of Chols in the second coordination shell with particular relative orientations. Splitting the -face into two sub-faces is fundamental for this ordering mechanism and its effect is remarkable considering the lateral liquid nature of the system.

The Chol biosynthetic pathway shows a systematic removal of methyl groups from the steroid ring. Each removal step optimizes sterol properties in terms of ordering and condensing effects[7]. This observation could lead to the wrong conclusion that the remaining methyl groups are just evolutionary fossils: however, recent studies[8-9] suggest that their presence regulates the sterol ring tilt in the bilayer, as an important factor to determine its condensing effect. Here, we provide an evidence of the importance of these methyl groups, in this case related to the molecular mechanism for a collective arrangement of cholesterol and lipid acyl chains. Such molecular self-assembly may be conceived as the initial stage for the formation of PC-Chol units resembling condensed complexes or even of a long-range superlattice structure. However, the connection of our results with both condensed complex and superlattice models cannot be directly established. MD technique used here neither does account for the chemical reactive processes involved in complex formation nor allow, so far, to perform simulations in sufficiently large systems to validate the long-range order of a superlattice structure. Moreover, due to the fluid nature of simulated membranes and the high selected temperature (338 K), the observed ordered structures are transient and local, rather than stable and long-ranged. Our results provide, however, compelling evidence for the tendency of Chols to self-assemble with PCs in particular configurations that may be relevant in lipid organization and ordering at larger length and longer time scales.

Other considerations about the ability of sterols to form ordered structures at longer ranges are given by the comparison with the simulations with Dchol. As a direct outcome of the sterol-sterol 2d-density plots, cholesterol promotes the formation of (dynamic) triangular networks whereas Dchol is more organized in ‘linear’ arrays. ‘Triangular’ configurations are more able to eventually expand into 2d sterol-rich regions than ‘linear’ patterns. Moreover, comparison of the membrane area between DSPC/Chol and DSPC/Dchol membranes reveals a counterintuitive finding: although Dchol allows a closer lateral approach (due to the absence of C18 and C19), the membrane area (for the same sterol content) is larger in DSPC/Dchol than in DSPC/Chol. Again, a three-fold symmetry in the lipid arrangement (Chol) is more able to condensate the membrane in a 2d-system than a two-fold configuration (Dchol).

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4 Meetings and conferences

Several contributions to scientific meetings have been based in my research visit. Doubtlessly, the work developed during my visit has improved and expanded my views on my expertise fields as well as lit has allowed me to be in touch with other fascinating fields. I would like to acknowledge the following persons:

- Ilpo Vattulainen (TUT, HUT)
- Mikko Karttunen (The Univ of Western Ontario)
- Emppu Salonen (Adjunct member at HUT)
- Tomasz Róg (TUT, HUT)
- Luca Monicelli (HUT, TUT)
- Teemu Murtola (HUT)
- Samuli Ollila (TUT)
- SanJa Pöyry (TUT)
- Touko Apajalahti (TUT)
- Alex H de Vries (University of Groningen)

Also as visitor member of the group I was entitled to assist to the Finish conference “National Lipid Meeting 2008” where I was able to present a poster with the title “Effect of double bond position in unsaturated phosphatidylcholine bilayers”

5 Future collaboration with host institution

The collaboration between the two groups because of my visit has been extended and strengthened. After my visit, we will continue with the current project developed which is far from been over. We are also thinking of extending our collaboration in new and exciting projects to reveal the subtle nature of the lipid membranes. In particular, we are thinking of expanding the force field parameters to use new lipids moieties. Also there is an additional possibility consisting in starting a new project which deal with some cross-grained models that will allow us to extend our results to longer time and size scales. This is very interesting since it will allow us to extend our understanding to new phenomena which are of current high interest on biology nowadays.

6 Projected publications/articles resulting or to result from your grant

From the present project we expect two publications in high impact factor journals. One publication will have a letter format and is nearly ready to be submitted at this moment. Later on, a regular paper will be edited containing a more extensive discussion of the really interesting finding we have done. Both papers will be submitted to you as soon as they will be published.

7 Other comments

I would like to acknowledge also to HUT (Technical University of Helsinki) to provide me a location to stay and access to the University facilities. This was needed as Vattulainen's group is split in between TUT (the host institution) and HUT.