Chain ordering of phospholipids in membranes containing cholesterol: What matters?

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Abstract

Cholesterol (CHOL) drives lipid segregation and is thus a key player for the formation of lipid rafts and thus for the ability of a cell to, e.g., enable selective agglomeration of proteins. The lipid segregation is driven by cholesterol’s affinity for saturated lipids, which stands directly in relation to the ability of cholesterol to order the individual phospholipid (PL) acyl chains. In this work, Molecular Dynamics simulations of DPPC (Dipalmitoylphosphatidylcholine, saturated lipid) and DLiPC (Dilinoleoylphosphatidylcholine, unsaturated lipid) mixtures with cholesterol are used to elucidate the underlying mechanisms of the cholesterol ordering effect. To this end, all enthalpic contributions, experienced by the PL molecules, are recorded as a function of the PL’s acyl chain order. This involves, the PL-PL, the PL-cholesterol interaction, the interaction of the PLs with water, and the interleaflet interaction. This systematic analysis allows one to unravel differences of saturated and unsaturated lipids in terms of the different interaction factors. It turns out that cholesterol’s impact on chain ordering stems not only from direct interactions with the PLs but is also indirectly present in the other energy contributions. Furthermore, the analysis sheds light on the relevance of the different entropic contributions, related to the degrees of freedom of the acyl chain.
Introduction

The functionality of Plasma Membranes (PM) of higher eukaryotic cells is controlled by a complex interplay of lipids of varying types, proteins, and other bioactive compounds. Within this interaction network, cholesterol (CHOL) is one of the most important ingredients exhibiting outstanding features as it is able to tune the mechanical properties and phase behavior of lipid bilayers. Ranging from concentrations of less than 10 mol% in organelle membranes to concentrations of 20-30 mol% of cholesterol in most of the PM, cholesterol leads to tighter lipid packing, making bilayers less permeable for smaller molecules and thus enhancing the PMs ability to shield the cytosol from the outside world.[1][9]

The formation of rafts is said to be directly linked to cholesterol’s affinity towards high-melting (saturated) lipids and thus has been well discussed in the literature. The idea that cholesterol’s effect on bilayer structure is universal and thus independent of composition was replaced by the notion that cholesterol’s affinity to a lipid decreases with the lipids unsaturation and varies with different head groups.[10][13] A recent study closer investigated the effects of cholesterol on unsaturated lipid bilayers, which were assumed to be unaffected by cholesterol by any means.[14] In this study, they indeed found a, though weaker, ordering effect of cholesterol on unsaturated lipids. Regen et al. used a technique coined nearest-neighbor recognition (NNR), in which pairs of like and unlike molecules are counted and hence a free energy of interaction can be calculated from it. In one approach, they compared kinked structures of cholesterol to compare the so-called template effect, where the rigid body of cholesterol is said to act as a template for ordering lipid acyl chains and the proposed umbrella model,[15] where PLs act as an umbrella, shielding the cholesterol body from unfavorable water interactions.[16][17] Their results point towards the template mechanism for the description of cholesterol’s ordering effect. In another study, they compared kinked and unkinked modifications of PLs to directly identify favorable interactions with saturated lipids vs unfavorable interactions with unsaturated lipids,[18] and via a Monte Carlo model, derived from NNR results, propose that this affinity is driven mainly by enthalpy
Figure 1: DPPC (left) and DLiPC (right) chain order parameter distribution in simulations varying in temperature and cholesterol concentration.

The important feature of domain registration is discussed to be highly dependent on composition. The degree of the relation of leaflet interdigitation on domain registration is discussed contradictory. While in a recent study the interdigitation is identified as a key driving force for domain registration another work negated any major relevance. In any way, leaflet interdigitation plays a role in ordering lipids. The higher the degree of unsaturation, the higher the leaflet interdigitation. Additionally, it is reported that cholesterol decreases leaflet interdigitation.

Cholesterol is known to decrease the water permeation by increasing acyl chain order. Even though the permeation is lowered and cholesterol does not have an impact on the PL head group region studies have shown that the hydrophobicity of the polar head group region is decreased. An early MD work indicates increased hydration of the head region. These findings are strongly related to the aforementioned umbrella model, which relates cholesterol’s ability to order acyl chains and properties at the bilayer-water interface.

All these examples show the complex nature of cholesterol’s impact on PL ordering. In this work we are taking a systematic look into each of the different sites cholesterol takes effect on from an energetic point of view. Specifically, we elucidate cholesterol’s effect on PL-PL interactions, PL-water interactions, interleaflet interactions, and, of course, the direct PL-cholesterol interaction. Each of these shed light on the interplay between those
different effects. For this purpose, we employed thorough Molecular Dynamics simulations of binary mixtures of DPPC (Dipalmitoylphosphatidylcholine) and DLiPC (Dilinoleoylphosphatidylcholine) with cholesterol, respectively, with concentrations ranging from pure PL systems to systems containing 30% cholesterol. The choice of a fully saturated (DPPC) and a polyunsaturated (DLiPC) lipid, even though rarely occurring in living organisms, provide the two extreme cases of cholesterol’s mode of action. We expect the behavior of cholesterol to change significantly between these limits. We used the well established CHARMM36 force-field, which has proven to very well reproduce lipid membrane properties and, in particular, acyl chain order.\textsuperscript{28,29} The resulting order parameter distributions for the different bilayer compositions simulated at different temperatures are shown in figure 1. In accordance with the expected properties of cholesterol, it shows a distinctively higher ordering effect on DPPC than on DLiPC. Which interactions are needed to correctly describe cholesterol ordering of saturated and unsaturated lipids? This is the key question that guides the analysis of this work.
Methods

Molecular dynamics simulations

The bilayer structures were prepared using the CHARMM-GUI Web-based graphical interface. All Molecular dynamics (MD) simulations were conducted using the CHARMM36 force-field and Gromacs 2018 MD software package. The systems were equilibrated using the established CHARMM-GUI parameter set. The TIP3P model was used as water model. To maintain the temperature the Nosé-Hoover algorithm was used with a coupling constant of 1 ps, coupling bilayer and solvent separately. To maintain the pressure at 1 atm the Parrinello-Rahman barostat was used with a coupling constant of 5 ps and a compressibility of 4.5x10^{-5} bar^{-1}. Hydrogen bonds were constraint using LINCS. Particle mesh Ewald electrostatics were used with a cutoff of 1.2 nm. The Lennard-Jones potential was shifted to zero between 1.0 and 1.2 nm, and a cutoff of 1.2 nm was used. The nonbonded interaction neighbor list was updated every 20 steps, using a cutoff of 1.2 nm. The temperature ranges, trajectory lengths, and compositions for each bilayer simulation are shown and summarized in table S1 in the supporting information.

Derivation of measured parameters

All analysis heavily relied on the MDAnalysis package for Python. All visualizations of the simulations were created using VMD.

The lipid chain order parameter $S$ is defined as $S = \langle 1.5 \cos^2 \theta - 0.5 \rangle$, where $\theta$ denotes the angle between the vector spanned by every second carbon atom in a lipid chain and average tilt vector per leaflet. Brackets indicate an average value over both chains and their carbon vectors.

Radial distribution functions (RDF) were calculated using gromacs analysis tools. All RDFs were calculated individually for each leaflet using in-plane distances and were
averaged. For the PL molecules and for CHOL the phosphor and the hydroxyl oxygen atoms respectively were used as reference positions.

The overlap score is the average overlap integral of groups of lipids based on the Bhattacharyya distance defined as

\[ BC_i(p, q) = \sum_{z \in Z} \sqrt{p_i(z) \cdot q_i(z)} \]

where \( p_i(z) \) and \( q_i(z) \) are the normalized distributions of the bilayer height (z-component) of the positions of the last 3 carbon atoms for a lipid group \( i \). In this manner, each PL molecule was assigned a group of neighboring PLs, with their P atoms located within 1 nm distance of the respective PL molecule’s P atom. Note that this definition is analogous to the nearest-neighbor \( N_N \) definition. The \( BC \) was then calculated for the z-position distributions of the lipid group and the lipids within the same radius but in the opposing leaflet. In this definition, a complete overlap of the last carbons in the PL chains would lead to an overlap score of 1. To relate the overlap to the order parameter, the order parameter of the host lipid within each group was used.

**Determination of enthalpic contributions**

![Figure 2: Identification of the different types of interaction for a PL (as CPK): nearest PL neighbors (purple), nearest CHOL neighbors (blue), non-nearest \( N_{out} \) neighbors (grey), and the surrounding water (blue shading).](image)
We separated the environment of a PL into different contributions to the ordering effect of PL chains, being interactions with its nearest neighbors ($N_n$), interactions with lipids of the outer neighboring shells (black region, $N_{out}$), the PL interactions with the opposing leaflet (white region) and the interaction with the surrounding water (blue region) (figure 2) and calculate the average sum of all interaction energy contributions of a PL with its surroundings as a function of the PL’s chain order. The interaction functions are averaged over simulations at different temperatures where the bilayers are found in an Ld/Lo state (290 K to 330 K for DLiPC, 330 K to 350 K for DPPC). Each energy is an average within an order parameter range of 0.1 and its standard deviation in each range lies at roughly 90 kJ/mol. The standard deviation is similar in all order parameter ranges, for both DPPC and DLiPC and in all CHOL compositions. Importantly, all averages presented in this work were determined with a numerical uncertainty of the mean smaller than 1 kJ/mol.
Results and discussion

PL-PL interaction

We start with the self-energy of a randomly chosen PL with order parameter $S$, determined as the average over its two acyl chains. For both PL the self-energy profiles exhibit an overall similar shape (figure 3) in the range of order parameters, accessible for both systems. Furthermore, even at the highest CHOL concentration no impact of CHOL is visible. Naturally, the interaction of the two acyl-chains dominates the dependence on the order parameter (data not shown). Note that for order parameters smaller than 0.4 the $S$-dependence is very weak.

When analyzing the interaction of different PL molecules, we distinguish nearest neighbor $N_n$ interaction from the interaction with outer neighbor shells $N_{out}$. They are separated by a distance cutoff taken from the position of the second minimum of the respective radial distribution functions with PL-phosphor and CHOL-hydroxy oxygen atoms as reference positions (figure S1 and S2).

For the $N_n$ interaction energy profiles in the pure PL bilayers, we hardly see a difference between DPPC and DLiPC in the range of $S|0.5$. Furthermore, the dependence on $S$ (figure 4).
Figure 4: A) Nearest neighbor $N_n$ PL interaction as a function of PL order. B) interaction of a PL with the outer neighbor shell $N_{out}$. The interaction functions are an average from simulations of the respective PL/CHOL mixture for temperatures of 290 to 320 K (DLiPC) and 330 to 350 K (DPPC).

A) is very weak. This insensitivity to $S$ reflects the fact that a PL chain is surrounded by PLs with a distribution of order parameters. Qualitatively, this can be expressed as a smearing-out of the self-energy curve. With a higher chain order parameter of DPPC, the interaction energy increases as is expected for more aligned acyl chains as already visible in the self-energy curves. Since the acyl chains of DLiPC are disordered, the range of high order parameters cannot be accessed here. A drastic difference becomes visible when observing the effect of CHOL: The DPPC interaction is only slightly influenced by the CHOL content up to a concentration of 20%. This observation sheds additional light on the well-known condensing effect, revealing a strong insensitivity of the DPPC-DPPC interaction upon the integration of CHOL in the membrane. In DPPC, for CHOL concentrations exceeding
20%, and in DLiPC for the complete range of CHOL concentrations, a decrease of PL-PL interaction is visible upon adding CHOL. This is a natural consequence of the decrease of the PL-density because here no condensing effect is present.

As expected the interaction from the outer neighbor shells $N_{out}$ is much smaller. However, due to its dependence on the order parameter and on the CHOL content, it cannot be neglected for a complete description of the enthalpic contributions in a membrane. The DPPC-DPPC interaction somewhat depends on the CHOL content even for small CHOL concentrations. This is in contrast to the $N_n$ interaction. Furthermore, the decrease of the interaction strength with increasing order parameter is opposite to the $N_n$ interactions. These observations are a consequence of the fact that interaction beyond the nearest neighborhood is particularly strong when both interacting lipids have a disordered acyl chain because of the increased probability of their encounter. This explains why for low order parameters of the central PL and low CHOL content, implying overall lower order parameters in the membrane, the interaction is strongest.

**PL-CHOL interaction**

One may expect that the PL-CHOL interaction is essential to understand the different impact of CHOL on the ordering of both lipids. The results for the DPPC-CHOL and DLiPC-CHOL $N_n$ interactions are shown in figure 5 A. Naturally, for higher CHOL concentration a stronger interaction is observed. This can be expressed even more quantitatively. It turns out that after normalization by the actual number of surrounding CHOL-molecules (for a given order parameter) the interaction energy per PL-CHOL pair does no longer depend on the number of surrounding CHOL-molecules (inset of figure 5). Stated differently, the PL-CHOL interaction is simply additive even for larger concentrations where a PL with, e.g., an order parameter around 0.5 is surrounded by approx. 2 CHOL-molecules.

Closer inspection shows that the DPPC-CHOL and DLiPC-CHOL $N_n$ interactions, both in absolute and relative terms, are very similar for order parameters up to 0.5 (figure 5 A).
Thus, for disordered chains the PL-CHOL interaction gives rise to an increase of PL order, in particular for higher CHOL concentrations. This is directly related to the linear increase in the average order parameters of the system.

For high order parameters the DPPC-CHOL and DLiPC-CHOL interactions seem to differ. Only the DPPC-CHOL \(N_n\) interaction strength exhibits a maximum (energy minimum) which is found at \(S=0.7\). This implies the presence of an optimum configuration of a saturated acyl chain, adapting to CHOL’s rigid body. This observation also reflects the unique ability of CHOL to inhibit gel formation for DPPC and supports the idea by Regen et al.\cite{Regen2011} that the CHOL body acts as a template for chain ordering and that any change of its structure will lead to a drastically different ordering capability. Interestingly, when con-
Figure 6: Interaction of PLs with lipids of the opposing leaflet as a function of the PL chain order parameter. The insets show the relation between a lipids overlap score, defined as the correlation of the position distributions of the last three carbon atoms, with its chain order parameter for DPPC and DLiPC bilayers.

Considering the interaction energy, normalized by the number of CHOL-neighbors, also DLiPC approaches such a maximum so that in analogy to DPPC there also exists an optimum interaction motif. Of course, due to the presence of double bonds in the acyl chains, order parameters significantly beyond that maximum cannot be explored.

In contrast to the PL-PL interaction, the PL-CHOL interaction has only a very small contribution to the overall energy of chain ordering (figure 5, B) which is in accordance with the discussion above.

**Interleaflet interaction**

Domain registration requires an interaction between the opposing leaflets of a bilayer. Here we investigate the interdependence of interleaflet interaction and the degree of leaflet intercalation and discuss possible additional factors that influence interleaflet interactions. Up to order parameters of approx. 0.5, both, DPPC and DLiPC interleaflet interaction energies increase with $S$ (figure 6). Interestingly though, the DPPC interleaflet interaction strength decreases at high order parameters whereas no reversal is observed for DLiPC. Changes in DLiPC order generally have a significantly stronger effect on the interaction than is the case for DPPC. In both cases, the addition of CHOL reduces the interleaflet interaction at a given
order parameter.

To understand the underlying sources of changed interaction we calculated the average overlap score of the PLs as a function of the PLs order parameter (insets of figure 6); see Methods. Lin et al. found that the higher the lipid unsaturation, the higher the leaflet interdigitation and, indeed, we find that the overall interdigitation is higher for DLiPC. While chain ordering of DPPC leads to a decrease in DPPC chain intercalation, the DLiPC intercalation even weakly increases with increasing DLiPC order parameter. In accordance with the work of Maibaum, the overlap score decreases for both lipids with increasing CHOL concentration.

To clarify the major differences between both PL, in the SI (figure S4) we show the interleaflet energy as a function of both overlap score and chain order parameter. For DLiPC the interaction strength increases both with overlap score (for fixed order parameter) and with order parameter (for fixed interaction strength). As, additionally, the average overlap score increases with chain order parameter, the S-dependence of DLiPC can be easily understood as a superposition of the direct chain order effect and the indirect overlap effect (overlap score increasing with chain order parameter). For DPPC the situation is more complex. As also shown in figure S4 the interleaflet interaction is again increasing with overlap score (for fixed order parameter) and order parameter (for fixed overlap score), although here the effect of order parameter is somewhat weaker than for DLiPC. Now two opposing effects have to be taken into account for DPPC. For an increasing order parameter, on the one hand, there is a direct increase of the interleaflet interaction strength. On the other hand, due to the decrease of the overlap score with order parameter, the interaction strength decreases indirectly. The first effect dominates at small S, the second effect at large S.

As again shown in figure S4 the addition of CHOL decreases the interleaflet interaction strength even for fixed order parameter and fixed overlap score. This observation, reflecting some complex impact of CHOL on the different interaction terms, naturally explains the dependence on the CHOL concentration for DPPC and DLiPC in figure 6.
Figure 7: Average number of water molecules around PL phosphor atoms as a function of the PL chain order parameter.

The influence of leaflet intercalation on domain registration is discussed contradictory by Shinoda et al. and Cheng et al.\textsuperscript{21,22} The former found a high influence of leaflet intercalation simulating different asymmetric SM/DOPC/CHOL bilayer compositions, while the latter found no influence of leaflet intercalation in POPC/DSPC/CHOL bilayers. It is reasonable to assume that the presence of domain registration goes along with a strong interleaflet interaction which, naturally, will depend on the local composition. In the SM/DOPC/CHOL setup a clear phase separation is visible with regions enriched in the unsaturated DOPC and depleted of CHOL. As concluded from our results, those regions exhibit a strong interleaflet interaction, as all three factors apply here (inherent disorder of chains of DOPC, increased intercalation and depletion of CHOL). On the other hand, the POPC/DSPC/CHOL system should show a more or less homogenous lateral distribution and thus, even though there is an overlap, should have a significantly weaker interleaflet interaction than is the case for the regions enriched in DOPC.

**PL-water interaction**

The effect of CHOL on the bilayer water interfacial structure has been widely discussed and can be conflated to the so-called umbrella model\textsuperscript{15} proposing an unfavorable interaction of water with the CHOL body and, accordingly, the PL head groups acting as an umbrella to
Figure 8: Interaction of a PL with water as a function of the PLs chain order parameter. The inset shows the ratio of water interaction and the respective average number of water molecules, thus constituting a PL-water pair energy.

shield CHOL from water. In this manner, increased CHOL concentration should lead to an increase in the interaction of PLs with water. Furthermore, as discussed in the introduction, CHOL influences the PL head group hydration. 26,27

We first looked at the effect of order parameter and CHOL content on the hydration of the PL head group region (figure 7). A neighbor cutoff was determined from the position of the first minimum of the PL-OH2 RDFs (figure S3). Surprisingly, both for DPPC and DLiPC the number of neighboring water molecules around the PL phosphor atom increases with order, though the hydration of the head group region is larger for DLiPC. Cholesterol has an opposite, albeit weak, effect on the two lipid types: While for DPPC the average number of water neighbors decreases with increasing CHOL content, it increases for DLiPC. This change of the number of water molecules with CHOL concentration is stronger for DPPC and, consequently, the trend is visible in the water interaction energies as well (figure 8).

To distinguish between the effects of the merely increased number of water molecules that interact with the PL and an optimization of a water-PL interaction configuration, we calculated the energy gain per water molecule in the first neighbor shell around the PL phosphor atoms (inset of figure 8). Remarkably, the energy contribution per water molecule no longer depends on the order of the PL nor on the CHOL concentration.

Thus, the dependence of the water interaction on lipid chain order parameter and CHOL
Figure 9: Sum of all interaction components $E_{PL-PL}$, $E_{PL-CHOL}$, $E_{\text{interleaflet}}$, $E_{\text{water}}$ and $E_{\text{self}}$ as a function of PL chain order parameter and CHOL concentration. The insets show the respective energy gain by changing the PL's chain order parameter from a disordered ($S=0.3$) to a more ordered state ($S=0.5$).

Concentration is solely a consequence of the slightly varying degree of hydration. We, therefore, found no evidence for a mechanistic effect of CHOL on the lipid water interaction as proposed by the umbrella model and agree with the literature in that CHOL does not affect the PL head group region, measured by the lipid interaction profile.

**Chain entropy**

Now we are in the position to estimate the overall enthalpic energy contribution for a randomly chosen PL by summing up all contributions, discussed so far. The result is shown in figure 9. We obtain two remarkable results. First, the results for DPPC and DLiPC hardly differ in the order parameter range between 0.3 and 0.5. This statement holds for the dependence on the order parameter as well as on the CHOL content. Note that individual energy contributions so far showed much larger differences between the saturated and the unsaturated lipids. For example, the interleaflet interaction showed very different dependencies on the order parameter for both lipids, whereas, e.g., the PL-CHOL interaction displayed a much larger dependence on CHOL concentration. Thus, we may conclude that there is a significant canceling effect.

In figure 1 we showed that CHOL has an ordering effect on DPPC roughly twice as
Figure 10: Estimation of the acyl chain entropy derived from the total sum of all enthalpic energy contributions and the order parameter distributions in the simulations for DPPC and DLiPC at two CHOL concentrations (left) and the difference between between the respective estimate for DPPC and DLiPC in bilayer composition from 0% to 30% CHOL.

strong as on DLiPC, with DPPC exhibiting overall higher order parameter values than DLiPC. Indeed, the energy gain for a transition from a disordered to a (more) ordered state (S=0.3 to S=0.5) increases linearly with CHOL concentration. However, the energy gain for the two PL types is very similar so that the impact of CHOL on PL order cannot solely be understood via its effect on the enthalpy gain.

For a complete description of the system also entropic effects have to be taken into account. An estimate \( Z(S) \) for the entropic contribution can be derived from the total interaction function \( H_{tot}(S) \) and the order parameter distribution \( p(S) \) in the simulations via the relation \( Z(S) \propto p(S) \cdot \exp \beta H(S) \) where \( \beta \) is the inverse product of the molar gas constant \( R \) and the temperature \( T \). Similar to the interaction enthalpy functions, the functions \( Z(S) \) were averaged over temperatures of 290 K to 330 K for DLiPC, 330 K to 350 K for DPPC and, for better comparability, were shifted to zero at \( S=0.35 \), which roughly lies in the order parameter region of the pure PL bilayers in the disordered phase (DPPC \( S=0.35 \), DLiPC \( S=0.25 \)) (figure 10, left).

Naturally, one observes that high order parameters are strongly disfavored. When comparing first the case without CHOL, the DLiPC entropy curve displays a somewhat stronger dependence on the order parameter. Of course, the difference would be even stronger for even higher values of \( S \) due to the strong reduction or absence of chain configurations with very high order parameters.
Since $Z(S)$ mainly reflects the degrees of freedom of the PL acyl chains, no significant dependence on the CHOL content is expected. To quantify the residual dependence on CHOL content, we have determined the differences $\Delta RT \ln Z(S)$ by comparing the entropy functions with those at zero CHOL content (figure 10, right). In the case of DPPC the differences are very small ($\Delta RT \ln Z(S)$ changes less than $\pm 1.5$ kJ/mol) and can be neglected (see also below). In the case of DLiPC, though, the addition of CHOL increases the entropic penalty for generating DLiPC chains of high order.

To estimate the impact of the residual dependence on the CHOL concentration, we recalculated the expected value of the order parameter under the assumption that $Z(S)$ does not change upon adding CHOL; see figure 11, left. In the case of DPPC the agreement with the actual average order parameter values is very good. Thus, as already mentioned just before, the weak dependence of entropy on the CHOL concentration is negligible. However, in the case of DLiPC the calculation leads to an overestimation of CHOL’s ordering effect. Thus, a prerequisite of the smaller dependence of the average order parameter on CHOL concentration for DLiPC as compared to DPPC is partly embedded in the dependence of the entropy on the CHOL content.

In a similar way one can check whether the difference of $H_{tot}(S)$ between DPPC and
Figure 12: Contributions of the total interaction enthalpy gain for ordering the respective acyl chains from $S=0.3$ to $S=0.5$. The dashed line separates contributions that are readily included in a lattice model (left), from the ones that typically are not included (right).

DLiPC or the difference of $Z(S)$ are more relevant. For this purpose, we combine the enthalpy function of one PL with the entropy function of the other PL and vice versa. If the enthalpy profiles are similar, the resulting calculated average order parameters should be mainly determined by the choice of $Z(S)$ and vice versa. Interestingly, it turns out that the predictions for the mixed combinations lie between the simulated data and, on average, are quite similar. Thus, we have to conclude that the differences between DPPC and DLPC are embedded in the enthalpy and the entropy to approx. equal parts.

**Implications for bilayer models**

The importance of nearest neighbor interactions to describe the lateral range of influence has been discussed in various simplistic models of lipid bilayers, strongly supporting the notion that lipids in a bilayer are only influenced by their nearest neighbors.\textsuperscript{11,13} The question arises whether this influence or lack thereof can effectively be observed in the interaction functions and how this observation deviates between the examined lipid types.

In this work, all energetic contributions of ordering PL chains can generally be distinguished between lateral, nearest-neighbor interactions, and the remaining interactions. In lattice models of bilayers typically the latter energetic contributions are discarded. They could be included through an additional ”effective” entropic contribution if the energies do
not depend on the CHOL concentration. To quantify the residual dependence on that concentration we display the energetic contributions for ordering from $S=0.3$ to $S=0.5$ for the different CHOL concentrations separately, distinguishing between the nearest-neighbor and non-nearest-neighbor components (figure 12).

It turns out that the dependence of all non-nearest-neighbor interactions on CHOL concentration is weak, only the PL-PL non-nearest-neighbor interaction exhibits a dependence on CHOL concentration. When comparing the extreme cases of 0% and 30% CHOL, these values vary at most by about 2.5 kJ/mol as compared to the (interpolated) value at 15% CHOL concentration for both types of PL. A scenario, where the maximum error might occur is, e.g., the modeling of lipid rafts where the CHOL concentration may spatially vary between a very low and a very high value. However, since the maximum error is even smaller than RT, models based on nearest-neighbor interaction should be able to reproduce the properties of CHOL-containing membranes.

The remaining challenge is to incorporate a chain entropy function that effectively takes into account the dependence on CHOL content. Since the chain entropy function characterizes a very local property of a chain, it is likely that one may take into account the number of neighboring CHOL molecules to specify the entropy. Work along this line is in progress.
Conclusion

In this work, we have derived the different contributions to the total interaction energy of cholesterol ordering of DPPC or DLiPC to understand the differing affinity cholesterol exhibits, as well as the underlying mechanism of cholesterol’s unique ordering capability. Having studied the prototype lipids DPPC and DLiPC as proxies for saturated and unsaturated lipids, we believe that our results provide important new insight for the interplay of the different enthalpy contributions of membranes containing cholesterol and phospholipids. A key observation is the maximum in interaction strength of the PL-CHOL interaction as a function of the order parameter which is a cornerstone for understanding the simultaneous effect of cholesterol to increase the order in the disordered phase and to fluidify the gel state. Furthermore, we could, e.g., rationalize why inherently disordered lipids display a stronger interaction between the bilayer leaflets or show that cholesterol only weakly influences the bilayer-water interaction, independent of the lipid type. This is relevant for the discussion of the umbrella model.

The results of this present work also serve as a valuable basis for the construction of a lattice model, taking into account the diverse effects of cholesterol. In light of this basis, we conclude that it is possible to formulate a bilayer lattice model based on enthalpic information not extending the nearest neighbors. Due to the lack of significant dependence on cholesterol concentration, the impact of the PL-interaction with water and the opposite leaflet can be directly taken into account by the use of an effective entropy function, as used, e.g., in. This model may help to better rationalize the impact of local interaction properties on the mesoscopic phase behavior of lipid mixtures.
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Supporting Information Available

Table S1: Parameters of the MD simulations.

| System               | Temperatures (K) | Lengths (ns) | #Phospholipids | #Cholesterol |
|----------------------|------------------|--------------|----------------|--------------|
| DPPC                 | 330-350          | >1000        | 204            | 0            |
| DLiPC                | 290-330          | >1000        | 204            | 0            |
| DPPC/CHOL10%         | 330-350          | >500         | 360            | 40           |
| DPPC/CHOL20%         | 330-350          | >800         | 280            | 70           |
| DPPC/CHOL30%         | 330-350          | >500         | 252            | 108          |
| DLiPC/CHOL10%        | 290-330          | >900         | 306            | 34           |
| DLiPC/CHOL20%        | 290-330          | >600         | 256            | 64           |
| DLiPC/CHOL30%        | 290-330          | >500         | 238            | 102          |

Figure S1: Radial distribution functions for lateral distances between P atoms. The RDFs were calculated individually for each leaflet and averaged.
Figure S2: Radial distribution functions for lateral distances between P and O atoms. The RDFs were calculated individually for each leaflet and averaged.

Figure S3: Radial distribution functions for distances between P and O atoms of surrounding water.
Figure S4: Interleaflet interaction as a function of both, the lipids overlap and its chain order parameter in respective bilayer DPPC/CHOL and DLiPC/CHOL mixtures from 0% to 30% CHOL.

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