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A molecular dynamics study of the lateral free energy profile of a pair of cholesterol molecules as a function of their distance in phospholipid bilayers

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Free energy profile of a pair of cholesterol molecules in a leaflet of 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) bilayers in the liquid-crystalline phase has been calculated as a function of their lateral distance using a combination of *NPT*-constant atomistic molecular dynamics calculations ($P = 1$ atm and $T = 310.15$ K) and the thermodynamic integration method. The calculated free energy clearly shows that the two cholesterol molecules form a dimer separated by a distance of 1.0−1.5 nm in POPC bilayers. Well depth of the free energy profile is about 3.5 kJ/mol, which is comparable to the thermal energy k_BT at 310.15 K. This indicates that the aggregation of cholesterol molecules in the bilayers depends on the temperature as well as the concentration of the system. The free energy function obtained here may be used as a reference when coarse grained potential model is investigated for this two-component system. Local structure of POPC molecules around two cholesterol molecules has also been investigated. © *2012 American Institute of Physics*. [http://dx.doi.org/10.1063/1.4704740]

I. INTRODUCTION

Cholesterol plays an important role in biological membranes. For example, the cholesterol molecules embedded in a membrane can change structural order of the membrane, membrane thickness, and membrane fluidity. In the case of membranes such as 1,2-di-palmitoyl-phosphatidylcholine, palmitoyl-sphingomyelin, and 1-palmitoyl-2-oleoyl-phos phatidylcholine (POPC) bilayers, added cholesterol gives rise to a new two-dimensional phase of the bilayer, i.e., the liquid-ordered (*lo*) phase under high concentrations of cholesterol, in addition to the original phase, i.e., the liquid-disordered (*ld*) phase at low concentrations.[1–8] This kind of phase separation in the bilayers is a basis of discussion of the existence of raft structure in the real cell membranes, which would consist mainly of the saturated phospholipids, cholesterol, and sphingolipids. However, the molecular details of the raft such as its size and lifetime are yet to be determined. Even whether it exists in the real membranes or not is not clear.

Some phenomenological models for the lateral arrangement of the cholesterol molecules such as the condensed complex model,[9] superlattice model,[10–12] and umbrella model,[13,14] have been proposed. These phenomenological models have succeeded in explaining the original experimental measurements, but they could not promote a deeper understanding of the raft. One of the reasons for this is the lack of information of the lateral interaction between the cholesterol molecules. Then, it is essential to evaluate quantitatively the lateral interactions between two cholesterol molecules embedded in the membranes if physicochemical properties of the raft are to be addressed using a statistical mechanical method. However, within our knowledge, no free-energy based investigation has been done for the interaction between them except for the one where a set of two-dimensional effective potential functions were proposed to reproduce the calculated lateral radial distribution functions from the three-dimensional atomistic molecular dynamics (MD) calculations.[15,16]

In the present paper, we evaluate the lateral free energy profile for two cholesterol molecules in POPC bilayers in the liquid-crystalline phase by calculating the mean force $\langle F(r)\rangle$ working between the two as a function of the lateral distance r between their centers of mass based on a series of 20 ns *NPT*-constant atomistic MD calculations ($P = 1$ atm and $T = 310.15$ K). To the best of our knowledge, this is the first quantitative investigation of the lateral potential of mean force between two cholesterol molecules in the lipid bilayers. To obtain $\Delta G(r)$, the lateral mean force $\langle F(r)\rangle$ was numerically integrated over r, i.e., the thermodynamic integration method.

II. CALCULATIONS

We carried out a series of *NPT*-constant atomistic MD calculations to calculate the lateral mean force $\langle F\rangle$ between two cholesterol molecules at ten distances in POPC lipid bilayers which is composed of a pair of leaflets, as follows:

The basic cell of each MD calculation contained 62 POPC molecules and two cholesterol molecules per leaflet, along with 3968 water molecules. This number of water molecules (31 water molecules per lipid) gives a fully hydrated POPC lipid bilayer in the multilamellar phase at 303.15 K.[17] We adopted a modified CHARMM27 potential of Hogberg *et al.*[18] and a rigid TIP3P model[19] for POPC and

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water, respectively. In our previous work,[20] we examined this new potential model and showed that it reproduces well the physical properties of fully hydrated POPC bilayers in the liquid-crystalline phase at ambient temperature. For example, the calculated membrane area per lipid which contains a pair of hydrocarbon chains was 65.3 Å^2, showing a good correspondence with the experimental value 68.3 Å^2.[17] For cholesterol, we adopted the potential model by Pitman *et al.*[21] The Lennard–Jones interaction was cut off at 13 Å with correction for the pressure and potential energy. The Coulomb interaction was calculated using the particle mesh Ewald method.[22] Production runs were carried out at constant pressure and temperature using the Parrinello–Rahman barostat and Nose–Hoover thermostat ($P = 1$ atm and $T = 310.15$ K).[23,24] Newton's equation of motion was numerically solved for each atom using the RESPA method with $\Delta t = 2$ fs. The length of chemical bonds where hydrogen atom is involved was constrained using the SHAKE/RATTLE/ROLL method.[25] When calculating the mean force between the two cholesterol molecules, the lateral distance between their centers of mass, r, was constrained, too, using the SHAKE/RATTLE method. The mean force was evaluated for the ten distances, that is, $r = 0.2$, 0.4, 0.7, 1.0, 1.3, 1.6, 1.9, 2.2, 2.5, and 2.8 nm.

Initial configurations for the calculation of lateral mean force were prepared as follows. A pure POPC bilayer system was equilibrated for 15 ns, and then a pair of POPC molecules which are separated by almost the same distance as the target value of r was replaced by two cholesterol molecules. After then, an additional 5 ns MD run was performed to stabilize the substituted cholesterol pair, during which the lateral distance between the two cholesterol molecules was adjusted exactly to the target value by slowly changing the constraint distance in accordance with the lateral self-diffusion coefficient of the cholesterol[26] ($10^{-12} - 10^{-11}$m^{2}s^{-1}). We carried out 20 ns production runs to average the lateral mean force for each system.

The two-dimensional mean force was calculated by averaging force working between the centers of mass of two cholesterol molecules defined by

$$F = \frac{1}{2}(\boldsymbol{F}_1 - \boldsymbol{F}_2) \cdot \boldsymbol{u}_{12}, \tag{1}$$

where $\boldsymbol{F}_1$ and $\boldsymbol{F}_2$ are the instantaneous forces working on the center of mass of each cholesterol molecule and $\boldsymbol{u}_{12}$ is the instantaneous lateral unit vector between the two centers of mass of the cholesterol molecule. It should be noted that $\boldsymbol{F}_1$ and $\boldsymbol{F}_2$ involve both cholesterol–cholesterol interactions and cholesterol–environment interactions, i.e., the surrounding POPC and water molecules. As there are two pairs of cholesterol molecules in a system, we assumed that each pair possessed independent statistics of the mean force. Thus, $\langle F \rangle$ was given by the average of two 20 ns trajectories. Further, for $r = 0.7$ nm and 2.2 nm, we prepared three additional independent 20 ns MD trajectories, i.e., four trajectories in total, in order to perform an error analysis for $\langle F \rangle$ using eight independent averages.

We estimated the two-dimensional excess Gibbs free energy profile $\Delta G^{\mathrm{ex}}(r)$ based on the thermodynamic integration method using the obtained $\langle F \rangle$ values for ten r's,

$$\begin{aligned}\Delta G^{\mathrm{ex}}(r) &= \int_{r_0}^{r} \left(\frac{\partial G(r')}{\partial r'} \right) dr' \\ &= \int_{r_0}^{r} \left(-\langle F(r') \rangle \right) dr', \end{aligned} \tag{2}$$

where $\Delta G^{\mathrm{ex}}(r = r_0 = 3.1\,\mathrm{nm})$ was set to be zero. The total free energy profile $\Delta G^{\mathrm{total}}(r)$ taking account of the ideal part, i.e., the Jacobian or area effect in the two-dimensional case, may be evaluated easily by

$$\begin{aligned}\Delta G^{\mathrm{total}}(r) &= \Delta G^{\mathrm{ex}}(r) + \Delta G^{\mathrm{id}}(r) \\ &= \Delta G^{\mathrm{ex}}(r) - k_{\mathrm{B}}T \ln \left(\frac{r}{r_0} \right). \end{aligned} \tag{3}$$

Since the symbol Δ represents the difference of thermodynamic state made by the translation of a cholesterol molecule of interest fixing the other one, a reference state, i.e., a reference distance r_0, must be defined even for the ideal part. In the present study, as stated above, r_0 has been adopted to be $r_0 = 3.1$ nm. In this case, the ideal part has positive value for $r < r_0$, whereas it shows negative value for $r > r_0$.

In order to understand the relation among these free energy functions, it is helpful to consider the lateral cholesterol–cholesterol radial distribution function $g(r)$ in this very dilute condition, i.e., two cholesterol molecules in the two-dimensional system. Then, each free energy function may be related to

$$\Delta G^{\mathrm{ex}}(r) = -k_{\mathrm{B}}T \ln \left(\frac{g(r)}{g(r_0)} \right), \tag{4}$$

$$\Delta G^{\mathrm{id}}(r) = -k_{\mathrm{B}}T \ln \left(\frac{2\pi r}{2\pi r_0} \right), \tag{5}$$

$$\Delta G^{\mathrm{total}}(r) = -k_{\mathrm{B}}T \ln \left(\frac{2\pi r g(r)}{2\pi r_0 g(r_0)} \right). \tag{6}$$

$\Delta G^{\mathrm{ex}}(r)$ obtained by Eq. (4) in the three-dimensional case was used as a starting potential function for the coarse-grained potential model, adopting a reference distance of $r_0 = \infty$.[27]

III. RESULTS AND DISCUSSION

In the present study, we are interested in the lateral properties of a cholesterol pair, where we assumed that the cholesterol pair of interest is located in a similar z-position within a leaflet of the bilayer. In order to test this assumption, first, the averaged z-position $\langle z \rangle$ of the center of mass of the cholesterol pair from the bilayer center was calculated and plotted in the upper panel of Fig. 1 as a function of their lateral distance r. The figure clearly shows that the averaged z-position depends little on r. This indicates that we may investigate the lateral properties of the cholesterol pair just by averaging the relevant lateral quantities.

The lower panel of Fig. 1 shows the calculated lateral mean force as a function of r, where the plus and minus signs on the vertical axis indicate repulsive and attractive forces, respectively. In the lower panel , the error bar at $r = 2.2$ nm is

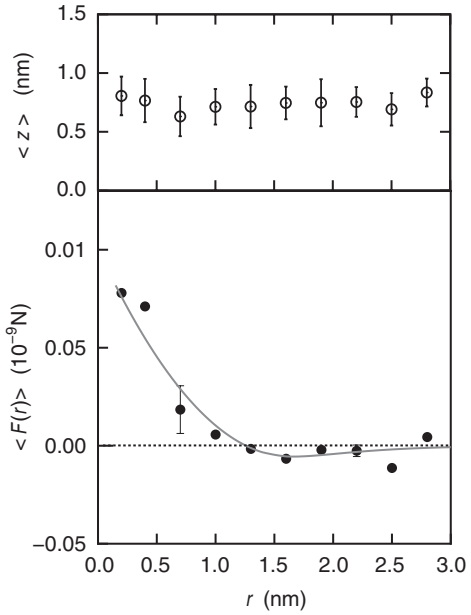

FIG. 1. The averaged z-position $\langle z \rangle$ of the center of mass of the cholesterol pair embedded in the POPC bilayers (upper panel) and the calculated lateral mean force working between them (lower panel) . The error bars for $\langle z \rangle$ represent the standard deviations over two 20 ns trajectories. The error bars for $\langle F(r) \rangle$ at $r = 0.7$ and 2.2 nm denote the standard error from eight samples. The gray line is a guide for the eye.

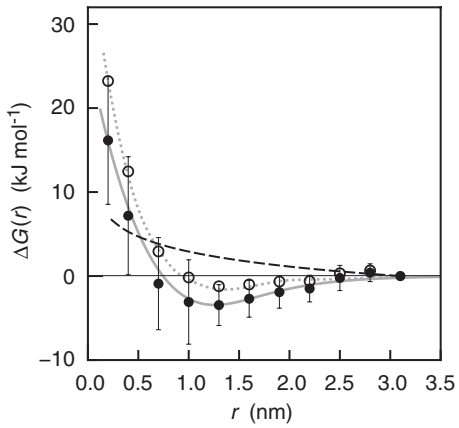

FIG. 2. The calculated free energy profile $\Delta G^{\mathrm{ex}}(r)$ (closed circles), $\Delta G^{\mathrm{id}}(r)$ (dashed line), and $\Delta G^{\mathrm{total}}(r)$ (open circles) as a function of the lateral distance between the two cholesterol molecules embedded in the POPC bilayers. The error bars denote the standard error for $\Delta G^{\mathrm{ex}}(r)$. The gray lines are a guide for the eye.

very small, whereas the one at $r = 0.7$ nm is large. The former case represents a good sampling for the relative orientation of the cholesterol pair over a period of 20 ns. The latter case comes from the close contact of two cholesterol molecules, inhibiting a good sampling of the relative orientation of the pair. In any case, they show that the statistics of the present calculations is satisfactory to discuss about the mean force.

Clearly, the lateral mean force is attractive for $r > 1.3$ nm, whereas it is repulsive for $r < 1.3$ nm. As expected, the smaller value of r, the more repulsive lateral mean force. The distance at which the mean force changes sign, around $r = 1.3$ nm, is much longer than the lateral diameter of a cholesterol molecule, 0.67 nm, as measured by Langmuir film experiments.[28] This implies that two cholesterol molecules in the POPC bilayers avoid direct contact with each other.

Figure 2 presents the calculated $\Delta G^{\mathrm{ex}}(r)$ together with the $\Delta G^{\mathrm{id}}(r)$ and $\Delta G^{\mathrm{total}}(r)$. A minimum is clearly found both for $\Delta G^{\mathrm{ex}}(r)$ and $\Delta G^{\mathrm{total}}(r)$ that results from the attractive mean force for long r. Although the well becomes a little shallow by taking account of the ideal term, the location of the minimum looks almost the same as each other, ranging$1.0 < r < 1.5$ nm, which is greater than the lateral diameter of a cholesterol molecule (0.67 nm) as stated above. On the other hand, the well depth of $\Delta G^{\mathrm{ex}}(r)$ is 3.5 kJ/mol, which is comparable to $k_{\mathrm{B}}T \sim 2.6$ kJ/mol at 310.15 K. In general, the attractive forces among molecules give rise to the liquid phase in addition to the gas phase. In the present case, the attraction in the potential of mean force among cholesterol molecules in POPC bilayers must have the system showing two phases as a function of concentration and temperature, i.e., the low-concentration phase of cholesterol and high-concentration phase. This is in good agreement with the experimentally observed phase diagram, i.e., the existence of *ld*- and *lo*-phases. At high temperatures and/or low concentrations, the cholesterol molecules may be dispersed in the bilayers. In contrast, when the temperature is low enough and/or the concentration is high enough, cholesterol molecules will form macroscopic lateral aggregations, showing phase separation.

The slope of repulsion is more gentle and reaches smaller r than the ordinary intermolecular interactions. This soft repulsion is caused by the definition of $F(r)$ where $F(r)$ is the lateral force between centers of mass of two cholesterol molecules. When two cholesterol molecules are located in the same z-position as each other as shown in Fig. 3(a), the repulsive potential curve must rise steeply at r corresponding to the lateral diameter of cholesterol molecule. However, in the present calculation, a cholesterol molecule can go onto the other one as shown in Fig. 3(b). Then, the repulsion is mild as a function of r and the two molecules can approach to each other to small r, i.e., the lateral distance between the centers of mass of the two cholesterol molecules.

Further, we analyzed the lateral arrangement of the POPC molecules around the cholesterol pair at $r = 1.3$ nm, where the system is most stable. Figure 4 shows the two-dimensional probability distribution function of the center of mass of the two tails of POPC. Average was taken over the 20 ns trajectories of the POPC molecules in one leaflet. In the figure, the origin was taken as the center of mass of the cholesterol dimer, and the x axis is in the same direction as the vector connecting two centers of mass of the cholesterol molecules. In the figure, we can observe two peaks between the cholesterol molecules above and below the x axis along the y axis. This indicates that the space between the two cholesterol molecules was occupied by two POPC molecules, as though they were stuck between the cholesterol molecules. Since $\Delta G^{\mathrm{ex}}(r)$ has its minimum around $r = 1.3$ nm, this structure stabilizes the dimer. The two peaks stated above for the center of mass of the two tails of POPC present a schematic illustration of a basic lateral structure of the two cholesterol molecules and the POPC molecules as shown in Fig. 5, where elliptic POPC

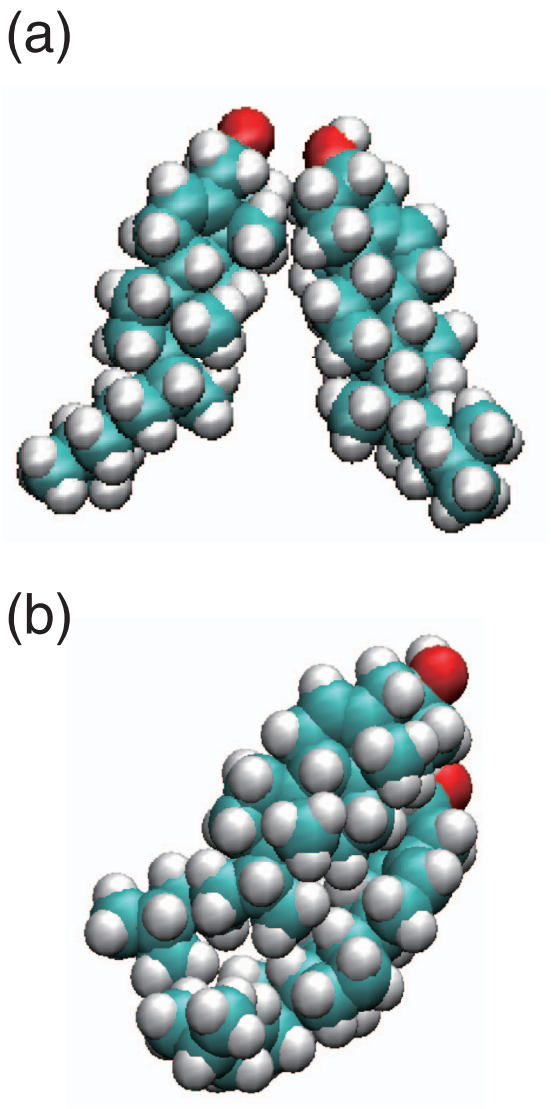

FIG. 3. Examples of relative structure of two cholesterol molecules at small r where (a) they are in the same level and (b) one go onto the other one.

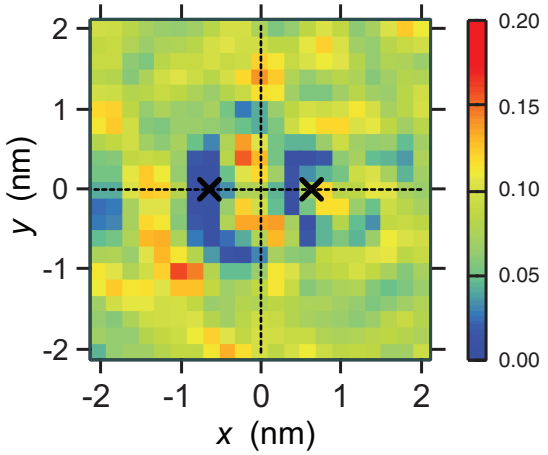

FIG. 4. The calculated probability distribution function of the center of mass of the two tails of POPC for configurations with the distance $r = 1.3$ nm between two cholesterol molecules. The origin is the center of mass of the cholesterol dimer, and the two cholesterol molecules are located at $(x, y) = (\pm 0.65, 0)$.

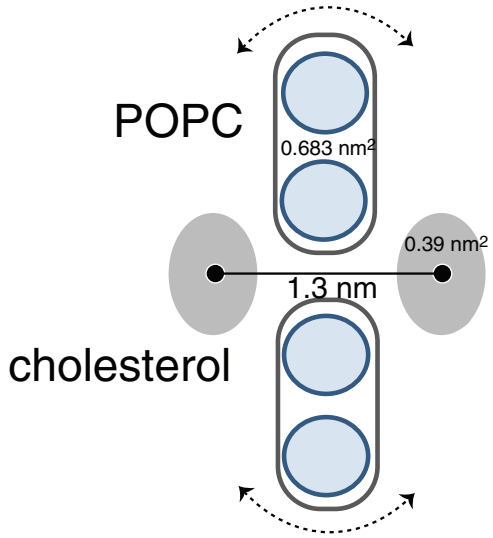

FIG. 5. A basic structural model for the local arrangement of POPC molecules between two cholesterol molecules with the most stable separation in POPC bilayers. The structure presented in the figure corresponds to a short-time average of this structural model where lipid molecules show translational libration as well as rotational one.

molecule with two acyl tails puts its one tail between two elliptic cholesterol molecules. The structure is, of course, considerably disordered in the actual bilayers where each lipid shows translational libration as well as rotational one.

IV. SUMMARY

We found that a weak but evidently attractive mean force acts between two cholesterol molecules for $r \geq 1.3$ nm in the POPC bilayers. The calculated $\Delta G^{\mathrm{ex}}(r)$ clearly shows a minimum at $r = 1.0$–1.5 nm, indicating that the two cholesterol molecules do not make a direct contact with each other, but form a dimer with the tails of the POPC molecules between them. The well depth of $\Delta G^{\mathrm{ex}}(r)$ is comparable to $k_{\mathrm{B}}T$. This implies that the lateral aggregation of the cholesterol molecules depends much on the temperature as well as the concentration. At high temperatures, cholesterol molecules are dispersed in the bilayers, i.e., the *ld* phase, whereas at low temperatures they aggregate to form a new phase, i.e., *lo* phase. Here, we must be careful that the potential of mean force is dependent on the concentration, temperature, etc. Direct use of the potential of mean force in the coarse-grained simulation may lead to a discrepancy between the simulated and real systems. The present free energy profile $\Delta G^{\mathrm{ex}}(r)$ may be used as a reference when two-dimensional coarse-grained potential model is investigated between two cholesterol molecules in an implicit POPC bilayer and when three-dimensional coarse-grained model[27] with explicit coarse-grained POPC and water is designed.

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