

Exploration of Molecular Interactions in Cholesterol Superlattices: Effect of Multibody Interactions

Juyang Huang

Department of Physics, Texas Tech University, Lubbock, Texas 79409 USA

ABSTRACT Experimental evidences have indicated that cholesterol may adapt highly regular lateral distributions (i.e., superlattices) in a phospholipid bilayer. We investigated the formations of superlattices at cholesterol mole fraction of 0.154, 0.25, 0.40, and 0.5 using Monte Carlo simulation. We found that in general, conventional pairwise-additive interactions cannot produce superlattices. Instead, a multibody (nonpairwise) interaction is required. Cholesterol superlattice formation reveals that although the overall interaction between cholesterol and phospholipids is favorable, it contains two large opposing components: an interaction favoring cholesterol-phospholipid mixing and an unfavorable acyl chain multibody interaction that increases nonlinearly with the number of cholesterol contacts. The magnitudes of interactions are in the order of kT . The physical origins of these interactions can be explained by our umbrella model. They most likely come from the requirement for polar phospholipid headgroups to cover the nonpolar cholesterol to avoid the exposure of cholesterol to water and from the sharp decreasing of acyl chain conformation entropy due to cholesterol contact. This study together with our previous work demonstrate that the driving force of cholesterol-phospholipid mixing is a hydrophobic interaction, and multibody interactions dominate others over a wide range of cholesterol concentration.

INTRODUCTION

Cholesterol is a major constituent of the mammalian plasma membranes. The molecular interactions between cholesterol and other lipid molecules have been the subjects of many studies (Finogold, 1993). We seek a general picture of cholesterol-phospholipids interaction, which can capture the key molecular interactions, and would allow us to understand a wide range of experimental results or even to predict new phenomena. In this study, we focus on the molecular interactions, which produce an interesting phenomenon in cholesterol containing lipid membranes: cholesterol superlattices.

Regular distribution of certain molecules in a lipid bilayer was initially proposed based on the observation of a series of “kinks” or “dips” in the ratio of excimer-to-monomer fluorescence of pyrene-phosphatidylcholine at some particular mole fractions (Somerharju et al., 1985; Tang and Chong, 1992; Chong et al., 1994). The bulky pyrene moieties were thought to form hexagonal superlattices to maximize separation from each other. Later, fluorescence data on cholesterol/phospholipid mixtures indicated that cholesterol molecules might also form superlattices in lipid bilayers (Chong, 1994; Virtanen et al., 1995; Liu et al., 1997). Recently, it has been suggested that lipid headgroups may also adopt superlattice distribution in phosphatidylethanolamine/phosphatidylcholine (PE/PC) bilayers (Cheng et al., 1997, 1999).

Numerous superlattice patterns have been suggested based on geometrical symmetry arguments, such as at cholesterol mole fraction (χ_{chol}) of 0.118, 0.154, 0.20, 0.25,

0.33, 0.40, and 0.5. Sugar et al. (1994) explored the formation of superlattice patterns using Monte Carlo simulations and introduced a long-range pairwise-additive repulsive interaction. They found that the long-range pairwise-additive repulsion can generate a superlattice pattern at $\chi_{\text{chol}} = 0.5$ but cannot produce large-scale superlattices of any other compositions. Thus, some key issues about the superlattices remain unsolved: How could such crystal-like structures exist in a bilayer without rigid chemical bonds between molecules? What kinds of molecular interactions are generally required to produce cholesterol superlattices? What are the magnitudes of the interaction energies? What are the physical origins of these interactions?

Recently, using x-ray diffraction and novel sample preparation procedures, we have measured the solubility limits of cholesterol in several different phospholipid bilayers (Huang et al., 1999). Interestingly, these solubility limits occur at cholesterol concentrations that correspond to well-defined cholesterol/phospholipid mole ratios: 1/1 for PE bilayers and 2/1 for PC bilayers.

We have developed a model of cholesterol-phospholipid interaction, which explains these discrete solubility limits. Our Monte Carlo simulations showed that pairwise-additive interaction was inadequate. Instead, the data can only be explained if cholesterol molecules take part in certain type of multibody interactions. The unfavorable cholesterol-cholesterol multibody interaction can be explained by our “umbrella model”: in a bilayer environment, nonpolar cholesterol must be covered by neighboring polar phospholipid headgroups to avoid the unfavorable free energy of exposing cholesterol to water. In a lattice model, this requirement can only be expressed in terms of multibody (or nonpairwise) interactions. At high cholesterol concentrations, this multibody interaction dominates all others. Thus, only those phospholipid/cholesterol lateral distributions, which meet

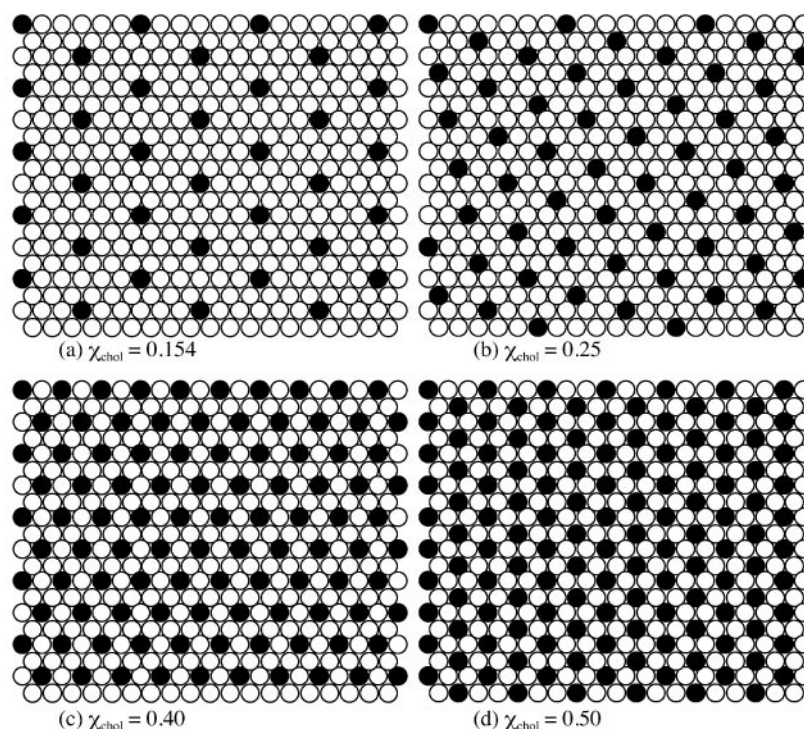
Submitted January 22, 2002, and accepted for publication April 3, 2002.

Address reprint requests to Juyang Huang, Department of Physics, Texas Tech University, Box 41051 Lubbock, TX 79409. Tel.: 806-742-4780; Fax: 806-742-1182; E-mail: juhuang@ttacs.ttu.edu.

© 2002 by the Biophysical Society

0006-3495/02/08/1014/12 \$2.00

FIGURE 1 Cholesterol superlattices at four cholesterol mole fractions. (○) Acyl chain; (●) cholesterol.



this coverage requirement, would be allowed. As the concentration of cholesterol increases, fewer and fewer lateral distributions become possible. Near the solubility limit, cholesterol and phospholipid molecules can only adapt some highly regular lateral distributions (i.e., superlattices). The solubility limit is reached when surrounding phospholipid headgroups can no longer completely cover any more cholesterol: the chemical potential of cholesterol jumps steeply, which leads to cholesterol crystal precipitation. Our model predicted that depending on the ability of phospholipid headgroups covering the neighboring cholesterol, cholesterol precipitation is most likely to occur near three discrete values of cholesterol mole fraction, 0.50, 0.57, and 0.67, which correspond to cholesterol/phospholipid mole ratios of 1/1, 4/3, and 2/1, respectively. Thus, the hydrophobic interaction has been implicated as the key driving force in the lateral organization of cholesterol in biomembranes (Huang and Feigenson, 1999).

Our work demonstrated that the regular distribution of cholesterol at high cholesterol concentration is generally resulted from multibody interactions. In this study, we extend this treatment to study the superlattices occurred at low cholesterol mole fractions. Fig. 1 shows the four cholesterol superlattices investigated in this study. They occur at cholesterol mole fraction of 0.154, 0.25, 0.4, and 0.5, corresponding to cholesterol/acyl chain ratio of 1/11, 1/6, 1/3, and 1/2, respectively. Interestingly, we found that to simulate the cholesterol superlattices at low cholesterol concentration, the interaction must be again in a form of multibody interaction (i.e., nonpairwise). This study not only estab-

lishes a theoretical foundation for superlattice formation in general but also reveals some unique interactions between cholesterol and phospholipid molecules. It shows that the overall interaction between cholesterol and phospholipids is favorable. But this favorable interaction contains two large opposing contributions: a very favorable interaction for mixing and an unfavorable interaction for phospholipid acyl chains making contact with cholesterol. This unfavorable interaction increases nonlinearly with the number of cholesterol contacts. The magnitudes of the interactions are in the order of kT . The physical origins of these interactions can be explained by our umbrella model (Huang and Feigenson, 1999): they most likely come from the requirement for polar phospholipid headgroups to cover the nonpolar cholesterol to avoid the exposure of cholesterol to water and from the sharp decreasing of acyl chain conformation entropy due to cholesterol contact. This study together with our previous work demonstrate that the driving force of cholesterol-phospholipid mixing is a hydrophobic interaction and multibody interactions dominate other interactions in a cholesterol-phospholipid bilayer from low cholesterol concentrations up to the cholesterol solubility limits.

THE MICROSCOPIC INTERACTION MODEL

A cholesterol/phospholipid bilayer is modeled as a two-dimensional triangular lattice. Each lattice site can be occupied by either a phospholipid acyl chain or by a cholesterol molecule. A phospholipid molecule occupies two

lattice sites. The Hamiltonian has two major components: one describing a large favorable interaction for cholesterol-phospholipid mixing, H_{chol} , and another for an unfavorable acyl chain multibody interaction with neighboring cholesterol, H_{chain} .

$$H_{\text{total}} = H_{\text{chol}} + H_{\text{chain}} \quad (1)$$

One of the basic requirements for cholesterol superlattices formation is that the overall interaction must be favorable for cholesterol-phospholipid mixing. Previously, we have shown that in high cholesterol concentration regime ($\chi_{\text{chol}} > 0.5$), the dominant interaction involving cholesterol is in a form of multibody interaction, which is characterized by an accelerating increasing of unfavorable interaction with the number of cholesterol-cholesterol contacts (Huang and Feigenson, 1999). In low cholesterol concentration regime ($\chi_{\text{chol}} \leq 0.5$), this type of multibody interaction becomes similar to a pairwise-additive interaction, because the chance of multiple cholesterol-cholesterol contacts is small. To simplify the model, we used a pairwise-additive interaction to describe the favorable cholesterol-phospholipid mixing. Following our early treatment (Huang and Feigenson, 1999),

$$H_{\text{chol}} = \frac{1}{2} \sum_{i,j} \Delta E_m (L_{\text{ai}} L_{\text{cj}} + L_{\text{ci}} L_{\text{aj}}) \quad (2)$$

in which L_{ai} and L_{ci} are the occupation variables (=0 or 1) of acyl chains and cholesterol, respectively. The summation i is over all lattice sites, and j is over the six nearest-neighbor sites of i only. ΔE_m is the pairwise-additive excess mixing energy of acyl chains and cholesterols, which is defined as

$$\Delta E_m = E_{\text{ac}} - (E_{\text{aa}} + E_{\text{cc}})/2 \quad (3)$$

in which E_{aa} , E_{cc} , and E_{ac} are the pairwise interaction energies between acyl chains, between cholesterol, and between acyl chain-cholesterol, respectively. The favorable cholesterol-phospholipid interaction can be expressed by a large *negative* value of ΔE_m , which is chosen to be sufficient large to prevent cholesterol clustering.

The unfavorable acyl chain multibody interaction with neighboring cholesterol is given by:

$$H_{\text{chain}} = \sum_i \sum_{s=0}^6 \Delta E_a A_s L_{\text{si}} L_{\text{ai}} \quad (4)$$

in which ΔE_a is the strength of the acyl chain multibody interaction, A_s are the energy-scaling factors, and L_{si} is the environment variable of a lattice site, which is defined as

$$L_{\text{si}} = \begin{cases} 1, & \text{if site } i \text{ has } s \text{ cholesterol} \\ & \text{as its nearest neighbor} \\ 0, & \text{otherwise.} \end{cases}$$

In Eq. 4, the summation s is over seven possible environments for the lattice site i : a site can have zero to six

cholesterols as its nearest-neighbors. We notice that the last possibility is impractical, because the second acyl chain from the same phospholipid molecule should occupy one of the nearest-neighbor sites. However, the last situation never occurs in our simulations because of low cholesterol concentration. If an acyl chain site is surrounded by s cholesterol molecules, then the multibody interaction energy for this chain will be $\Delta E_a A_s$. No energy difference is assumed for the different arrangements of these s cholesterol molecules among the nearest-neighbor sites. Seven energy-scaling factors (A_0, A_1, \dots, A_6) define the relative magnitude of the multibody interaction in the seven possible situations, and ΔE_a determines the overall strength of the acyl chain multibody interaction. In this study, ΔE_a is chosen to be positive, which implies that the multibody interaction of acyl chains with their nearest-neighbor cholesterol is unfavorable.

If the interaction between an acyl chain and its nearest-neighbor cholesterol is pairwise and additive, then the seven energy-scaling factors would become ($A_0, A_1, A_2, A_3, A_4, A_5, A_6$) = (0, 1, 2, 3, 4, 5, 6), i.e., the interaction energy increases *linearly* with number of cholesterol contacts. A multibody interaction generally does not have this linearity, and the magnitude of the interaction is determined by all six nearest-neighbors together. For example, ($A_0, A_1, A_2, A_3, A_4, A_5, A_6$) can be (0, 1, 3, 6, 10, 15, 21) or (0, 0, 1, 3, 6, 7, 8).

SIMULATION METHOD

Simulations were performed on a triangular lattice with a standard periodical boundary condition. Neighboring cholesterols and acyl chains can exchange their position with a probability given by the Metropolis method (Metropolis et al., 1953). All simulations started from an ideal mixture of given composition. The typical equilibrium time was 15,000 to 30,000 Monte Carlo steps. At a superlattice composition, multiple superlattice domains (with different orientations) usually form at beginning. Eventually, one domain would takeover all others and cover the entire simulation lattice. From that point on, the superlattices become quite stable with almost no defect.

Simulating superlattices encounters a unique simulation size requirement: Each superlattice pattern has its own periodicity. A perfect superlattice pattern can only be generated if the size of the simulation lattice is multiple of the pattern periodicity. Otherwise, defects will be introduced. The periodicities for superlattices at cholesterol mole fraction 0.154, 0.25, 0.4, and 0.5 are 12, 14, 4, and 6, respectively. Thus, the lowest common denominator is 84. The simulation lattice used in this study is 84×84 , which can accommodate all four superlattice patterns in Fig. 1. Comparing the trial simulations using 168×168 and 336×336 lattices, the energy differences were less than 0.4%.

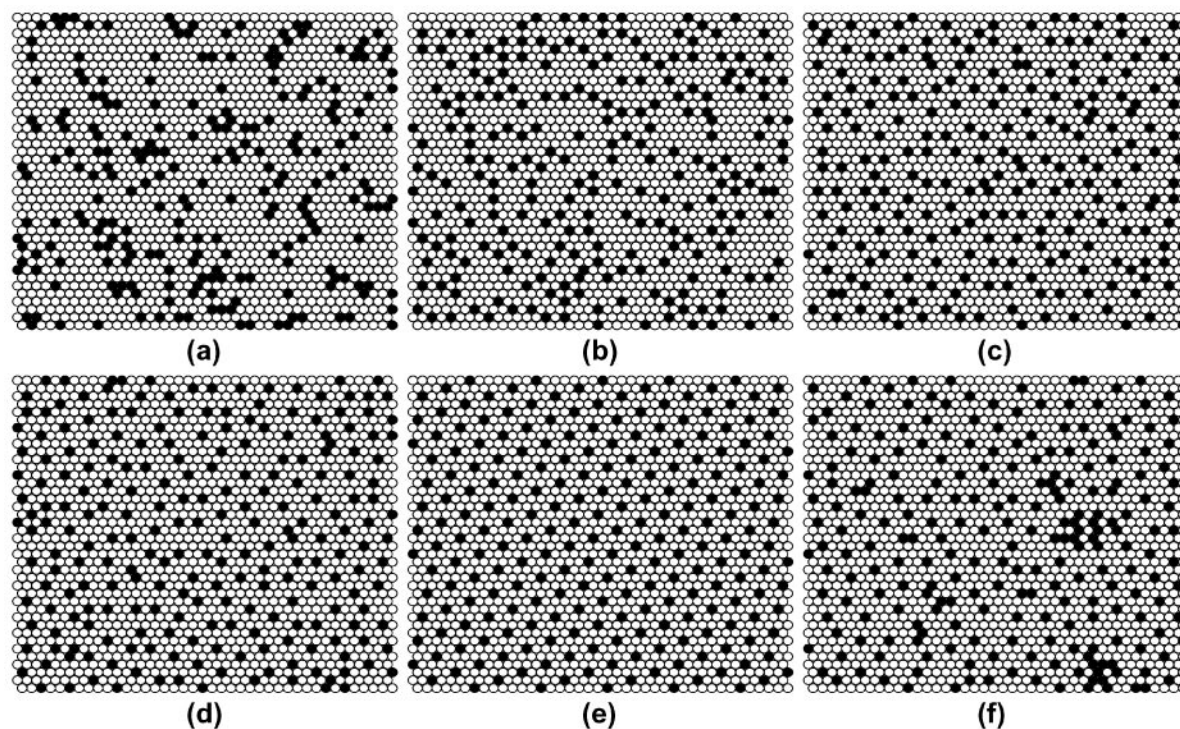


FIGURE 2 Snapshots of cholesterol and phospholipid lateral distributions at $\chi_{\text{chol}} = 0.25$ simulated with various combinations of interaction energies. The energy-scaling factors are $(A_0, A_1, A_2, A_3, A_4, A_5, A_6) = (0, 0, 1, 1, 1, 1, 1)$. (a) Random distribution; (b) $\Delta E_m = -2$ kT and $\Delta E_a = 0$; (c) $\Delta E_m = -2$ kT and $\Delta E_a = 1.5$ kT; (d) $\Delta E_m = -2$ kT and $\Delta E_a = 2.0$ kT; (e) $\Delta E_m = -2$ kT and $\Delta E_a = 2.5$ kT; (f) $\Delta E_m = -1$ kT and $\Delta E_a = 2.5$ kT. (○) Acyl chain; (●) cholesterol.

RESULT

A careful examination of Fig. 1, *b* to *d* would confirm that in all three cases, the local environments of cholesterol are quite similar: Every cholesterol molecule is surrounded by six acyl chains. However, the local environments of acyl chains are very different in these superlattice patterns: The number of cholesterol surrounding each acyl chain is one, two, and three in Fig. 1, *b*, *c*, and *d*, respectively. This analysis is crucial to understand the formation of superlattices. First, it indicates that it is the local environment of acyl chains that distinguishes different superlattices. Second, it suggests that these three superlattice patterns could belong to a same family of superlattices. Finally, to generate these superlattice patterns, the key interaction is the interaction of acyl chains with their nearest neighbors. We will discuss the microscopic interaction energies required to generate each pattern in detail.

Superlattice at $\chi_{\text{chol}} = 0.25$

Fig. 2 illustrates the type of microscopic interactions required to generate the superlattice pattern at $\chi_{\text{chol}} = 0.25$. Fig. 2 *a* shows a snapshot of random mixing of acyl chains with cholesterol at $\chi_{\text{chol}} = 0.25$. By random mixing, some cholesterol molecules form small clusters. Because super-

lattices require no cholesterol-cholesterol contact, a strong unfavorable interaction between cholessterols, or equivalently, a strong favorable interaction between cholesterol and acyl chains is needed, which corresponds to a large negative value of ΔE_m in Eq. 2. Fig. 2 *b* is a snapshot simulated with $\Delta E_m = -2$ kT. In this distribution, most cholesterol clusters are eliminated, but cholesterol distribution shows no long-range order.

We extensively tested the possible values of ΔE_m and found that no value of ΔE_m could produce a large-scale superlattice at this composition. The interactions in Eq. 2 and 3 are limited to the nearest-neighbors only. Sugar et al. (1994) used a long-range repulsive energy between pyrene-labeled acyl chains to simulate superlattices. Similarly, no large-scale superlattice formed at this concentration. These results led us to an inescapable conclusion: if the interactions between molecules are pure pairwise-additive, regardless their magnitudes and the range of interaction, no superlattice could form at this composition.

Could other types of interactions required for the superlattice formation? We discovered that to generate the superlattice, a second type of interaction is required. In Fig. 2, *a* and *b*, acyl chains have variable number of cholesterol contacts, ranging from 0 to 4. The superlattice pattern at $\chi_{\text{chol}} = 0.25$ (Fig. 1 *b*) is characterized by each acyl chain

having exact one cholesterol as its nearest neighbor. To produce the superlattice pattern, we found that in addition to a large favorable interaction for cholesterol-phospholipid mixing, acyl chains must have an unfavorable multibody interaction with their nearest-neighbor cholesterols. Eq. 4 describes such interaction. The superlattice pattern at $\chi_{\text{chol}} = 0.25$ can be produced if a high energy penalty is assigned whenever an acyl chain has two or more cholesterol contacts. A simple way to achieve this is to assign the seven energy-scaling factors as $(A_0, A_1, A_2, A_3, A_4, A_5, A_6) = (0, 0, 1, 1, 1, 1, 1)$, i.e., if an acyl chain has zero or one cholesterol contact the interaction energy is low, and if an acyl chain has two or more cholesterol contacts, a high energy penalty is paid. Fig. 2 *c* to *e* show snapshots of cholesterol lateral distributions simulated with a fixed pairwise-additive interaction ($\Delta E_m = -2$ kT), and various strength of acyl chain multibody interaction. As the strength of the acyl chain multibody interaction increases, the number of acyl chains having two or more cholesterol contacts decreases, and cholesterol molecules become more uniformly distributed. At $\Delta E_a = 2.5$ kT (Fig. 2 *e*), a perfect superlattice pattern appears.

There is more than one way to choose the energy-scaling factors. The key is that A_0 and A_1 must be much smaller than A_2 to A_6 . For example, $(A_0, A_1, A_2, A_3, A_4, A_5, A_6) = (0, 1, 4, 5, 6, 7, 8)$ will work as well. This nonlinear increase can only be expressed as a multibody interaction in a lattice model.

Fig. 2 *f* shows a snapshot of cholesterol-acyl chain distribution simulated with $\Delta E_m = -1$ kT and $\Delta E_a = 2.5$ kT. It demonstrates that when the favorable mixing energy ΔE_m is too small, superlattice will not form and cholesterol clustering can occur. The acyl chain multibody interaction is unfavorable for cholesterol-phospholipid mixing. If it were the only interaction in a simulation, it would create many huge cholesterol clusters. Therefore, to generate a superlattice, there must be a delicate balance between the favorable cholesterol-phospholipid mixing energy and the unfavorable acyl chain multibody interaction energy.

Superlattice at $\chi_{\text{chol}} = 0.4$

The superlattice pattern at $\chi_{\text{chol}} = 0.40$ (Fig. 1 *c*) is characterized by each acyl chain having exact two cholesterol as its nearest neighbors. Similar to the superlattice at $\chi_{\text{chol}} = 0.25$, it requires a large favorable cholesterol-phospholipid mixing energy as well as an unfavorable acyl chain multibody interaction (see Fig. 4, *a-c*). The favorable mixing energy can be expressed by a large negative value of ΔE_m , and the acyl chain multibody interaction can simply be expressed by assigning the seven energy-scaling factors as $(A_0, A_1, A_2, A_3, A_4, A_5, A_6) = (0, 0, 0, 1, 1, 1, 1)$, i.e., the energy penalty is high when an acyl chain has three or more cholesterol as its nearest-neighbors. Again, the key is that A_0 to A_2 must be much smaller than A_3 to A_6 . For example,

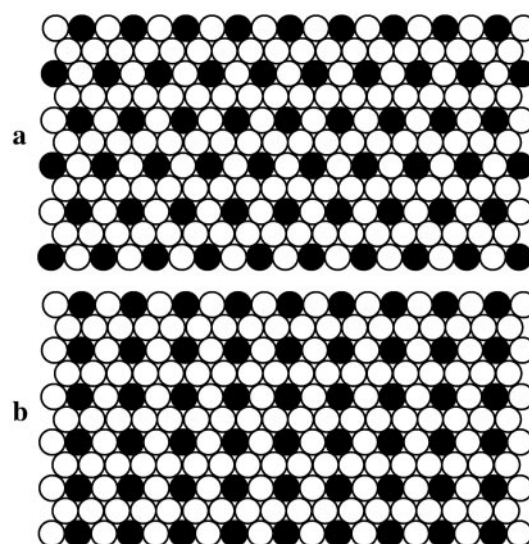


FIGURE 3 Two possible regular distribution patterns at $\chi_{\text{chol}} = 0.40$. (○) Acyl chain; (●) cholesterol.

$(A_0, A_1, A_2, A_3, A_4, A_5, A_6) = (0, 1, 2, 5, 6, 7, 8)$ will also work.

The simulations were complicated by the fact that there are two possible regular distribution patterns at $\chi_{\text{chol}} = 0.40$ (Fig. 3). In both patterns, each acyl chain has exact two cholesterols as its nearest-neighbors. Thus, the energies are identical in both patterns according to our Hamiltonian. The pattern in Fig. 3 *a* is a hexagonal distribution, and that in Fig. 3 *b* is a rectangular one. The computer-simulated pattern in Fig. 4 *c* contains domains of both patterns in Fig. 3, which reflects the energy degeneracy in our model.

Superlattice at $\chi_{\text{chol}} = 0.5$

The superlattice pattern at $\chi_{\text{chol}} = 0.5$ (Fig. 1 *d*) has a special significance for cholesterol in PE bilayers. Using x-ray diffraction, we found that the highest equilibrium cholesterol mole fraction can be reached in a PE bilayer is 0.5. Above that, excess cholesterol precipitate and form cholesterol monohydrate crystals (Huang et al., 1999). Our Monte Carlo simulations showed that at $\chi_{\text{chol}} = 0.5$, cholesterol form a superlattice pattern in PE bilayers, and the chemical potential of cholesterol jumps steeply (Huang and Feigenson, 1999).

This particular superlattice pattern (Fig. 1 *d*) was first simulated by Sugar et al. (1994) using a long-range pairwise-additive repulsive interaction. We have shown that this pattern can also be produced by a nearest-neighbor favorable mixing interaction (Huang and Feigenson, 1999). Similar to the energy formulations at $\chi_{\text{chol}} = 0.25$ and 0.4, the superlattice can be generated with a large negative value of ΔE_m , and an acyl chain multibody interaction, such as $(A_0, A_1, A_2, A_3, A_4, A_5, A_6) = (0, 0, 0, 0, 1, 1, 1)$. The effect of

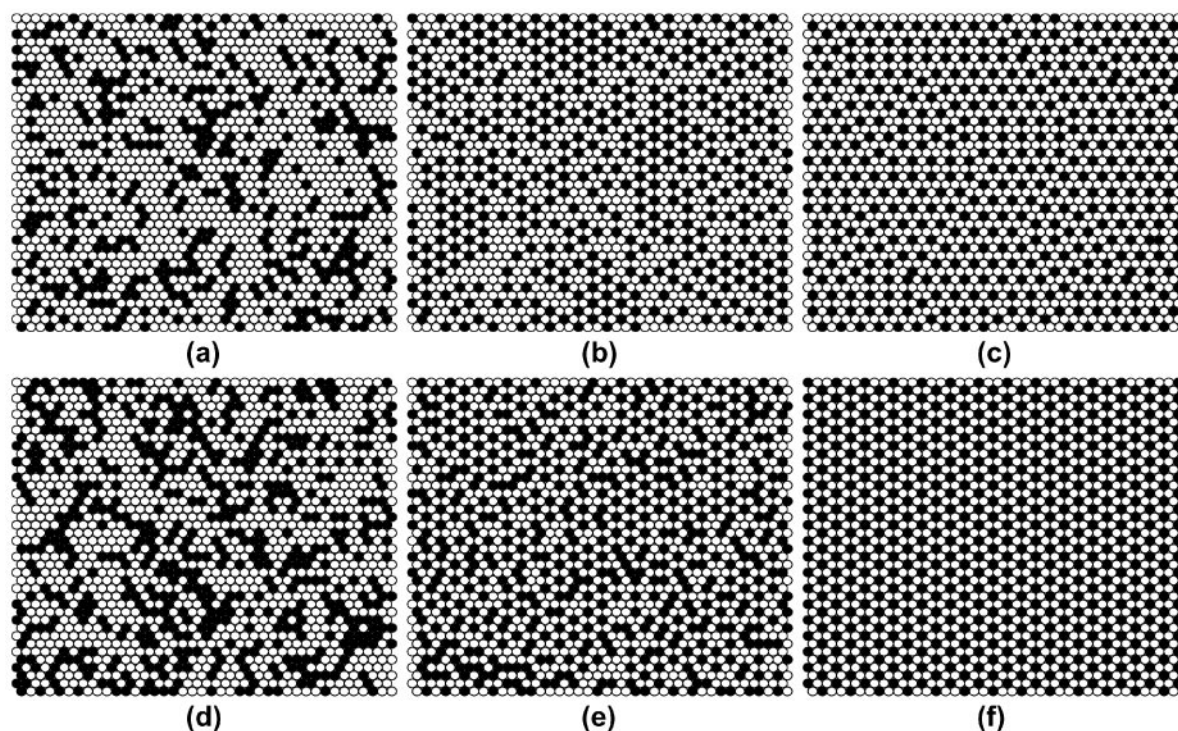


FIGURE 4 Snapshots of cholesterol and phospholipid lateral distributions simulated with various combinations of interaction energies. (a–c) Distributions at $\chi_{\text{chol}} = 0.40$ simulated with the energy-scaling factors $(A_0, A_1, A_2, A_3, A_4, A_5, A_6) = (0, 0, 0, 1, 1, 1, 1)$. (a) Random distribution; (b) $\Delta E_m = -2.5$ kT and $\Delta E_a = 0$; (c) $\Delta E_m = -2.5$ kT and $\Delta E_a = 2.5$ kT. (d–f) Distributions at $\chi_{\text{chol}} = 0.50$ simulated with a pairwise interaction only. (d) Random distribution; (e) $\Delta E_m = -1.3$ kT; (f) $\Delta E_m = -2.5$ kT. (○) Acyl chain; (●) cholesterol.

the acyl chain multibody interaction is to ensure each acyl chain having a maximum of three cholesterol contacts. However, this superlattice pattern is the *only pattern* at this composition that each cholesterol molecule has no contact with other cholesterol, and at the same time each acyl chain has exact three cholesterol contacts. Thus, the effect of the acyl chain multibody interaction can also be achieved by a large negative value of ΔE_m alone. The distinction between the multibody and pairwise interactions disappears at this particular composition. Generally, forming a superlattice requires a multibody interaction. The only exception is this one, which can be generated with a pairwise-additive interaction alone. This explains the early simulation result by Sugar et al. (1994).

The fact that pairwise interactions cannot generate superlattice at $\chi_{\text{chol}} < 0.5$ indicates that the Gibbs free energy is not at minimum if a system stays at a superlattice distribution. A large pairwise cholesterol-cholesterol repulsive interaction tends to separate cholesterol. However, corresponding to a given average separation (or a given average total energy of the system), there are many possible cholesterol lateral distributions, especially at low cholesterol concentration. A system is unlikely to stay at the lowest energy distribution (i.e., a superlattice), because with a slightly higher total energy, many other (nonsuperlattice) distributions become possible. Thus, by staying away from a su-

perlattice distribution, the Gibbs free energy is minimized due to the gain in entropy.

Superlattice at $\chi_{\text{chol}} = 0.154$

Unlike the superlattice patterns at $\chi_{\text{chol}} = 0.25, 0.40$, and 0.5 , the superlattice pattern at $\chi_{\text{chol}} = 0.154$ (Fig. 1 a) requires a second nearest-neighbor interaction. In this pattern, each cholesterol molecule has six acyl chains forming a nearest-neighbor shell, and there is also a network of acyl chains with no cholesterol contact evenly separating these shells from each other. We found that to simulate this superlattice, three interactions are required: 1) a strong favorable cholesterol-phospholipid mixing energy; 2) an unfavorable acyl chain nearest-neighbor multibody interaction; and 3) for an acyl chain with no cholesterol contact, the energy is lowered (by ~ 0.3 kT) if it is in contact with other acyl chains, which do have cholesterol contacts. The third interaction mimics a long-range repulsive interaction between cholesterol, i.e., energy is lowered if the separation between cholesterol increases. Sugar et al. (1994) studied such long-range interactions with a cutoff radius up to the 30th neighbor. The justification for the repulsive interaction is that bulky molecules, like cholesterol, may induce steric elastic strain in the acyl chain lattice. Our treatment is

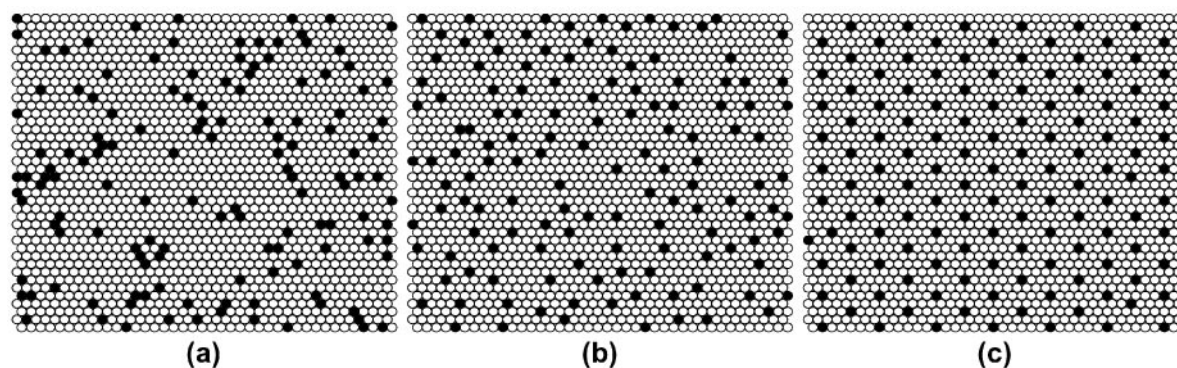


FIGURE 5 Snapshots of cholesterol and phospholipid lateral distributions at $\chi_{\text{chol}} = 0.154$. The energy-scaling factors are $(A_0, A_1, A_2, A_3, A_4, A_5, A_6) = (0, 0, 1, 1, 1, 1, 1)$. (a) Random distribution; (b) $\Delta E_m = -1$ kT and $\Delta E_a = 2.5$ kT, without the second nearest-neighbor interaction; (c) $\Delta E_m = -1$ kT and $\Delta E_a = 2.5$ kT, with the second nearest-neighbor interaction. (○) Acyl chain; (●) cholesterol.

equivalent to a cutoff distance at the second nearest neighbor. Fig. 5 *a* shows a random mixing of acyl chains with cholesterol at $\chi_{\text{chol}} = 0.154$. Fig. 5 *b* shows a distribution simulated with the first two interactions stated above but without the third interaction. Cholesterol molecules have no long-range order, because acyl chains with no cholesterol contact can distribute freely. Fig. 5 *c* shows a superlattice pattern simulated with all three interactions together. It is possible that the first and the third interactions can be combined as one long-range cholesterol-cholesterol repulsive energy as did by Sugar et al. (1994). More study is underway for this superlattice and others at $\chi_{\text{chol}} < 0.25$.

Hamiltonian for multiple superlattices

In the above examples, a set of interaction parameters can generate one superlattice at one particular composition but not at others. Experimentally, there are indications that superlattices could occur at several different compositions for a given phospholipid/cholesterol system. Could there be a Hamiltonian, which can generate a series of superlattices? We found that it is possible to generate the superlattices at $\chi_{\text{chol}} = 0.25, 0.4$, and 0.5 with a same Hamiltonian. The basic requirement is that there must be a strong favorable cholesterol-phospholipid mixing energy and an unfavorable acyl chain multibody interaction, which has an *accelerating* increase in its magnitude with the number of cholesterol contacts, specifically, from A_1 to A_3 . For example, if we assign the seven energy-scaling factors of acyl chain multibody interaction as $(A_0, A_1, A_2, A_3, A_4, A_5, A_6) = (0, 1, 3, 6, 10, 15, 21)$ or $(0, 0, 1, 3, 6, 7, 8)$, it could generate all three superlattices. The key is that A_2 must be much larger than A_1 to generate the superlattice at $\chi_{\text{chol}} = 0.25$, and A_3 must be much larger than A_2 to generate the superlattice at $\chi_{\text{chol}} = 0.40$. The values of A_4 to A_6 are not critical because even pairwise-additive interaction can generate the superlattice at $\chi_{\text{chol}} = 0.50$.

Fig. 6 shows snapshots of cholesterol/phospholipid lateral distribution at various cholesterol compositions, simulated with $\Delta E_m = -6.5$ kT, $\Delta E_a = 2.3$ kT, and the energy-scaling factors $(A_0, A_1, A_2, A_3, A_4, A_5, A_6) = (0, 0, 1, 3, 6, 7, 8)$. The large negative value of ΔE_m promotes cholesterol-phospholipid mixing and prevents the formation of cholesterol clusters. At $\chi_{\text{chol}} = 0.21$ (Fig. 6 *a*), all cholesterol are well separated from each other, but no long-range order is present due to low cholesterol concentration. At $\chi_{\text{chol}} = 0.25$ (Fig. 6 *b*), a superlattice pattern forms, which is characterized by each acyl chain having exact one cholesterol contact. The relative magnitude of energy-scaling factors A_1 and A_2 is directly responsible for the formation of superlattice at this composition. $A_1 = 0$ implies low energy penalty for any acyl chain with one cholesterol contact; whereas $A_2 = 1$ implies that the energy penalty for an acyl chain having two cholesterol contacts is one ΔE_a . To lower the energy, acyl chains avoid two or more cholesterol contacts. The superlattice pattern in Fig. 6 *b* is a distribution at this composition in which every acyl chain has only one cholesterol contact. If more cholesterol molecules are added to the mixture, it would force some acyl chains to have two cholesterol contacts. This is illustrated in Fig. 6 *c*. At $\chi_{\text{chol}} = 0.28$, the long-range order of cholesterol is disrupted. As the cholesterol concentration continues to increase (Fig. 6, *d* and *e*), the superlattice pattern of $\chi_{\text{chol}} = 0.4$ begins to form, which is characterized by each acyl chain having exact two cholesterol contacts. Similarly, the relative magnitude of scaling factors A_2 and A_3 is directly responsible for the formation of superlattice at $\chi_{\text{chol}} = 0.4$. Because A_3 is three times larger than A_2 , a much larger energy penalty would have to be paid if an acyl chain has three cholesterol contacts instead of two. To lower the energy, acyl chains avoid three or more cholesterol contacts, which results a superlattice distribution at $\chi_{\text{chol}} = 0.4$ (Fig. 6 *e*). Fig. 6, *f* to *i* shows snapshots of cholesterol distributions as the concentration of cholesterol continuously in-

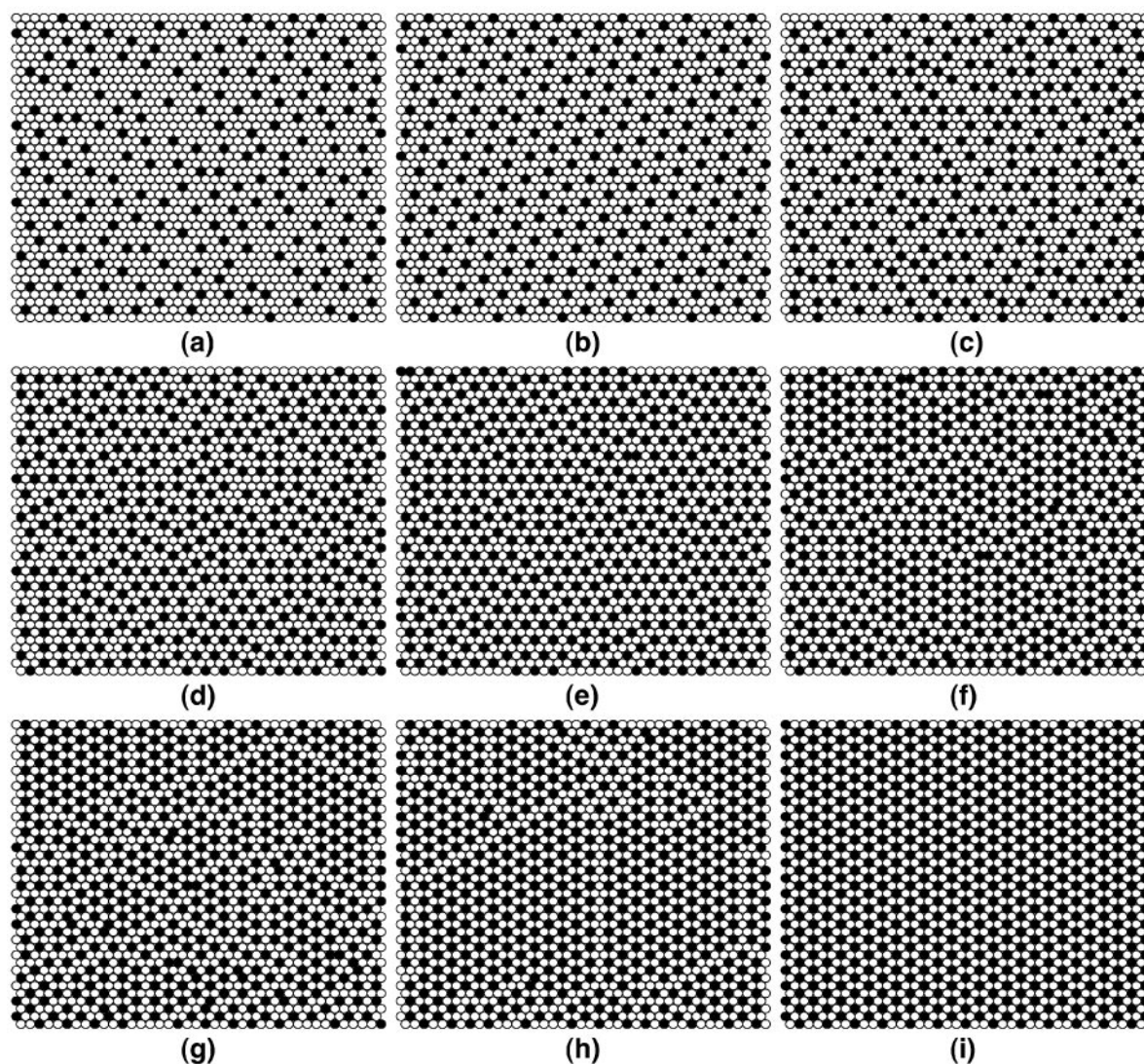


FIGURE 6 Snapshots of cholesterol and phospholipid lateral distributions at various cholesterol mole fractions simulated with a same set of interaction parameters: $(A_0, A_1, A_2, A_3, A_4, A_5, A_6) = (0, 0, 1, 3, 6, 7, 8)$, $\Delta E_m = -6.5$ kT, and $\Delta E_a = 2.3$ kT. (a) $\chi_{\text{chol}} = 0.21$; (b) $\chi_{\text{chol}} = 0.25$, a superlattice; (c) $\chi_{\text{chol}} = 0.28$; (d) $\chi_{\text{chol}} = 0.37$; (e) $\chi_{\text{chol}} = 0.40$, a superlattice; (f) $\chi_{\text{chol}} = 0.43$; (g) $\chi_{\text{chol}} = 0.45$; (h) $\chi_{\text{chol}} = 0.47$; (i) $\chi_{\text{chol}} = 0.50$, a superlattice. (○) Acyl chain; (●) cholesterol.

creases up to $\chi_{\text{chol}} = 0.5$. At $\chi_{\text{chol}} = 0.43$ (Fig. 6 *f*), the size of superlattice domains of $\chi_{\text{chol}} = 0.4$ becomes small. Above $\chi_{\text{chol}} = 0.45$, domains of the superlattice of $\chi_{\text{chol}} = 0.5$ increase in size and percolate at $\chi_{\text{chol}} = 0.5$. Above $\chi_{\text{chol}} = 0.5$, our Hamiltonian (Eq. 2) becomes invalid, because a cholesterol-cholesterol multibody interaction should be used to represent a strong hydrophobic interaction (Huang and Feigenson, 1999).

DISCUSSION

Possible physical origin of the interactions

A number of molecular interactions were suspected to be responsible for superlattice formation (Tang and Chong,

1992; Cheng et al., 1997; Somerharju et al., 1999). In this study, we show that only certain types of interaction with certain magnitude could contribute. To form a cholesterol superlattice, two opposing types of interactions are needed: a large interaction that favors the cholesterol-phospholipid mixing and a smaller unfavorable acyl chain multibody interaction that increases nonlinearly with the number of cholesterol contacts. The combined effect of both interactions must still favor the cholesterol-phospholipid mixing. The magnitude of the interactions should be in the order of kT.

There are several possible sources of molecular interaction, which can contribute to the favorable interaction for cholesterol-phospholipids mixing. It has been proposed that

when an acyl chain is substituted by a guest molecule (e.g., a pyrenylacyl or a cholesterol), which has a larger cross-sectional area, it imposes a steric strain in the acyl chain matrix. To minimize the strain, the bulkier guest molecules tend to maximize their separation, which provides a driving force for cholesterol-phospholipid mixing (Chong, 1994; Virtanen et al., 1995). Another possible source is from the imbalance between the effective head group and acyl chain cross-sectional areas. The effective size of the head group of a typical PC is significantly larger than that of its two acyl chains. A regular distribution of bulky cholesterol in acyl chain region can provide the maximum relief to the packing frustration (Somerharju et al., 1999). However, the magnitudes of the above interactions in fluid-phase bilayers remain to be determined.

Recently, we proposed the umbrella model, which suggests that hydrophobic interaction is a strong driving force for cholesterol-phospholipid mixing. In a bilayer environment, nonpolar cholesterol must be covered by neighboring polar phospholipid headgroups to avoid the unfavorable free energy of exposing cholesterol to water. The requirement promotes the mixing of cholesterol with phospholipids. The magnitude of the interaction could reach several kT. This hydrophobic interaction dominates any other interactions at high cholesterol concentration and dictates the solubility limit of cholesterol in a bilayer. At low cholesterol concentration, the coverage for cholesterol is still required. This interaction should be particularly strong in cholesterol-PE bilayers. We have shown that PE molecules are not able to cover any cholesterol clusters at all due to the small size of their headgroups. In PC bilayers, the magnitude may be smaller, due to larger headgroup size of PC (Huang and Feigenson, 1999).

The presence of a large unfavorable acyl chain multibody interaction arises from this study. It is an essential requirement for superlattice formation at low cholesterol concentration. This interaction has following properties: 1) the interaction is on acyl chains; 2) it increases steeply with the number of cholesterol contacts; 3) the magnitude is approximately several kT. What could be the physical origin of this interaction? We believe that the answer can be found in our umbrella model: it most likely comes from the reduction of acyl chain conformation entropy due to cholesterol contact. Although mixing cholesterol with phospholipids must have an overall favorable free energy, from the point of view of acyl chains, cholesterol molecules are aggressive *invaders*. Cholesterol molecules have to partially live under the headgroups of phospholipids and occupy the lateral spaces that would otherwise be available to acyl chains. The rigid, bulky bodies of cholesterol can significantly reduce the number of possible conformations of neighboring acyl chains, which is evidenced by increasing chain order parameter when cholesterol is added to a bilayer and by the “cholesterol condensing effect” (Leathes, 1925; Demel et al., 1967; Stockton and Smith, 1976; Vist and Davis, 1990).

We can roughly estimate the free energy increase associated with it. An acyl chain with 16 to 18 carbons could have more than thousands possible conformations. If contacting with cholesterol reduces the number by a factor of 10, it would result a $k \times \ln 10$ ($=2.3 k$) reduction in entropy or a 2.3-kT increase in free energy. Our lattice model does not explicitly contain the detail information of acyl chain conformations. Thus, the increase in free energy can only be phenomenologically expressed as an unfavorable energy. In addition, the increase in free energy is unlikely to be linear with the number of cholesterol contacts. Thus, it can only be expressed as a multibody interaction (or nonpairwise-additive) in a lattice model.

Our interpretation of the source of the acyl chain multibody interaction is also consistent with differential scanning calorimetry studies on cholesterol/phospholipid mixtures. Spink et al. (1999) found that the free energies of transfer of the cholesterol from a gel to a fluid phase (ΔG_{tr}) are small and negative (i.e., preferring fluid phases). But it is resulted from a huge negative entropy and enthalpy. For example, in DPPC bilayers at the gel-fluid phase transition temperature, the transition enthalpy ΔH_{tr} is -36 kcal/mol, the entropy contribution $-T\Delta S_{tr}$ is $+35.3$ kcal/mol, and the net transition free energy ΔG_{tr} is only -0.7 kcal/mol (Spink et al., 1999). Thus, reduction of acyl chain conformation entropy in fluid phases (due to cholesterol contact) has a huge positive contribution to the free energy.

Delicate balance of interactions

As demonstrated in the Result section, forming cholesterol superlattices requires a delicate balance between two opposing interactions. The effect of the large favorable cholesterol-phospholipid mixing interaction is to prevent cholesterol clustering. However, superlattices will not form by this energy term alone, as shown in Fig. 2 *b*. On the other hand, the effect of acyl chain multibody interaction is to minimize the number of cholesterol contacts for acyl chains. If this interaction becomes dominant, it will result clustering of cholesterol, as shown in Fig. 2 *f*. When cholesterol form clusters, less number of acyl chains would need to have cholesterol contacts. When these two interactions reach a right balance, it can result a distribution in which there is no cholesterol clustering and each acyl chain has a minimum number of cholesterol contacts, that of course is a superlattice, such as the one in Fig. 2 *e*.

Superlattice formation has the characteristics of a discontinuous phase transitions. Once the interaction parameters reach critical values, the long-range order suddenly appears. The long-range order is only limited by the simulation lattice size (J. Huang, manuscript in preparation). Below the critical value, no obvious superlattice pattern exists, such as in Fig. 2 *d* or *f*. Thus, formation of superlattices requires not only the right type of interactions, but also the right magnitudes. The strict requirement on interactions suggests that

formation of superlattice should be sensitive to experimental conditions.

In this study, all superlattice patterns, except the one at $\chi_{\text{chol}} = 0.5$, require the unfavorable acyl chain multibody interaction. Our interpretation of the source of this interaction (i.e., acyl chain entropy contribution) is consistent with the experimental observation that superlattices do not form in a gel-phase bilayer, in which entropy change should be minimum. In addition, it is also consistent with the finding that the double bond position on acyl chains can affect cholesterol superlattice formation (Wang et al., 2002). Wang and Chong discovered that if a *cis* double bond is located between C9 carbon and the carboxyl carbon of acyl chains, superlattices become undetectable. A double bond next to the cholesterol position can make the acyl chains less “compressible,” which can make cholesterol-phospholipid mixing less favorable, reduce the magnitude of acyl chain conformation change, and diminish the entropy contribution. Without a significant acyl chain multibody interaction, superlattice cannot form.

The relationship between the nonlinear increase of the acyl chain multibody interaction and the entropy contribution can be understood as follows: let us assume that when an acyl chain has only one cholesterol contact, the reduction on its conformation entropy is only modest. The energy-scaling factor A_1 should be small. When an acyl chain has two cholesterol contacts, the number of acyl chain conformation is greatly reduced, and the entropy effect gets much larger, which results in a big jump in A_2 . This jump could provide the necessary condition for the formation of superlattice at $\chi_{\text{chol}} = 0.25$. Let us further assume that when an acyl chain has three cholesterol contacts, the acyl chain motion is so restricted that it can only adapt nearly all-*trans* conformations. Then it creates another sharp decrease in entropy and a big jump in A_3 . This would provide the necessary condition for the formation of superlattice at $\chi_{\text{chol}} = 0.4$. After that, even if the entropy can only change slightly (i.e., $A_3 \approx A_4 \approx A_5 \approx A_6$), the favorable cholesterol-phospholipid mixing energy could generate the superlattice at $\chi_{\text{chol}} = 0.5$ by itself.

Superlattices at other compositions

We have successfully simulated the superlattice patterns at $\chi_{\text{chol}} = 0.154$, 0.25, 0.4, and 0.5. We also explored the possible combinations of ΔE_m , ΔE_a , and the multibody energy-scaling factors, and found that our Hamiltonian does not generate superlattice patterns at other compositions, such as at 0.33 or 0.2, which have been observed in number of experiments (Chong, 1994; Virtanen et al., 1995; Liu et al., 1997). Unlike the superlattices have been simulated in this study, one common feature of those superlattice patterns is that the acyl chains do not have a uniform cholesterol contact number. Thus, it is still unclear that what type of molecular interactions could generate those superlattices.

It is possible that other types of multibody interactions or long-range interactions are required in those cases. However, a concrete conclusion from this study is that a superlattice pattern, other than the one at $\chi_{\text{chol}} = 0.5$, cannot be generated from pure pairwise-additive interactions.

No two-phase region between two superlattice compositions

It has been suggested that there should be a two-phase coexisting region between two superlattice compositions (Somerharju et al., 1999). The snapshots of lipid distribution in Fig. 6 may even appear to support it. However, our thermodynamic calculation of superlattices shows that there could not be a *thermodynamic* two-phase coexisting region between two superlattice compositions. If two phases coexist, the chemical potential of the cholesterol and phospholipids must remain constant. Our calculation shows that the chemical potentials are not constant between two superlattices (J. Huang, manuscript in preparation). In fact, the chemical potential of cholesterol jumps steeply at each superlattice composition (Huang and Feigenson, 1999).

Superlattice and cholesterol phase diagram

The superlattice at $\chi_{\text{chol}} = 0.154$ has a special significance. It is in direct conflict with some published cholesterol-phospholipid phase diagrams. Many of the phase diagrams show a two-phase region between $\chi_{\text{chol}} \approx 0.05$ and 0.25 (Vist and Davis, 1990; Almeida et al., 1993; Zuckermann et al., 1993). Therefore, a mixture with $\chi_{\text{chol}} = 0.154$ should contain one phase with $\chi_{\text{chol}} \approx 0.05$ and another with $\chi_{\text{chol}} \approx 0.25$. The amount of each phase can be calculated using the lever rule (Moore, 1962). However, if a superlattice does exist at $\chi_{\text{chol}} = 0.154$, it would imply that the mixture has only one phase: a superlattice can never be formed by adding two phases of different composition together.

Recently, Feigenson and Bulboltz constructed a ternary phase diagram of dipalmitoyl-PC/dilauroyl-PC/cholesterol based on fluorescence microscopy, fluorescence resonance energy transfer, and dipyrone-PC excimer/monomer measurements (Feigenson and Bulboltz, 2001). They found an abrupt change of phase at $\chi_{\text{chol}} \approx 0.16$ as well as at 0.25. There was no evidence of coexisting phases between these two compositions. These findings are consistent with the superlattice model.

Umbrella model and cholesterol-phospholipid interactions

The umbrella model was originally proposed to explain the cholesterol solubility limit data in PE and PC bilayers (Huang and Feigenson, 1999). Cholesterol is largely a non-

polar molecule. In water, cholesterol forms cholesterol monohydrate crystals, not a bilayer, because the polar hydroxyl is not big enough to cover the rest nonpolar body. In a bilayer, cholesterol molecules rely on phospholipid headgroups to cover the nonpolar part of cholesterol to avoid the unfavorable free energy of exposing cholesterol to water. The umbrella model emphasizes that this coverage requirement is the dominant driving force of cholesterol-phospholipid interaction.

With the umbrella model, one can explain a wide range of experimental data or even make certain predictions: 1) Because cholesterol molecules need to squeeze into the acyl chain region and partially hide under the phospholipid headgroups, it will restrict the motions of acyl chains or even force acyl chain to adapt nearly all-*trans* conformations. The acyl chain order parameter should increase (Stockton and Smith, 1976; Vist and Davis, 1990). 2) The acyl chain region could become so tightly packed that the membrane permeability should decrease (Kinsky et al., 1967). 3) The actual area occupied by an acyl chain should decrease, i.e., the “cholesterol condensing effect” (Leathes, 1925; Demel et al., 1967). 4) Cholesterol should have little tendency to cluster in a bilayer. Otherwise, the coverage requirement would be difficult to meet. 5) As the cholesterol concentration increases, the phospholipid headgroups need to reorient to cover more interfacial area (per headgroup).

Many of the descriptions above are consistent with recent molecular dynamics simulations. For examples, it has been shown that cholesterol molecules are covered by DPPC headgroups in a bilayer and conformation of acyl chains next to cholesterol is restricted (Smondyrev and Berkowitz, 1999; Tu et al., 1998; Chiu et al., 2001).

We have used the umbrella model to understand two seemingly unrelated phenomena in cholesterol containing bilayers: cholesterol solubility limits and cholesterol superlattices. In the umbrella model, the solubility limit of cholesterol in a bilayer is interpreted as the composition at which phospholipid headgroups can no longer cover any additional cholesterol. The model successfully explained the solubility limit difference between PE and PC bilayers, and the precise discrete values of the solubility limits (Huang and Feigenson, 1999). In this study, we found that the interactions required for superlattice formation can also be explained by the umbrella model. The coverage requirement for cholesterol provides a strong favorable interaction for cholesterol-phospholipid mixing; the restriction of the acyl chain motion by cholesterol can create a large entropy effect and generate an unfavorable multibody interaction, which could lead to the formation of cholesterol superlattices. Although the focus of this study is the molecular interactions and their magnitudes in cholesterol superlattices, because the magnitudes of these uncovered interactions are so large, they should reflect a general picture of cholesterol-phospholipids interactions in a bilayer. The two large opposing interactions discussed above should exist in

various cholesterol-phospholipid bilayers, even if in some cases they do not reach the critical balance to form cholesterol superlattices.

The usefulness of the umbrella model suggests that it captures the key molecular interactions between cholesterol and phospholipids. Thus, the umbrella model emerges as a simple but powerful tool to understand the cholesterol-phospholipid interactions and to explain a wide range of experimental data, starting from low cholesterol concentrations up to the cholesterol solubility limits.

Role of multibody interactions

Interactions between lipid molecules have been often modeled as pairwise interactions (Finegold, 1993). As one enjoys the simplicity of lattice models, one should also beware of other consequences of reducing a complex three-dimensional lipid molecule into a point. The phenomena of cholesterol superlattice and cholesterol solubility limit provided us some unique opportunities to reveal the complicity of the molecular interactions. In both cases, we found that the interactions are decisively multibody. It is even more alarming that in both cases the magnitudes of the multibody interactions are in the order of kT, which can easily dominate many other interactions. This study together with our previous work demonstrates that in a wide range of cholesterol compositions, the dominant interactions between cholesterol and phospholipids are multibody type.

The author thanks Dr. Gerald W. Feigenson for many productive discussions. This work was supported by the National Science Foundation Grant MCB-9722818 and the Petroleum Research Fund Grant PRF-34872-AC7.

REFERENCES

- Almeida, P. F. F., W. L. C. Vaz, and T. E. Thompson. 1993. Percolation and diffusion in three-component lipid bilayers: effect of cholesterol on an equimolar mixture of two phosphatidylcholines. *Biophys. J.* 64: 399–412.
- Cheng, K. H., M. Ruonala, J. Virtanen, and P. Somerharju. 1997. Evidence for superlattice arrangements in fluid phosphatidylcholine/phosphatidylethanolamine bilayers. *Biophys. J.* 73:1967–1976.
- Cheng, K. H., J. Virtanen, and P. Somerharju. 1999. Fluorescence studies of dehydroergosterol in phosphatidylethanolamine phosphatidylcholine bilayers. *Biophys. J.* 77:3108–3119.
- Chiu, S. W., E. Jakobsson, and H. L. Scott. 2001. Combined Monte Carlo and molecular dynamics simulation of hydrated lipid-cholesterol lipid bilayers at low cholesterol concentration. *Biophys. J.* 80:1104–1114.
- Chong, P. L.-G. 1994. Evidence for regular distribution of sterols in liquid crystalline phosphatidylcholine bilayers. *Proc. Natl. Acad. Sci. U.S.A.* 91:10069–10073.
- Chong, P. L.-G., D. Tang, and I. P. Sugar. 1994. Exploration of physical principles underlying lipid regular distribution: effects of pressure, temperature, and radius of curvature on E/M dips in pyrene-labeled PC/DMPC binary mixtures. *Biophys. J.* 66:2029–2038.
- Demel, R. A., L. L. M. van Deenen, and B. A. Pethica. 1967. Monolayer interactions of phospholipids and cholesterol. *Biochim. Biophys. Acta.* 135:11–19.

- Feigenson, G. W., and J. T. Buboltz. 2001. Ternary phase diagram of dipalmitoyl-PC/dilauroyl-PC/cholesterol: nanoscopic domain formation driven by cholesterol. *Biophys. J.* 80:2775–2788.
- Finegold, L., editor, 1993. Cholesterol in membrane models. CRC Press, Boca Raton, FL.
- Huang, J., J. T. Buboltz, and G. W. Feigenson. 1999. Maximum solubility of cholesterol in phosphatidylcholine and phosphatidylethanolamine bilayers. *Biochim. Biophys. Acta.* 1417:89–100.
- Huang, J., and G. W. Feigenson. 1999. A microscopic interaction model of maximum solubility of cholesterol in lipid bilayers. *Biophys. J.* 76: 2142–2157.
- Kinsky, S. C., S. A. Luse, D. Zopf, L. L. M. van Deenen, and J. Haxby. 1967. Interaction of filipin and derivatives with erythrocyte membranes and lipid dispersions: electron microscopic observations. *Biochim. Biophys. Acta.* 135:844–861.
- Leathes, J. B. 1925. Role of fats in vital phenomena. *Lancet.* 208:853–856.
- Liu, F., I. P. Sugar, and P. L.-G. Chong. 1997. Cholesterol and ergosterol superlattices in three-component liquid crystalline lipid bilayers as revealed by dehydroergosterol fluorescence. *Biophys. J.* 72:2243–2254.
- Metropolis, N., A. W. Rosenbluth, M. N. Rosenbluth, and A. Teller. 1953. Equation of state calculations by fast computing machines. *J. Chem. Phys.* 21:1087–1092.
- Moore, W. J. 1962. Physical Chemistry, 3rd Ed. Prentice-Hall, Englewood Cliffs, NJ. 128.
- Smondyrev, A. M., and M. L. Berkowitz. 1999. Structure of dipalmitoylphosphatidylcholine/cholesterol bilayer at low and high cholesterol concentrations: molecular dynamics simulation. *Biophys. J.* 77: 2075–2089.
- Somerharju, P., J. A. Virtanen, and K. H. Cheng. 1999. Lateral organization of membrane lipids: the superlattice view. *Biochim. Biophys. Acta.* 1440:32–48.
- Somerharju, P., J. A. Virtanen, K. K. Eklund, P. Vainio, and P. K. Kinnunen. 1985. 1-Palmitoyl-2-pyrenedecanoyl glycerophospholipids as membrane probes: evidence for regular distribution in liquid-crystalline phosphatidylcholine bilayers. *Biochemistry.* 24:2773–2781.
- Spink, C. H., S. Manley, and M. Breed. 1999. Thermodynamics of transfer of cholesterol from gel to fluid phases of phospholipid bilayers. *Biochim. Biophys. Acta.* 1279:190–196.
- Stockton, B. W., and I. C. P. Smith. 1976. A deuterium NMR study of the condensing effect of cholesterol on egg phosphatidylcholine bilayer membranes. *Chem. Phys. Lipids.* 17:251–261.
- Sugar, I. P., D. Tang, and P. L.-G. Chong. 1994. Monte Carlo simulation of lateral distribution of molecules in a two-component lipid membrane: effect of long-range repulsive interactions. *J. Phys. Chem.* 98: 7201–7210.
- Tang, D., and P. L.-G. Chong. 1992. E/M dips: evidence for lipids regularly distributed into hexagonal super-lattices in pyrene-PC/DMPC binary mixtures at specific concentrations. *Biophys. J.* 63:903–910.
- Tu, K., M. L. Klein, and D. J. Tobias. 1998. Constant-pressure molecular dynamics investigation of cholesterol effects in a dipalmitoylphosphatidylcholine bilayer. *Biophys. J.* 75:2147–2156.
- Virtanen, J. A., M. Ruonala, M. Vauhkonen, and P. Somerharju. 1995. Lateral organization of liquid-crystalline cholesterol-dimyristoylphosphatidylcholine bilayers: evidence for domains with hexagonal and centered rectangular cholesterol superlattices. *Biochemistry.* 34: 11568–11581.
- Vist, M., and J. H. Davis. 1990. Phase equilibria of cholesterol/dipalmitoylphosphatidylcholine mixtures: ^2H nuclear magnetic resonance and differential scanning calorimetry. *Biochemistry.* 29:451–464.
- Wang, M. M., I. P. Sugar, and P. L.-G. Chong. 2002. Effect of double bond position on dehydroergosterol fluorescence intensity dips in phosphatidylcholine bilayers with saturated sn-1 and monenoic sn-2 acyl chains. *J. Phys. Chem.*, In press.
- Zuckermann, M. J., J. H. Ipsen, and O. G. Mouritsen. 1993. Theoretical studies of the phase behavior of lipid bilayers containing cholesterol. In Cholesterol in Membrane Models. L. Finegold, editor. CRC Press, Boca Raton, FL. 223–257.