Cholesterol Superlattice Model Is Compatible with the Calorimetric Behavior of Cholesterol/Phosphatidylcholine Bilayers

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Previous studies have demonstrated that addition of cholesterol to bilayers of saturated phosphatidylcholine (PC) has dramatic effects on the gel-to-liquid phase-transition characteristics of the bilayer. In particular, the enthalpy of the original sharp transition diminishes strongly with increasing cholesterol concentration and a new, broad transition component appears. The enthalpy of the sharp component becomes essentially zero close to the cholesterol mole fraction of 0.25, while the enthalpy of the broad component achieves a maximum near this mole fraction. The broad component typically becomes undetectable when the cholesterol mole fraction is increased to 0.5. In this study we show that these previous experimental findings are fully compatible with a simple model proposing that (i) the cholesterol molecules tend to adopt a regular, superlattice-like lateral distribution within the PC matrix and (ii) the contribution of an acyl chain to the sharp and broad components is critically dependent on its distance from the closest cholesterol molecule. Furthermore, the parameters for PC chain length dependency of cholesterol interactions given by the model are intuitively feasible and in accordance with previous spectroscopic and modeling studies. These findings provide strong support for the cholesterol superlattice model, so far largely based on data obtained with potentially perturbing probes.

Introduction

Cholesterol is a major component of most mammalian membranes where it has both structural and functional roles.[1] The principles of interaction of cholesterol with other membrane constituent have been the subject of innumerous studies, but still many crucial issues remain to be resolved.[2] Among the many different techniques employed, calorimetry has been perhaps the most popular one and disaturated phosphatidylcholines (PC) have typically been chosen as the phospholipid.

In the absence of cholesterol such saturated PC bilayers undergo a highly cooperative gel-to-liquid phase transition at a characteristic temperature that depends on the length of the acyl chains.[3] Addition of cholesterol to such bilayers leads to a decrease in the enthalpy of the original sharp transition and to the appearance of a new, broad one. The enthalpy of the sharp component typically vanishes at a x_c of 0.25, while the enthalpy of the broad component achieves a maximum at or near the same mole fraction. The enthalpy of the broad component usually becomes essentially zero close to x_c of 0.5. The broad component has been associated with acyl chains that are in contact with cholesterol and the sharp one with chains apart from cholesterol.[4]

One currently popular model proposes that addition of cholesterol to the PC bilayer leads to formation of so-called liquid ordered domains that coexist with liquid disordered domains over a wide composition range.[5] Although this model can reasonably well reproduce the observed enthalpic behavior of some PC/cholesterol systems, it does not explain why critical events have been observed at several cholesterol mole fractions (see ref 6). Moreover, diffraction and NMR studies have failed to show any phase separation in the liquid-crystalline phase when x_c is less than 0.5.[7−9] Accordingly, it appears that a simple domain-segregation model may not offer a complete description of phosphatidylcholine/cholesterol bilayers.

Recently, it has been proposed by several investigators that cholesterol and some other sterols tend to adopt a regular, superlattice-like lateral distribution in phosphatidylcholine/cholesterol bilayers.[6,10−13] This superlattice model (SL model) predicts that several critical compositions, each corresponding to a cholesterol superlattice with a particular lattice constant, exist.[14,15] Notably, regular distributions of membrane components have also been considered by others (e.g., refs 4, 16, 17). For instance, Owicki and McConnell showed that the experimentally observed effects of proteins and cholesterol on the phase-transition temperature and enthalpy are well simulated by assuming that these molecules are regularly distributed in the bilayer.[17] In the present study we have examined whether the cholesterol superlattice model is compatible with the established calorimetric behavior of PC/cholesterol bilayers.

Theory

Superlattice Model. A key feature of the superlattice model (SL model) is that there are a number of so-called critical compositions, each corresponding to a guest (cholesterol in this case) superlattice with a particular lattice constant. Between two consecutive critical compositions superlattice domains corresponding to either critical composition coexist together with more or less randomly organized domains.[18] We will now derive

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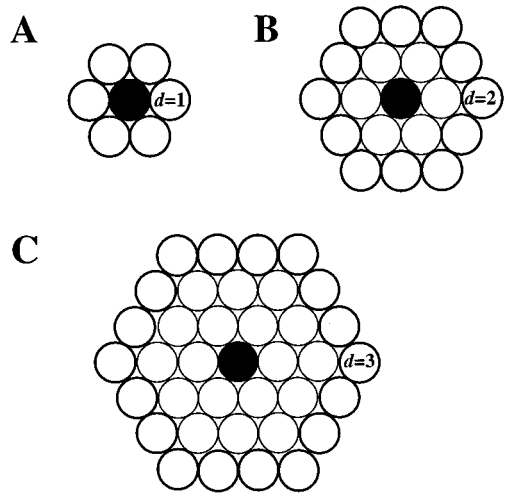

Figure 1. Definition of the acyl chain layers around cholesterol. The closed and open circles represent phospholipid acyl chains and cholesterol, respectively. Symbol *d* indicates the distance of a layer (shell) from the closest cholesterol molecule. In parts A, B, and C the first, second, and third acyl chain layers are emphasized, respectively.

equations allowing the analysis of the calorimetric behavior of cholesterol/phospholipid bilayers in terms of the superlattice model.

Sharp Endotherm. When the cholesterol mole fraction (x_c) is less than 0.25, three different categories of acyl (or alkyl) chains are proposed to exist (cf. Figure 1): (i) chains that are in contact with and thus strongly perturbed[31] by a cholesterol molecule (Figure 1A), (ii) chains that reside in the next hexagonal layer and are somewhat perturbed[19] (Figure 1B), and (iii) chains in the third and subsequent layers that are not significantly perturbed by cholesterol (Figure 1C). The equations giving the mole fractions of molecules in each of these categories as a function of x_c are derived first.

When $0 \le x_c \le 0.1$, there are six phospholipid molecules for each cholesterol in the second layer; i.e., the fraction of phospholipid in this layer (y_2) is $6x_c$. When $x_c = 0.1$, the whole bilayer can be considered to consist of unit cells with $y_2 = 0.60$, depicted in Figure 1B. Within the range $0.1 \le x_c \le 0.25$ all phospholipid molecules are either in the first or in the second layer and the fraction of phospholipid in the first layer is equal to $3x_c$ (cf. Figure 1). Subtracting this fraction from the total phospholipid mole fraction (x_{PL}) yields $y_2 = x_{PL} - 3x_c$. For $x_c \ge 0.25$, all phospholipids (acyl chains) are proximal to cholesterol and thus $y_2 = 0$. These results can be summarized as

$$\begin{aligned} y_2 &= 6x_c && \text{for } 0.00 \le x_c \le 0.10 \\ &= x_{PL} - 3x_c = 1 - 4x_c && \text{for } 0.10 \le x_c \le 0.25 \\ &= 0 && \text{for } 0.25 \le x_c \end{aligned} \quad (1)$$

Finally, the fraction of phospholipid molecules in the third and subsequent layers is obtained simply by subtracting the first and second layer fractions from the total phospholipid fraction (x_{PL}). When $x_c \ge 0.1$, there are no acyl chains with $d \ge 3$. Accordingly, we obtain

$$\begin{aligned} \sum_{d\ge 3} y_d &= x_{PL} - 9x_c = 1 - 10x_c && \text{for } 0.00 \le x_c \le 0.10 \\ &= 0 && \text{for } 0.10 \le x_c \end{aligned} \quad (2)$$

When the fractional contribution of an acyl chain in layer *d* to the sharp enthalpic component is denoted by s_d and when the transition enthalpy of the neat phospholipid bilayer is denoted by ΔH_{PL}, the enthalpy of the sharp component in the presence of cholesterol (ΔH_s) will be

$$\Delta H_s = \sum_{d\ge 1} s_d y_d \Delta H_{PL} \quad (3)$$

It is assumed that the acyl chains in the first layer are so strongly perturbed by cholesterol that their contribution to the sharp transition is negligible; i.e., $s_1 = 0$. However, this assumption is not essential, since the fits give $s_1 = 0$ in all cases. The chains in the second layer are probably only partially perturbed and should thus contribute somewhat to the sharp transition, i.e., $s_2 > 0$. Finally, the chains in the third and subsequent layers should be virtually unperturbed by cholesterol, and therefore, we assumed that $s_d = 1$ when $d \ge 3$. Equation 3 can thus be presented as

$$\Delta H_s = (s_2 y_2 + \sum_{d\ge 3} y_d)\Delta H_{PL} \quad (4)$$

Substituting the partial mole fractions of eqs 1 and 2 in eq 4 yields

$$\begin{aligned} \Delta H_s &= (1 - 10x_c + 6x_c s_2)\Delta H_{PL} && 0.00 \le x_c \le 0.10 \\ \Delta H_s &= (1 - 4x_c)s_2 \Delta H_{PL} && 0.10 \le x_c \le 0.25 \end{aligned} \quad (5)$$

Equation 5 predicts that if $s_2 = 1$, ΔH_s is a linear function of the cholesterol mole fraction when $0 \le x_c \le 0.25$, while if $s_2 < 1$, there would be a kink at $x_c = 0.1$. Furthermore, the sharp component should disappear when $x_c = 0.25$. Note that these critical mole fractions are intrinsic to the superlattice model and therefore not adjustable. Experimental enthalpies are typically given per mole of phospholipid. This can be obtained simply by dividing the total enthalpy by the phospholipid mole fraction:

$$\Delta H_{PL,s} = \frac{\Delta H_s}{x_{PL}} = \frac{\Delta H_s}{1 - x_c} \quad (6)$$

Broad Endotherm. We assume that the broad transition endotherm mainly derives from low cooperativity melting of the acyl chains in contact with, and thus strongly perturbed by, cholesterol. However, probably also the chains in the second layer are somewhat perturbed by cholesterol,[19] and therefore, they contribute to the broad transition (when $x_c \le 0.25$). Analogous to the sharp transition, the enthalpy of the broad transition can be expressed as

$$\Delta H_b = (y_2 b_2 + y_{1,1} b_{1,1} + y_{1,2} b_{1,2} + y_{1,3} b_{1,3})\Delta H_{PL} \qquad 0 \le x_c \le 0.50 \quad (7)$$

The first term on the right side of eq 7 represents residual melting of the acyl chains in the second layer; i.e., those chains (or parts of chains) that did not melt upon the sharp transition. The partial mole fractions $y_{1,n}$ ($n = 1$, 2, or 3) relate to phospholipid molecules in contact with one, two, or three cholesterol molecules, respectively, while the coefficients $b_{1,n}$ ($n = 1$, 2, or 3) indicate the fraction of the corresponding phospholipids taking part in the broad transition (Figure 2). The partial mole fraction y_2 is given by eq 1. Therefore, only $y_{1,1}$, $y_{1,2}$, and $y_{1,3}$ need to be calculated.

According to the SL model, an acyl chain is in contact with either *n* or $n + 1$ cholesterol molecules ($n = 0$, 1, or 2), depending on the cholesterol concentration. (Note that in a

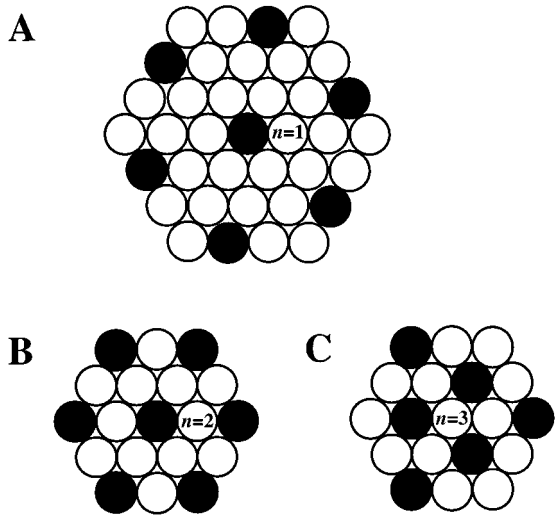

Figure 2. Definition of the three acyl chain classes based on the number of cholesterol contacts. Phospholipid acyl chains can be divided into subclasses depending on the number of cholesterol contacts (n). (A) Each chain is in contact with one cholesterol molecule ($n = 1$). (B) Each chain is in contact with two cholesterols ($n = 2$). (C) Each chain is in contact with three cholesterols ($n = 3$).

random lattice, an acyl chain could simultaneously make contact with zero to six cholesterol molecules at any nonzero cholesterol concentration.) The number of these acyl chains is denoted by N_n and N_{n+1}, respectively. Accordingly, the acyl chains have altogether $nN_n + (n + 1)N_{n+1}$ contacts to cholesterol. On the other hand, there are altogether $6N_c$ cholesterol to acyl chain contacts (N_c = the number of cholesterol molecules) when $x_c \leq 0.50$ (cf. Figure 2). Because the number of acyl chain to cholesterol contacts must be the same as the number of the cholesterol to acyl chain contacts, we obtain

$$6N_c = nN_n + (n + 1)N_{n+1} \tag{8}$$

The total number of acyl chains, N_a, is

$$N_a = N_n + N_{n+1} \tag{9}$$

These two equations allow N_n and N_{n+1} to be solved:

$$N_n = (n + 1)N_a - 6N_c$$
$$N_{n+1} = -nN_a + 6N_c \tag{10}$$

for $0 \leq x_c \leq 0.50$. By taking into account that a phospholipid molecule contains two acyl chains and that $x_{PL} + x_c = 1$, eqs 10 yields

$$y_{1,n} = -(n + 4)x_c + n + 1$$
$$y_{1,n+1} = (n + 3)x_c - n \tag{11}$$

where

$$n = 0 \quad \text{if} \quad 0.00 < x_c < 0.25$$

$$n = 1 \quad \text{if} \quad 0.25 < x_c < 0.40$$

$$n = 2 \quad \text{if} \quad 0.40 < x_c < 0.50$$

When $n = 0$, only the latter equation of eq 11 is applicable (cf. eq 1). Equation 7 can be combined with eqs 1 and 11 to give the enthalpy of the broad component for each critical cholesterol mole fraction interval:

$$\Delta H_b = (6x_c b_2 + 3x_c b_{1,1})\Delta H_{PL} \qquad 0.00 \leq x_c \leq 0.10$$
$$\Delta H_b = [(1 - 4x_c)b_2 + 3x_c b_{1,1}]\Delta H_{PL} \quad 0.10 \leq x_c \leq 0.25$$
$$\Delta H_b = [(2 - 5x_c)b_{1,1} + (4x_c - 1)b_{1,2}]\Delta H_{PL} \qquad 0.25 \leq x_c \leq 0.40$$
$$\Delta H_b = [(3 - 6x_c)b_{1,2} + (5x_c - 2)b_{1,3}]\Delta H_{PL} \qquad 0.40 \leq x_c \leq 0.50 \tag{12}$$

As was the case with the sharp transition (eq 6), the enthalpy per mole of phospholipid is obtained simply by dividing the total enthalpy with the phospholipid mole fraction. The values of s_2, b_2, $b_{1,1}$, $b_{1,2}$, and $b_{1,3}$ can now be found by fitting eqs 5 and 12 to the experimental data.

There are two aspects of the present model that need to be emphasized. First, the model assumes that the enthalpy varies in a systematic manner (linearly, in essence) between two consecutive critical cholesterol mole fractions. Therefore, each data point, independent of whether it is lying on a critical concentration or not, has an equal weight in the fitting process. Deviations (changes in the slope) can occur only at a critical mole fraction.

Second, the present model considers only four critical cholesterol mole fractions, i.e., 0.10, 0.25, 0.40, and 0.50, while the previous superlattice models have proposed the additional critical mole fractions of 0.118, 0.143, 0.154, 0.20, 0.222, and 0.33 for x_c of 0.10−0.50.[6,10] The omission of these latter "critical" cholesterol mole fractions in the present model is justified because significant changes in the enthalpic behavior of phospholipid acyl chains (due to change in the number acyl chain-cholesterol contacts) are expected to take place only when passing one of the critical cholesterol mole fractions included in the present model but not when passing any of those excluded. For example, when the cholesterol mole fraction exceeds the value of 0.25 or higher, each acyl chain must be in contact with cholesterol, while this is not the case below this value. In contrast, no such abrupt changes in the number acyl chain/cholesterol contacts occur at the cholesterol mole fractions of 0.20 and 0.222. While minor enthalpic effects might occur also at these mole fractions, due to a small predicted increase in the overall bilayer packing density,[13] the experimental data point density is far too low and the experimental errors are far too high to allow one to include these "less critical" mole fractions in the present model. More detailed calorimetric studies need to be carried out to determine whether enthalpic deviations occur also at these cholesterol mole fractions.

Results and Discussion

Effect of Cholesterol on the Sharp and Broad Transition Components. Figure 3 displays the normalized enthalpies of both the sharp and broad transition components for DMPC, DPPC, DSPC, and DAPC bilayers versus cholesterol content as given in refs 20 and 21**.** The lines represent fits to the data according to the SL model. Reasonably good fits are generally observed for both components. Some deviations are observed, particularly at low cholesterol concentrations, but these could well be due to problems in dissecting the relative contributions of the two components by deconvolution. Supporting this possibility, such deviations were not observed when the total enthalpy was plotted vs cholesterol mole fraction (data not shown).

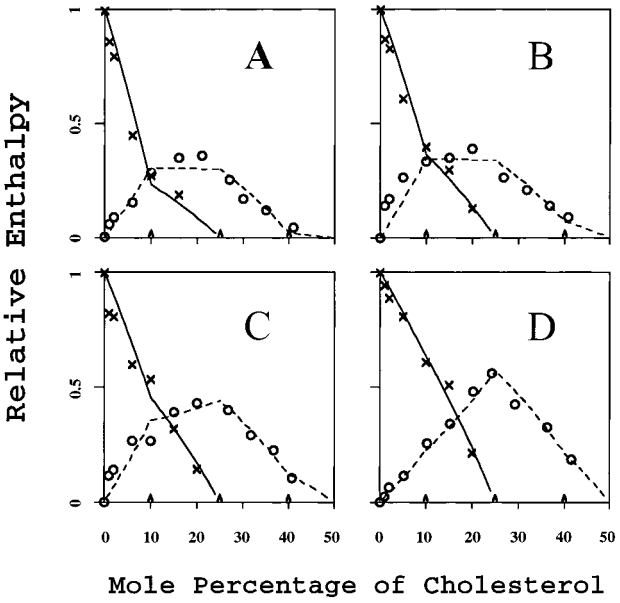

Figure 3. Effect of cholesterol content on the enthalpies of the sharp and broad component of the main phase transition of phosphatidylcholine/cholesterol bilayers: (A) DMPC (di-14/0-PC); (B) DPPC (di-16/0-PC); (C) DSPC (di-18/0-PC); (D) DAPC (di-20/0-PC). The closed and open circles refer to the sharp and broad transition components, respectively, obtained from refs 20 and 21. The lines represent best fits of eq 5 (sharp component) and eq 12 (broad component) to the data.

TABLE 1: Fractional Contribution of the Acyl Chains to the Transitional Subprocesses According the SL-model[a]

N	s_2	b_2	$s_2 + b_2$	$b_{1,1}$	$b_{1,2}$	$b_{1,3}$
14	0.35	0.31	0.66	0.30	0.02	0.00
16	0.55	0.35	0.90	0.34	0.07	0.00
18[b]	0.68	0.31	0.99	0.44	0.12	0.00
18[c]	0.72	0.25	0.97	0.47	0.23	0.00
20	0.95	0.05	1.00	0.57	0.21	0.00

[a] N indicates the number of carbon atoms in the PC acyl chains, and s and b refer to the sharp and broad transition components, respectively. The first subscript refers to the position of the acyl chain layer relative to the closest cholesterol molecule and the second subscript to number of cholesterol neighbors of the acyl chain. For further details see Theory. [b] From ref 20. [c] From ref 21.

The fractional enthalpies of the acyl chains in the different layers as given by the model (eqs 5 12) are listed in Table 1. The first notable feature is that the contribution to the sharp transition is strongly dependent on the length of the acyl chains. Thus, in DAPC/cholesterol bilayers the acyl chains in the second layer contribute fully ($s_2 = 0.95$) to the sharp component, while in DMPC/cholesterol bilayers the contribution is much less ($s_2 = 0.35$). This indicates that short acyl chains in the second layer are much more perturbed by cholesterol than long acyl chains. Supporting this, the contribution of the second layer chains to the sharp and the broad transitions combined ($s_2 + b_2$) is close to 1 for DAPC and DSPC but only 0.66 for DMPC.

Regarding the contribution of the first layer acyl chains to the broad transition (Table 1), an analogous tendency is observed: the shorter the acyl chains, the higher the fractional perturbation by the proximal cholesterol molecule(s). As expected, the perturbation is also proportional to the number of proximal cholesterols, i.e., $b_{1,1} > b_{1,2} > b_{1,3}$. In fact, $b_{1,3}$ should be equal to zero, since the total experimental enthalpy always extrapolates to zero when $x_c = 0.50$ or slightly less (see Figure 3).

TABLE 2: Number of Acyl Chain Carbons Not Taking Part in the Indicated Transition Process[a]

N	$(1 - s_2)N$	$(1 - s_2 - b_2)N$	$(1 - b_{1,1})N$	$(1 - b_{1,2})N$
14	9.1	4.8	9.8	13.7
16	7.2	1.6	10.6	14.9
18	5.8	0.2	10.1	15.8
18	5.0	0.5	9.5	13.9
20	1.0	0.0	8.6	15.8

[a] The symbols are explained in footnote *a* of Table 1 and in Theory.

We note that while we have emphasized the compatibility of the SL model with the calorimetric behavior of disaturated PC/cholesterol bilayers, other single-phase models (eqs 5 17) could be compatible with the data as well. However, this would not probably be the case with models proposing phase separation in the liquid-crystalline state.

Transversal Distribution of the Perturbation of PC Acyl Chains by Cholesterol. The present data also allow us to make some suggestions regarding the transversal distribution of the conformational perturbation of phospholipid acyl chains by cholesterol. We calculated, on the basis of the data given in Table 1, the number of acyl chain carbons that seem to contribute neither to the sharp nor the broad transition (see Table 2). The results suggest several interesting things**.** First, regarding the chains in the second layer, it appears that the number of acyl carbons *not* contributing to the sharp enthalpic component increases from 1 to 9 when chain length decreases from 20 to 14. Analogously, the number of carbons contributing neither to the sharp nor the broad transition component $[(1 - s_2 - b_2)N]$ decreases systematically with chain length (Table 2). In contrast, if one considers the acyl chains in contact with a single cholesterol molecule (and therefore contributing only to the broad component), it appears that a constant number of carbons, i.e., 9.6 ± 1, is perturbed by cholesterol independent of the length of the chain. Notably, the length of the (rigid) 5-ring structure of cholesterol is equivalent to that of approximately 10 methylene units. Thus, the effect of a single proximal cholesterol molecule can be rationalized as being due to perturbation of the acyl chain segment that is in contact with the rigid part of cholesterol, the rest of the chain remaining essentially unperturbed.

When one considers the acyl chains that are in contact with two cholesterol molecules $[(1 - b_{1,2})N]$, it appears that again a constant number of carbons, i.e., 14.8 ± 1, is perturbed by cholesterol independent of PC acyl chain length (Table 2). This number closely corresponds to the length of the cholesterol molecule (excluding the OH moiety), thus suggesting that when there are two proximal cholesterol molecules, also the acyl chain segment in contact with the aliphatic side chain of cholesterol is fully perturbed.

Altogether, these results are intuitively feasible and also in a good agreement with results obtained in previous calorimetric, spectroscopic, and modeling studies on PC/cholesterol bilayers,[2,4,22] thus supporting the validity of the SL model of cholesterol/PC bilayers.

Driving Force for Superlattice Formation in PC/Cholesterol Bilayers. Critical phenomena have been observed previously at particular cholesterol mole fractions, such as 0.25, 0.33, 0.50, and 0,67 (cf. refs 6, 23). Initially, the existence of such "critical" concentrations was proposed to indicate the formation of cholesterol/phosphatidylcholine complexes of a particular stoichiometry, such as 1:3, 1:2, 1:1 or 2:1.[23] However, no feasible explanation regarding the molecular interactions responsible for the formation of complexes with so many different

stoichiometries has been provided. Furthermore, spectroscopic studies have largely failed to support the idea of complex formation.[2]

The SL model does not invoke any complex formation between PC and cholesterol. Rather, it is proposed that (i) neat PC bilayers are "strained" or "frustrated" because the (too) big phosphocholine headgroups hamper close packing of the acyl chains, (ii) this frustration diminishes when PC mixes with cholesterol, a lipid with a very small polar moiety, and (iii) the maximal effect is obtained when the cholesterol molecules adopt an even, SL-like lateral distribution in the PC matrix.[6] Notably, cholesterol as been proposed to act as a PC headgroup "spacer" previously (e.g., ref 24). Thus, there would be an important *enthalpic* contribution to superlattice formation due enhanced van der Waals interactions between the acyl chains in the presence of cholesterol. However, it seems likely that there is also a significant entropic component. This would derive from increased rotational freedom of the bulky phosphocholine headgroups in the presence of cholesterol. Importantly, many recent studies have provided compelling evidence that entropy (i.e., free volume) can drive systems toward remarkable positional order.[25,26] The explanation for this perhaps unexpected result is that the entropy gain (increased free volume) in the ordered state more than compensates for the entropy loss due to increased positional order in that state.[27]

The putative PC/cholesterol superlattices could be similar the "hexatic" phase[28] or the recently discovered smectic A′ phase that exists between the hexatic and liquid phases.[29] A characteristic feature of the hexatic phase is that despite a long-range orientational order, no long-range translational order exists. The smectic A′ phase does not possess any kind of long-range order but has significant *local* order and is therefore different from a normal liquid phase. A superlattice membrane with a hexatic or smectic A′-like character would be "soft", but importantly, the characteristic stoichiometry of the lattice components would be maintained.

The most important biological implication of the cholesterol superlattice model is that it could provide, by proposing the existence of a limited number of "critical", energetically favorable composition, a feasible answer to the puzzling question of what is the primary signal controlling the cholesterol content of cellular membranes (cf. ref 30).

Conclusions

The present study demonstrates that the cholesterol superlattice model is fully compatible with the previously documented effects of cholesterol on the transition enthalpy of bilayers of disaturated phosphatidylcholines. The model also provides parameters for the interaction of cholesterol with phospholipid acyl chains of different length that are intuitively feasible as well as in accordance with previous experimental and modeling data. These results provide further support for the superlattice model, so far largely based on data obtained with probe methods.

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Glossary

PC	phosphatidylcholine
SL	superlattice
T_m	gel-to-liquid-phase transition temperature
x_c	cholesterol mole fraction

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