Phase Diagram of a Ternary Mixture of Cholesterol and Saturated and Unsaturated Lipids Calculated from a Microscopic Model

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We employ a molecular model to study a ternary mixture of saturated lipid, with tails of 16 carbons, a monounsaturated lipid with tails of 18 carbons, and cholesterol. The model, solved within mean-field theory, produces several forms of phase diagrams depending upon the relative strengths of interactions, but only one that shows the coexistence of two liquid phases observed in experiment. The lipids in the phase rich in cholesterol are more ordered than those in the other. The binary cholesterol, saturated lipid system also exhibits liquid, liquid coexistence.

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There has been enormous interest in the hypothesis that the lipids comprising the plasma membrane are distributed inhomogeneously, with regions rich in cholesterol and saturated lipids floating, like rafts, in a sea of unsaturated lipids. Such rafts have been implicated in many biological processes including endocytosis, transcription and transduction processes, and viral infection. The extent of the interest is reflected in the number of recent books and reviews [1–5]. It has spurred numerous *in vitro* investigations of ternary systems of cholesterol and a high-melting point (HMP) lipid, either sphingomyelin or a saturated glycerolipid, and a glycerolipid whose melting point is lower, either because it is unsaturated [6–8] or because, although saturated, it is relatively short [9,10]. A major question the ternary studies could address is whether the aggregation phenomenon indicated *in vivo* might simply be attributed to liquid-liquid phase separation. If so, it could be observed *in vitro*. The assumption was that one fluid, the liquid disordered (LD) phase, would be rich in the low-melting-point (LMP) lipid, while the other, the liquid-ordered (LO) phase, would be rich in cholesterol and the HMP lipid. The nomenclature ''liquid ordered'' [11] signifies that there are fewer thermally excited *gauche* bonds in the hydrocarbon tails of the lipid chains in this phase than in the liquid disordered phase. In addition to these two phases, a gel phase occurs below the melting, or main-chain transition temperature, of the HMP lipid when the concentration of this lipid is large. This phase is the most ordered of the three.

While there is some agreement on broad features in the interior of the ternary diagrams, there is also considerable uncertainty. In particular, the approach to the cholesterol, HMP lipid binary system, and the phase diagram of that system itself remain a matter of controversy. The disagreement centers on whether there is coexistence of two liquid phases over some range of temperature even in this binary system. Evidence of such coexistence derives from several studies using different probes: electron spin resonance [12], freeze fracture electron microscopy [13], nuclear magnetic resonance (NMR) [14], fluorescence resonance energy transfer [15], and other fluorescence studies [8]. Perhaps the strongest evidence, however, is indirect, coming from calorimetry [14,16]. In the cholesterol, dipalmitoylphosphatidylcholine (DPPC) system, these measurements clearly show a very large and narrow specific heat signal at a temperature which is rather constant over a range of compositions, the standard indicator of a triple point. The signal was so interpreted by Vist and Davis [14]. Given the undisputed existence of a gel phase which coexists with a liquid phase at low temperature, the observation of a triple point leads to the conclusion of the existence of a gel and two liquid phases at higher temperatures. There is NMR evidence for such a triple point in another cholesterol, HMP lipid system as well [17].

There are also arguments against the existence of two liquid phases in the cholesterol, HMP lipid system. First, the sharp specific heat signals have been interpreted as *not* arising from three-phase coexistence by McElhaney and co-workers [16], and they also dispute the conclusion drawn by Vist and Davis from their NMR experiments. Indeed the NMR work of Huang *et al.* [18] found no such coexistence, nor did those using fluorescence recovery after photo bleaching [19]. Fluorescence microscopy also does not observe phase separation in these systems [7]. Even if the behavior of this binary system were clear, its relation to that of the canonical 1:1:1 raft composition would not be.

To clarify the ternary system and its evolution from the binary one, we have carried out a study based upon a microscopic model. Previous theoretical investigations of these mixtures have been limited to the binary systems of cholesterol and HMP lipid [11,20,21], or HMP and LMP lipids [22]. Notable among the former is the pioneering study of Ipsen *et al.* which coined the description ''liquid-ordered phase'' [11].

Our ternary mixture consists of cholesterol, a lipid with two saturated tails of 16 carbons, C16:0, and another with two monounsaturated tails of 18 carbons, C18:1. The lipids

tails are described using the Flory rotational isomeric states representation [23]. One introduces the local volume fraction per unit length for a lipid tail in a particular configuration, $\hat{\phi}_\sigma(z) = \sum \hat{\phi}_{\sigma,k}(z)$, where $\hat{\phi}_{\sigma,k}(z) \equiv \nu_\sigma(k) \times \delta[z - z_k]$, the sum runs from $k = 1$ to n_σ, the z direction is perpendicular to the plane of the bilayer, and $\sigma = s$ or u is an index indicating whether the lipid is saturated or unsaturated. The $\nu(k) = 28$ Å$^3 \equiv \nu_0$ is the volume of the kth monomer if it is a CH_2, or 56 Å^3 if a CH_3 group, and $n_s = 15$, $n_u = 17$. The local orientation of the chain is conveniently specified by the unit normal to the plane determined by the kth CH_2 group, $\mathbf{u}_{\sigma,k} = \delta\mathbf{r}_k/|\delta\mathbf{r}_k|$, $\delta\mathbf{r}_k \equiv \mathbf{r}_{k-1} - \mathbf{r}_{k+1}$, with $k = 1, \ldots, n_s - 1$. Just as for the lipids, the configuration of the cholesterol is completely specified by the location of all of its carbon atoms and of the angles between them. The orientation of the small acyl chain of the cholesterol is specified in the same manner as are the lipid chains. The orientation of its rigid rings are specified by the unit vector, $\mathbf{u}_c$, from the third to the 17th carbon in the molecule, using the conventional labeling, and a normal vector to the plane of those rings. Knowing the location of all of its carbon atoms in a given configuration, such as one shown in the inset of Fig. 1, one introduces a local volume fraction, $\hat{\phi}_c(z)$, just as for the lipids, above, with $n_c = 27$ and $\nu_c(k) \approx 21.0$ Å^3. In earlier theories [24], the effect of the short-range repulsive and long-range attractive interactions between elements was accounted for approximately by replacing them by a constraint that the density within the hydrophobic region be constant locally. The free energy of the system can then be written in terms of the $\hat{\phi}$. It is for this reason that our volumetric description of all molecules, above, is taken to be atomically accurate. While accounting well for many properties of bilayers, this theory cannot lead to a main-chain transition to a gel phase. Hence we have added [22] an additional separable interaction per unit volume which tends to align the elements with each other and with the bilayer normal $\mathbf{c}$:

$$V_{\sigma,\sigma'} = -(J_{\sigma,\sigma'}/\nu_0) g(\mathbf{u}_\sigma \cdot \mathbf{c}) g(\mathbf{u}_{\sigma'} \cdot \mathbf{c}), \quad (1)$$

$$g(\mathbf{u}_\sigma \cdot \mathbf{c}) \equiv (m + 1/2)(\mathbf{u}_\sigma \cdot \mathbf{c})^{2m}. \quad (2)$$

For large m, $g \approx m \exp(-m\theta^2)$, where θ is the angle between the two unit vectors. Matching lipid parameters, we have taken $m = 18$. Consequently, the interaction is well approximated by

$$V_{\sigma,\sigma'}(\theta, \theta') \approx -(m^2 J_{\sigma,\sigma'}/\nu_0) \exp[-m(\theta - \theta')^2] \times \exp[-2m\theta\theta']. \quad (3)$$

The first factor is isotropic and of short range. The second expresses the fact that the interior of the bilayer is a nematic, because the bilayer normal direction is singled out. Thus the interaction is favorable only if both bonds are relatively well aligned with one another and also with the bilayer normal. We take the strength of the local interactions between bonds in lipid tails to be the same, $J_{ss} = J_{uu} = J_{su} \equiv J_{ll}$, irrespective of whether the bonds are in a saturated or unsaturated chain. Thus the difference between saturated and unsaturated lipids is not built into the Hamiltonian, but arises solely from the fact that the configurations of these lipids differ due to the presence of the double bond in the latter. As a consequence, unsaturated lipids have fewer configurations which are well oriented with one another and with the bilayer normal. Hence the energy of these configurations are not as low as those of saturated chains, a difference reflected in their lower main-chain transition temperatures [22]. Similarly, the local interaction between cholesterol and lipids depends only on the local orientation of the links in the lipid tail and the local orientation of the links of the small acyl chain of the cholesterol or of the orientation, $\mathbf{u}_c$, of the rigid rings. The strength of this interaction, J_{lc}, is the same between cholesterol and links of saturated and unsaturated lipids. Again the difference between the interaction energy of cholesterol and saturated or unsaturated lipids is not built into the Hamiltonian, but arises from the differences in the configurations of the saturated and unsaturated tails. This difference will manifest itself in a preference for the cholesterol to be associated with saturated lipids which have more configurations which pack more easily with the cholesterol. Finally the local interaction between choles-

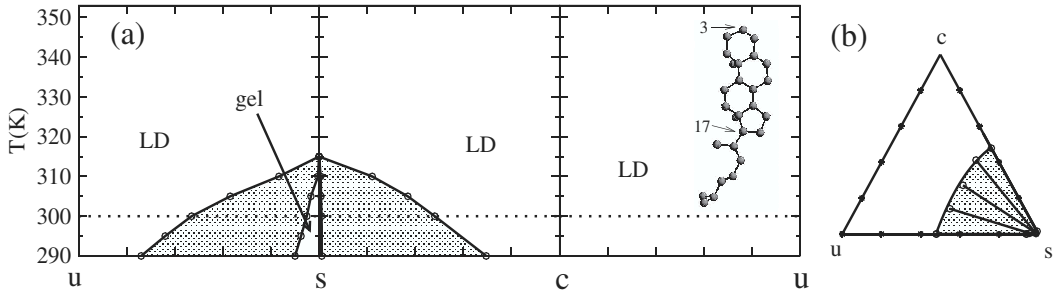

FIG. 1. Panel (a): Calculated phase diagrams of the three binary mixtures of cholesterol (c), saturated (s), and unsaturated (u) lipids in temperature-composition space for $J_{ll}(m + 1/2)^2/k_B T^* = 1.44$ and $J_{lc} = J_{cc} = 0.0$. These binary diagrams form the sides of the Gibbs prism, a cut through which at 300 K produces the Gibbs triangle shown in panel (b). Regions of two-phase coexistence are shaded, and some tie lines are shown. Inset: One of the configurations of our model cholesterol.

terols, of strength J_{cc}, depends only on the local orientations of the links of their acyl chains, or on the orientation, $\mathbf{u}_c$ of the axis of their rings. Within mean-field theory, which we will employ to solve our model, the interaction energy does not depend on the vector normal to the plane of the rings.

Because the interaction between elements depends upon the local orientation of these elements with respect to the bilayer normal, it is convenient to introduce a function, $\hat{\xi}_\sigma(z)$, which measures the local density of elements with a given orientation [25]: $\hat{\xi}_\sigma(z) = \sum \hat{\phi}_{\sigma,k}(z) g(\mathbf{u}_\sigma \cdot \mathbf{c})$, where the sum runs from $k = 1$ to $n_\sigma - 1$. The Helmholtz free energy per unit area, $f_A(T, \rho_s, \rho_u, \rho_c)$, with $\rho_\sigma = N_\sigma/A$, $\sigma = s$, or u, the areal density of saturated or unsaturated chains, and $\rho_c = N_c/A$ the areal density of cholesterol, can now be obtained directly within mean-field theory. One finds

$$\beta f_A = -\frac{1}{\nu_0}\int\Big\{(\beta/2)\sum_{\sigma,\sigma'} J_{\sigma\sigma'}\rho_\sigma\langle\hat{\xi}_\sigma\rangle\rho_{\sigma'}\langle\hat{\xi}_{\sigma'}\rangle + \sum_\sigma \rho_\sigma[\langle\hat{\xi}_\sigma\rangle B_\sigma(z) + \langle\hat{\phi}_\sigma\rangle\Pi(z)]\Big\}dz - \sum_\sigma \rho_\sigma \ln Q_\sigma + \frac{\rho_s}{2}\ln\frac{\rho_s}{\rho_0} + \frac{\rho_u}{2}\ln\frac{\rho_u}{\rho_0} + \rho_c \ln\frac{\rho_c}{\rho_0}, \quad (4)$$

with $\rho_0 \equiv V/A\nu_0$, $\beta \equiv 1/k_B T$. Here $\langle O_\sigma(z)\rangle = \mathrm{Tr} P_\sigma \hat{O}_\sigma(z)$, the average in a single chain ensemble, with

$$P_\sigma = \frac{1}{Q_\sigma}\exp\{-\mathcal{E}_\sigma\} \equiv \frac{1}{Q_\sigma}\exp\Big\{-\beta H_1 - \frac{1}{\nu_0}\int[\hat{\phi}_\sigma(z)\Pi(z) + \hat{\xi}_\sigma(z)B_\sigma(z)]dz\Big\},$$

$Q_\sigma = \mathrm{Tr}\exp\{-\mathcal{E}_\sigma\}$, and H_1 contains the intrachain energy arising from the presence of *gauche* bonds. The three unknown fields $B_s = B_u \equiv B_l$, B_c, and Π are determined by three local self-consistent equations obtained from minimization of the free energy with respect to the $\langle\hat{\xi}_\sigma\rangle$ and $\langle\hat{\phi}_\sigma\rangle$.

The heart of the above method, and its difficulty, is the evaluation of the single molecule partition functions Q_σ. To evaluate them, we have generated on the order of 10^7 configurations of each molecule. Finally the effect of the water, lipid interface is taken into account via a contribution to the free energy per unit area of γ_0, set equal to the oil, water tension. Thus the total free energy per unit area, or surface tension, is $f_{\mathrm{tot}}(T, \rho_s, \rho_u, \rho_c) = f_A + \gamma_0$. We look for phases for which this surface tension vanishes. Phase equilibria is determined by standard thermodynamic equalities [22].

We now turn to the results of our calculation. The binary phase diagram of unsaturated and saturated lipids [22] depends upon packing constraints and the lipid-lipid interaction strength J_{ll}, which we set by matching the main-chain transition of the saturated lipid to $T^* = 315$ K, that of DPPC. Below this temperature there is a gel phase, rich in the saturated lipid, and a disordered liquid phase rich in the unsaturated lipid. The main-chain transition temperature of the unsaturated lipid is below 0 °C. The nature of the ternary diagram depends upon the relative strengths, J_{lc} and J_{cc}, of the lipid-cholesterol and cholesterol-cholesterol aligning interactions. We find essentially three classes of diagrams.

The first occurs when the other two interactions are weak compared to J_{ll}. For the binary cholesterol, saturated lipid system, this results only in gel and LD phases, and therefore no liquid, liquid coexistence, consistent with one side of the controversy noted earlier. The three binary phase diagrams as a function of temperature and composition are shown in Fig. 1(a). A ternary diagram at a temperature below the main-chain temperature is shown in Fig. 1(b). There is *no* region of liquid-liquid coexistence. As such regions can be seen directly in the experimental ternary systems [6,7], this choice of interactions does not apply to the cholesterol, lipid system. It could apply, however, to the lanosterol, lipid system, as suggested in Ref. [17], as that system does not exhibit liquid, liquid coexistence.

A second class of diagram occurs if the lipid-cholesterol aligning interaction, J_{lc}, is dominant. This produces a gel phase which is swollen with cholesterol. As there is no

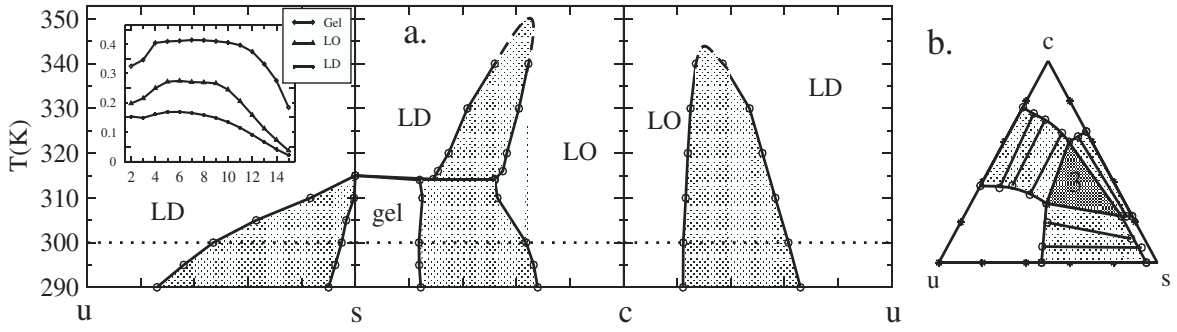

FIG. 2. Panel (a): Binary phase diagrams for $J_{lc} = 0.78 J_{ll}$ and $J_{cc} = 0.73 J_{ll}$, and J_{ll} as in Fig. 1. The saturated lipid-cholesterol mixture has a triple point very near the main-chain transition temperature, so that the gel, LD coexistence region is very narrow. Dashed lines are extrapolations. The ternary mixture at $T = 300$ K is shown in panel (b). Inset: Order parameters of saturated tails in the three coexisting phases at 300 K.

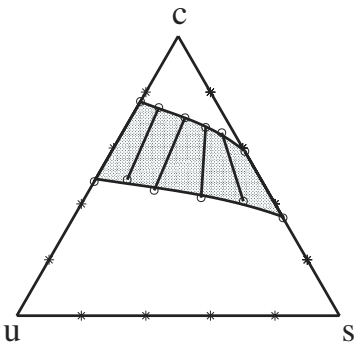

FIG. 3. Phase diagram of the ternary mixture at $T = 320$ K. Interactions are the same as in Fig. 2.

experimental evidence for this, we do not consider this choice of interactions further.

A third class of diagram occurs when the interaction between cholesterols is at least comparable to that between lipids. Binary phase diagrams based on our calculations are shown in Fig. 2(a). In the cholesterol, saturated lipid system, there is coexistence of two liquid phases over a range of high temperatures, and a temperature of three-phase coexistence with a gel phase, consistent with Refs. [12–15]. A cut through the ternary diagram at a temperature of $T = 300$ K below the triple temperature is shown in Fig. 2(b).

There are three regions of two-phase coexistence which extend to the binary axes. The coexistence region between LO and LD liquids would be identified as the region in which rafts could exist. The diagram is topologically identical to that reported in Ref. [8]. The degree of order, S_{CD}, in the lipid chains is given in terms of the second Legendre polynomial $|S_{CD}| = |P_2(\theta_k)|/2$, where θ_k is the angle between the normals to the bilayer and to the plane of the kth CH_2 group. The order parameter for the saturated lipids is shown in the inset of Fig. 2 for each of the three phases which coexist in the phase diagram of Fig. 2(b). As can be seen, the chains in the LO phase are indeed more ordered than those in the LD phase. This increased order is due to the interactions of the lipids with cholesterol, which is itself well ordered. The effect of the larger cholesterol concentration is evident in the plateau corresponding to ordering about cholesterol's ring structure. A similar plateau is seen in simulations [26]. We have also verified that the addition of cholesterol tends to disorder the gel phase, in agreement with experiment [14].

The evolution of the ternary diagram with temperature is simple. As the temperature decreases from 300 K to the freezing point of water, the region of three-phase coexistence expands, but there are no topological changes. As the temperature increases from 300 K, the region of three-phase coexistence shrinks and vanishes at the triple temperature of the binary cholesterol, HMP lipid system. Above the main-chain temperature of this lipid, there is a two-phase region of LO and LD coexistence which stretches from one binary system with cholesterol to the other, as seen in Fig. 3 for $T = 320$ K. At higher temperatures, this region detaches from the binary cholesterol, LMP lipid axis at a critical point, and the region shrinks with further increase of temperature until it vanishes at a critical point on the cholesterol, HMP lipid axis. In sum, we have solved, within mean-field theory, a model of ternary mixtures of lipids and cholesterol which includes packing effects. Within it, liquid-liquid coexistence in such systems originates from the incompatibility of cholesterol and lipid packing, and is intimately tied to the existence of such coexistence in at least one of the cholesterol, lipid binary systems.

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