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## Lateral phase separation in mixtures of lipids and cholesterol

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**Abstract.** – In an effort to understand “rafts” in biological membranes, we propose phenomenological models for saturated and unsaturated lipid mixtures, and lipid-cholesterol mixtures. We consider simple couplings between the local composition and internal membrane structure, and their influence on transitions between liquid and gel membrane phases. Assuming that the gel transition temperature of the saturated lipid is shifted by the presence of the unsaturated lipid, and that cholesterol acts as an external field on the chain melting transition, a variety of phase diagrams are obtained. The phase diagrams for binary mixtures of saturated/unsaturated lipids and lipid/cholesterol are in semi-quantitative agreement with the experiments. Our results also apply to regions in the ternary phase diagram of lipid/lipid/cholesterol systems.

*Introduction.* – Biological membranes typically contain various components such as lipid mixtures, sterols, and proteins that are indispensable to cell functions [1]. Rather than being uniformly distributed in the membrane, there is growing evidence that some intra-membrane components are incorporated in domains arising from lateral lipid segregation in membranes. This phenomenon has attracted great interest in the context of “rafts” [2], *i.e.*, liquid domains rich in cholesterol, saturated lipids (typically sphingomyelin lipids), and in some cases particular proteins [3]. Moreover, cholesterol-rich domains have been directly observed in model membranes composed of lipid mixtures and cholesterol, using advanced fluorescence microscopy [4–10].

Prior to the notion of “rafts” in biological membranes, the role of cholesterol was investigated in binary lipid-cholesterol membranes [11–13], where phase separation was observed using NMR and calorimetry [14,15]. Lipid membranes undergo a freezing or gel-like transition, in which the hydrocarbon tails order. Addition of cholesterol has several effects. It suppresses the gel transition below physiologically relevant temperatures, and can lead to coexistence of two liquid phases with very different orientational order. It is now believed that model

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membranes containing two phospholipids (saturated and unsaturated) and cholesterol [4–10] exhibit “rafts” which are *liquid-ordered* ( $L_o$ ) domains, coexisting with a surrounding background in a *liquid-disordered* ( $L_d$ ) state [3].

From a physical viewpoint, a strategy for understanding the basic structure of “rafts” in biological membranes is as follows [16]. First, it is necessary to have a simple model for binary mixtures of saturated and unsaturated lipids. Second, a minimal model describing binary lipid-cholesterol systems is required in order to understand the effects of cholesterol on membrane phase behavior. Finally, these two viewpoints could be combined to fully investigate three-component systems. Here we focus on the first two steps, and propose simple phenomenological models for lipid-lipid and lipid-cholesterol binary mixtures. Such an approach is quite useful since we can predict, at least qualitatively, the complex phase behavior (shown, *e.g.*, in fig. 2(a) below) of the ternary lipid-lipid-cholesterol system from the three binary sub-systems.

It is generally believed that the gel phase transition is driven by the freezing of lateral motion as well as conformational ordering of lipids. However, because these two degrees of freedom are strongly coupled, the gel phase transition occurs at a single temperature for pure lipid systems. One of our major assumptions is that the membrane state, even for lipid mixtures, can be described by one internal degree of freedom, coupled to the lateral phase separation. Our main result is phase diagrams for two-component systems, which reproduce experimental ones, without specifying the detailed microscopic state of the lipids. Moreover, we shed light on recent phase diagrams obtained for ternary mixture [8, 9].

*Model for saturated-unsaturated lipids systems.* – If different lipids exhibit only a small difference in their gel transition temperature, their phase diagram in terms of temperature-composition parameters will include a “cigar-like” shape of liquid-gel coexistence (as in fig. 1(a)). As the gel transition temperature difference between the two lipids is increased, a gel-gel coexistence region appears below the liquid-gel coexistence. At even larger difference, the phase diagram becomes more complex as the two coexistence regions partially overlap [17].

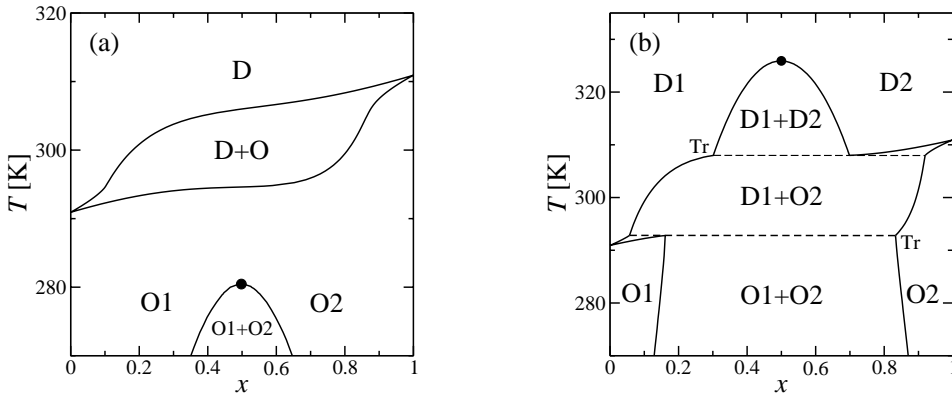


Fig. 1 – Calculated phase diagrams of binary mixtures of saturated and unsaturated lipids as a function of mole fraction of the saturated lipid  $x$  and temperature  $T$  for (a)  $J = 4.0 k_B T_s^*$ , and (b)  $J = 5.0 k_B T_s^*$ . The other parameters are  $a'_2 = 174 k_B$ ,  $a_3 = -307 k_B T_s^*$ ,  $a_4 = 613 k_B T_s^*$ ,  $T_u^* = 240$  K,  $T_s^* = 260$  K as estimated in [18]. The gel transition temperature is  $T_g = 291$  K and 311 K for  $x = 0$  (fully unsaturated) and 1 (fully saturated), respectively. The ordered (large  $\psi$ ) and disordered ( $\psi = 0$ ) phases are, respectively, denoted by O and D. The critical point is indicated by a filled circle, and the triple point by Tr. The critical points are located at (a)  $x_c = 0.498$ ,  $T_c = 280$  K, and (b)  $x_c = 0.5$ ,  $T_c = 326$  K, respectively.

We consider a single bilayer membrane composed of  $x$  mole fraction of saturated lipid and  $(1 - x)$  of unsaturated lipid. The two lipids are taken to have different gel transition temperatures originating from different chain length, degree of saturation, or hydrophilic head group. We assume the same area per molecule for both species, and ignore lipid exchange with the surrounding solvent. The total free energy  $f^{\ell\ell} = f_1^{\ell\ell} + f_2^{\ell\ell}$  comprises i) the free energy  $f_1^{\ell\ell}$  of mixing and binary interactions, and ii) the “stretching” energy  $f_2^{\ell\ell}$ , which controls changes in bilayer thickness.

The free energy per site  $f_1^{\ell\ell}$  is the sum of the entropy of mixing and enthalpy. It can be written within a Bragg-Williams (mean-field) theory as

$$f_1^{\ell\ell}(x) = k_B T [x \log x + (1 - x) \log(1 - x)] - \frac{1}{2} J x^2, \quad (1)$$

where  $k_B$  is the Boltzmann constant,  $T$  is the temperature,  $J > 0$  is an attractive interaction parameter that enhances demixing. Linear terms in  $x$  can be disregarded because they merely shift the origin of the chemical potential.

To describe the gel transition, which involves chain ordering and stiffening, we introduce a rescaled membrane thickness  $\psi \equiv (\delta - \delta_0)/\delta_0$  as an order parameter, where  $\delta$  is the actual membrane thickness and  $\delta_0$  is the constant membrane thickness in the disordered phase corresponding to the liquid phase [18]. Note that  $\psi$  summarizes changes in various degrees of freedom, including the conformations of the hydrocarbon chains and their inter-chain correlations. Since the gel transition is first order, an appropriate phenomenological Landau expansion of the “stretching” free energy per site in powers of  $\psi$  is [18]

$$f_2^{\ell\ell}(x, \psi) = \frac{1}{2} a'_2 [T - T^*(x)] \psi^2 + \frac{1}{3} a_3 \psi^3 + \frac{1}{4} a_4 \psi^4, \quad (2)$$

where  $a'_2 > 0$ ,  $a_3 < 0$ , and  $a_4 > 0$ . For a single component membrane (*i.e.*,  $x = 0$  or  $1$ ),  $T^*$  is a reference temperature, and the first-order gel transition temperature is given by  $T_g = T^* + 2a'_2/(9a'_2a_4)$ . For a binary mixture, the transition temperature depends on the composition  $x$ ; for convenience, we describe the reference temperature  $T^*(x)$  as a linear interpolation between the two pure limits:

$$T^*(x) = xT_s^* + (1 - x)T_u^*, \quad (3)$$

where  $T_s^*$  and  $T_u^*$  are the reference temperatures of the pure saturated and unsaturated lipids, respectively. With all else the same (head group size, interactions, and chain length, etc.), we expect  $T_s^* > T_u^*$ , because unsaturated lipids break up the crystallizing tendencies. Note that eq. (3) leads to a coupling term  $x\psi^2$  [19]. We have neglected a lower-order bilinear term  $x\psi$ , which induces a small temperature and composition dependence in  $\delta_0$ . Another possible coupling term  $x^2\psi$  simply renormalizes the interaction parameter  $J$ .

Combining eqs. (1)-(3), we obtain the total free energy  $f^{\ell\ell}$ . After minimizing with respect to  $\psi$ , the two-phase region is obtained by the Maxwell construction. In fig. 1, we show two typical phase diagrams of binary lipid mixtures. For small  $J$  (fig. 1(a)), which corresponds experimentally to mixtures with weak segregation tendency, a cigar-like coexistence region is obtained between a disordered phase (D) where  $\psi = 0$  (liquid) and an ordered phase (O) where  $\psi > 0$  (gel). At temperatures below the cigar-shape region, there is another coexistence region between two ordered phases, O1 + O2, with  $\psi_1 \lesssim \psi_2$ . This type of phase diagram was experimentally observed for DEPC-DPPC binary lipid systems [20]. When  $J$  is increased, the O1 + O2 coexistence region extends into and beyond the D + O coexistence region (fig. 1(b)). In this more complex phase diagram, there are two triple points (denoted as Tr) at which the

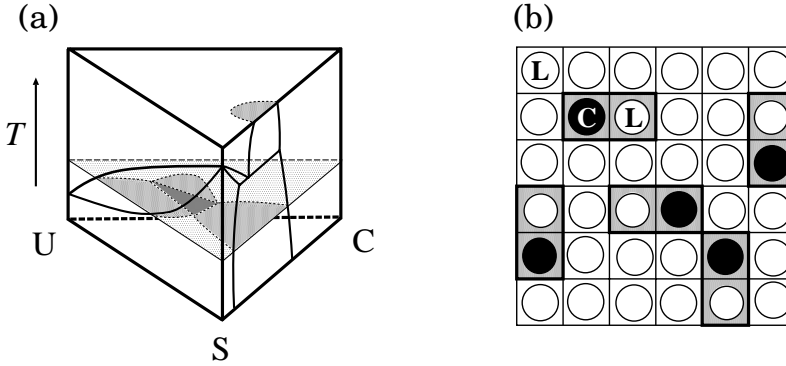


Fig. 2 – (a) Schematic phase prism of a ternary system consisting of saturated lipid (S), unsaturated lipid (U), and cholesterol (C). The gray and black regions on the constant-temperature triangle plane are two-phase and three-phase coexisting regions, respectively. (b) Lattice model for a lipid-cholesterol mixture. Each cholesterol molecule ( $\bullet$ ) forms a dimer with a neighboring lipid molecule ( $\circ$ ).

two disordered phases and ordered phase (D1 + D2 + O2), or the disordered phase and two ordered phases (D1 + O1 + O2) coexist. Many features of this phase diagram can be seen for DEPC-DPPE lipid mixtures which have a stronger segregation tendency because both head and tail moieties are different [20].

*Model for lipid-cholesterol systems.* – Next we discuss the role of sterols, such as cholesterol and lanosterol, on the phase behavior. On the one hand, a small amount of cholesterol destabilizes the gel phase in favor of a *liquid-disordered* ( $L_d$ ) phase [14, 15]. Substantial amount of cholesterol, on the other hand, stabilizes a *liquid-ordered* ( $L_o$ ) phase in which the lipid hydrocarbon tails are extended, but maintain high lateral mobility. This reflects the dual molecular mechanism of a cholesterol molecule: it can act i) as an “impurity” and weakens the inter-lipid interactions for ordering, or ii) as a “chain rigidifier” and induces conformational order in neighboring lipid chains [13]. Moreover, recent experiments using atomic force microscopy showed that the cholesterol-rich domains are thicker than the cholesterol-poor regions [21, 22], supporting the assumption that the local membrane thickness can be taken as the order parameter.

Based on these observations, we propose a model for lipid-cholesterol binary mixtures. Consider a membrane with cholesterol mole fraction  $c$  and lipid mole fraction  $(1 - c)$ . There are three contributions to the total free energy  $f^{\ell c} = f_1^{\ell c} + f_2^{\ell c} + f_3^{\ell c}$ : i) the entropy of mixing  $f_1^{\ell c}(c)$  between lipid and cholesterol; ii) the “stretching” energy  $f_2^{\ell c}(\psi)$  of lipid molecules; and iii) direct coupling terms between lipid and cholesterol,  $f_3^{\ell c}(c, \psi)$ , which take into account the effect of cholesterol on the bilayer thickness.

We note that the coexistence region in the lipid-cholesterol system is typically found only for  $c \leq 0.5$  [8, 14, 15]. For cholesterol concentration above roughly 0.5, the entire bilayer becomes unstable. To account for such observations we assume that each cholesterol molecule irreversibly dimerizes with a single lipid molecule forming a “condensed complex”, as has previously been inferred from experiments [23] and simulation [24]. Since all cholesterol molecules are assumed to form dimers, as shown in fig. 2(b), the entropy of mixing is taken between lipid-cholesterol dimers and the remaining monomeric lipids, and is given by a Flory-Huggins form:

$$f_1^{\ell c}(c) = k_B T [c \log 2c + (1 - 2c) \log(1 - 2c)]. \quad (4)$$

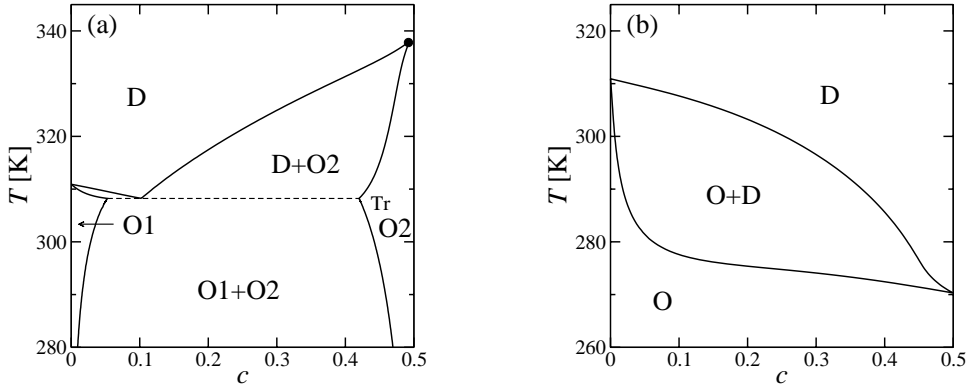


Fig. 3 – Phase diagrams of binary lipid-sterol mixtures as a function of mole fraction  $c$  of sterol and temperature  $T$  for (a) cholesterol,  $\Gamma_1 = 11 k_B T^*$ ,  $\Gamma_2 = 47 k_B T^*$ ; and (b) lanosterol,  $\Gamma_1 = 28 k_B T^*$ ,  $\Gamma_2 = 20 k_B T^*$ . The other parameters are the same as in fig. 1, except  $T^* = 260$  K. The gel transition temperature at  $c = 0$  is  $T_g = 311$  K. The ordered (large  $\psi$ ) and disordered (non-zero small  $\psi$ ) phases are, respectively, denoted by O and D. The critical point indicated by a filled circle in (a) is located at  $c_c = 0.492$ ,  $T_c = 338$  K.

This free energy is valid only for  $0 \leq c \leq 0.5$ , since each cholesterol molecule is paired with a neighboring lipid, and the monolayer loses its stability above about  $c = 0.5$ . The first term is the entropy of dimers having an area fraction of  $2c$ , while the second term accounts for the entropy of lipid monomers of area fraction  $(1 - 2c)$ . Although extensions of the model to include excess free cholesterol and/or formation of trimers, etc. is possible [25], the present situation is enough to describe the qualitative phase behavior.

The Landau free energy describing the structural phase transition of the membrane is given by  $f_2^{\ell c}(\psi)$ , and is the same as  $f_2^{\ell \ell}$  of eq. (2):

$$f_2^{\ell c}(\psi) = \frac{1}{2}a_2'(T - T^*)\psi^2 + \frac{1}{3}a_3\psi^3 + \frac{1}{4}a_4\psi^4, \quad (5)$$

where the order parameter  $\psi$  is again the relative bilayer thickness, and  $T^*$  is the reference temperature of the pure lipid system.

The simplest free energy to account for the previously mentioned dual effects of cholesterol can be expressed by the following phenomenological coupling terms:

$$f_3^{\ell c}(c, \psi) = \frac{1}{2}\Gamma_1 c\psi - \frac{1}{2}\Gamma_2 c^2\psi, \quad (6)$$

where  $\Gamma_1 > 0$  and  $\Gamma_2 > 0$  are the coupling constants. The first term expresses the fact that a small amount of cholesterol ( $c > 0$ ) acts as an “impurity”. It interferes with the crystalline ordering, and enhances disordered chains (smaller  $\psi$ ). This term is necessary because, as shown in the experimental phase diagram [14,15], the gel- $L_d$  coexistence temperature decreases upon adding cholesterol to the pure lipid system. The second term corresponds to the “chain rigidifying” effect reflecting the fact that a larger amount of cholesterol favors ordered tail states and hence larger  $\psi$ . Meanwhile, this coupling induces lipid-cholesterol phase separation since it is proportional to  $c^2$  with a negative coefficient. In other words, the effective lipid-cholesterol interaction depends on the conformational states of the lipid chains.

Adding eqs. (4)-(6), we first minimize  $f^{\ell c}$  with respect to  $\psi$  and then construct the phase diagrams, as shown in fig. 3. The topology of the phase diagram depends on the relative

values of the two parameters,  $\Gamma_1$  and  $\Gamma_2$ . This can be seen in fig. 3, where two different sets of values are taken to model (a) lipid-cholesterol and (b) lipid-lanosterol interactions. In fig. 3(a), there are three different coexisting regions. Due to the  $\Gamma_1$  coupling term in eq. (6), the region of the disordered (D) phase, characterized by a non-zero small value of  $\psi$ , widens at the expense of the ordered (O1) phase, characterized by a large value of  $\psi$ , upon the addition of a small amount of cholesterol ( $c < 0.1$ ). Note that D- and O1-phases, respectively, correspond to  $L_d$  and gel phases in the experiments. For larger  $c$ , however, the  $\Gamma_2$  coupling term overcomes the first term, and the region of the D-phase narrows in favor of O2-phase ( $c > 0.1$ ), which corresponds to the  $L_o$  phase where the order parameter  $\psi$  takes a value much larger than that in the D-phase. The obtained phase diagram for lipid-cholesterol systems agrees semiquantitatively with the experimental one [14,15]. The D- and O2-phases have the same symmetry and are continuously connected, with a critical point at  $c_c = 0.492 < 0.5$ . The appearance of the critical point can be understood by noticing that the coupling terms in eq. (6) are linear in  $\psi$ , and act as an external field coupled to  $\psi$ . In general, the first-order transition becomes continuous when the applied external field is strong enough.

For larger  $\Gamma_1$  and smaller  $\Gamma_2$  we obtain the phase diagram shown in fig. 3(b). In this case, the effective external field is too weak to eliminate the first-order transition, and the critical point does not exist. This phase diagram resembles that of binary lipid-lanosterol mixtures [26]. Compared to cholesterol, lanosterol has three additional methyl groups and has a structurally rougher hydrophobic part, leading to a weaker enhancement of lipid tail stretching (smaller  $\Gamma_2$ ). Moreover, an extra double bond in the lanosterol hydrocarbon tail can be expected to inhibit crystallinity (larger  $\Gamma_1$ ). In fact, lanosterol is considered to be a precursor to cholesterol in the evolutionary pathway [26]. Although cholesterol can stabilize the region of coexistence between the D- and O2-phases (*i.e.*,  $L_d + L_o$ ) as shown in fig. 3(a), such a coexistence has not been identified for the lipid-lanosterol mixtures [26]. The fact that the coexistence region ends at  $c = 0.5$  is due only to the artificial truncation of the model at  $c = 0.5$ . If free excess lanosterol were allowed, the phase boundaries would continue until the membrane loses its integrity. However, such a break-up of the membrane is not considered here. We note that although the values of the coupling constants are chosen in an attempt to reproduce the experimental phase diagrams, their order of magnitude is in agreement with that obtained from molecular level theory [27].

*Discussion.* – Within a few simplifying assumptions we have obtained phase diagrams that successfully mimic the experimental ones. A few points merit further discussion. We used only one order parameter  $\psi$  (related to local membrane thickness) to describe the membrane internal degrees of freedom of chain ordering and crystallinity. This assumption is well motivated for pure lipid membrane where both ordering and crystallization occur simultaneously. Although addition of cholesterol may separate the chain ordering and freezing transitions and stabilize the  $L_o$  phase, the thickness  $\psi$  is sufficient here to distinguish between the gel (O1),  $L_o$  (O2), and  $L_d$  (D) phases. Note that the symmetry of the D- and O2-phases is the same (hence the critical point), and differs from the solid-like and local crystalline order of the O1-phase. As an extension to the present model, it may be of interest to use two distinct order parameters.

Finally, we discuss the qualitative behavior of the full ternary phase diagram for saturated (S) and unsaturated (U) lipids in the presence of cholesterol (C). The phase diagrams in figs. 1(a) and 3(a) correspond to the two side views of the schematic compositions-temperature phase prism in fig. 2(a), namely the US- $T$  and the SC- $T$  planes, respectively. A topologically similar phase diagram to fig. 3(a) is expected for the UC- $T$  plane [26].

Consider a cut of the phase prism at a fixed temperature as shown in fig. 2(a). Both the D + O coexistence region on the US- $T$  plane and the O1 + O2 coexistence region on the

SC-*T* plane extend onto this triangle plane, and may form a three-phase coexisting region (D + O1 + O2, *i.e.*,  $L_d + \text{gel} + L_o$ ) when they meet each other. If the phase separation does not occur between U and C for this temperature, a critical point between D and O2 is expected to appear. As the temperature is increased, the line of critical points may end when the three-phase region disappears. These arguments are consistent with recent experimental studies of the phase morphology inside the phase prism [6–9]. Further calculations of the full ternary phase diagrams are in progress. “Rafts”, reported to be in the  $L_o$  (O2) phase, may correspond to nm- $\mu\text{m}$  sized domains similar to the macroscopic phase separation seen in ternary mixtures at biologically relevant temperatures and compositions [3]. The small length scales associated with “rafts” *in vivo* remain unexplained.

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